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The Toxicity of Hydrogen Peroxide to Rainbow Trout *Oncorhynchus mykiss* and Cutthroat Trout *Oncorhynchus clarki* Fry and Fingerlings

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Abstract

Fungal and parasitic infections of fish can significantly impact the survival of cultured fish. Formalin is currently used to control such infections; however, concern has arisen over its safety to users and to the environment. Hydrogen peroxide has been designated as a low priority fungicidal drug by the United States Food and Drug Administration (FDA), yet, little information is available on treatment concentrations or its toxicity to trout. Rainbow trout *Oncorhynchus mykiss* and cutthroat trout *Oncorhynchus clarki* fry and fingerlings were exposed to hydrogen peroxide concentrations of 0, 70, 170, 280, 420 and 540 ppm for 30, 60 or 120 min at 15 C to determine the chemical's toxicity. Rainbow trout fry and fingerlings experienced elevated mortalities (>20%) during treatments using 420 and 540 ppm for 30 min; 280, 420 and 540 ppm for 60 min; and ≥170 ppm for 120 min. Cutthroat trout fry experienced elevated mortalities (>23%) during treatments using 540 ppm for 30 min; 420 and 540 ppm for 60 min; and ≥170 ppm for 120 min. Cutthroat trout fingerlings experienced elevated mortalities (>60%) during treatments using 540 ppm for 60 min and ≥280 ppm for 120 min. No control mortalities were encountered for both life stages of either species. The lethal concentrations (LC_{50}) of both age classes and species for each of the three durations ranged from 514–636 ppm for 30 min treatments, 322–506 ppm for 60 min treatments, and 189–280 for 120 min treatments. Mortalities for all four toxicity tests which occurred during a 96-h post-treatment period were centered around the following treatments: 30 min, 540 ppm; 60 min, 280–540 ppm; 120 min, 170 ppm. Tissue damage to gills was found only among fish that did not survive the initial chemical exposure. Test concentrations proved to be relatively stable during a 24-h period, retaining better than 85% of their original strength for all five dilutions. At a water temperature of 15 C concentrations should not exceed 280 ppm for a 30-min treatment.

The treatment of diseased fish and the therapeutic application of drugs is an aspect of fish culture that requires significant time and money. Infections of eggs caused by aquatic fungi can greatly reduce hatching success and seriously impact survival of juvenile and adult fish. Historically malachite green was used with success to control fungus on eggs, and in combination with formalin, to control external protozoan parasites. Malachite green was withdrawn from use by the U.S. Food and Drug Administration (FDA) in 1991 because of its potential teratogenic qualities (Meyer and Jorgenson 1983). Formalin is currently approved for use by the FDA as a fungicide on salmonid and esocid eggs and as a parasiticide for several cold and cool water fish species (Beaulieu et al. 1992). However, the use of formalin has caused concern about human safety and the impact of its discharge into the environment. As a result of this, substantial effort has been put into the search for effective alternate fungicidal and parasiticidal treatments.

Investigators have compared the antifungal activities of various chemicals including hydrogen peroxide and salt to that of formalin and malachite green. Hydrogen peroxide has been found to be effective for controlling fungus on rainbow trout *Oncorhynchus mykiss* eggs (Marking et al. 1994; Rach 1995; Schreier et al. 1996) and fall

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chinook salmon *Oncorhynchus tshawytscha* eggs (Waterstrat and Marking 1995). Even though salt (NaCl) appears to be a relatively effective fungicide (Edgell et al. 1993; Waterstrat and Marking 1995; Schreier et al. 1996) the quantities required to achieve a desired effect (≥15,000 ppm) may make its use prohibitive when large numbers of eggs are to be treated. Hydrogen peroxide has therefore risen to the forefront as an alternative fungicide because of its apparent effectiveness in controlling fungus and because it breaks down into benign constituent components, water and oxygen. The FDA has designated hydrogen peroxide as a drug of low regulatory priority (LRP) when it is administered at concentrations of 250–500 mg/L to control fungus on eggs and all life stages of fish (Beaulieu et al. 1992).

In addition to being an effective fungicide, hydrogen peroxide also has potential as a treatment of external bacterial infections and parasites. Hydrogen peroxide has been shown to effectively immobilize and/or kill sea lice *Lepeophtheirus salmonis* on Atlantic *Salmo salar* and chinook salmon (Johnson et al. 1993; Bruno and Raynard 1994). Hydrogen peroxide may also be effective in treating bacterial gill disease in cutthroat *Oncorhynchus clarki* and rainbow trout (Roberts 1995).

To determine the ability of hydrogen peroxide to act as an effective external treatment, information was required on the chemical concentrations and duration of treatments that were lethal to the target animals at various temperatures. The toxicity level for adult spring chinook salmon appears to be between 100 ppm and 250 ppm at 12–13 C (treatment duration unknown) (Fitzpatrick et al. 1995). In another study (Johnson et al. 1993), when chinook (20–49 g/fish) and Atlantic salmon (18–40 g/fish) were treated with 1,500 ppm hydrogen peroxide for 20 min at 11, 14, or 18 C the fish treated at 11 and 14 C exhibited 0–5% mortality one day post-treatment (dpt) compared to 100% at 18 C. When fish were treated with 3,000 ppm for 20 min at 11 C, mortality increased to 25% for the chinook salmon and 10% for the Atlantic salmon.

When Bruno and Raymond (1994) exposed post-smolt Atlantic salmon to 1,230 ppm hydrogen peroxide for 20 min at 13.5 C, 35% of the fish died within 2 h. However, when the Atlantic salmon were exposed to 1,260 ppm for 20 min at 10 C, no mortalities resulted. Brown trout *Salmo trutta* exhibited 45% and 100% mortalities, respectively, during treatments of 15 min and 45 min of 3,000 ppm hydrogen peroxide at 12 C (Rach et al. 1995). Under the same conditions, lake trout *Salvelinus namaycush* exhibited mortalities of 3% and 100%, respectively. The purpose of this study was to determine the toxic concentration of hydrogen peroxide to two life stages of rainbow and cutthroat trout treated at 15 C.

Materials and Methods

Test Animals

Bear Lake cutthroat *Oncorhynchus clarki utah* eggs were obtained from the Mantua State Hatchery, Mantua, Utah, USA and hatched at the Fisheries Experiment Station Logan, Utah. Rainbow trout of the Fish Lake-DeSmet strain were obtained from the J. Perry Egan State Brood Station, Bicknell, Utah and transferred to the Fisheries Experiment Station as eyed eggs. For both trials, fry toxicity tests were started approximately 2 wk after first feeding (cutthroat = 0.15 g/fish, rainbow = 0.26 g/fish). Cutthroat trout from the same lot of fish as the fry were used for the fingerling portion of the study when the fish had reached a mean weight of 7.5 g. The rainbow trout fingerling portion of the study was conducted when the fish had reached a mean weight of 7.3 g.

Experimental Design

Test dilutions for the four trials were: 0 (control), 70, 170, 240, 420, and 540 ppm, and each concentration was tested for a duration of 30, 60, or 120 min. Tests were conducted in six 75-L glass aquaria (60 ×

30 × 28 cm) which were supplied with supplemental aeration. Aquaria were lighted from above with fluorescent lights and from the rear with a single incandescent light. Water was supplied by a well and had the following properties: temperature, 15 C; hardness, 248 mg/L; alkalinity, 257 mg/L; pH, 7.5. Dilutions were made from stabilized 35% hydrogen peroxide (Dyce Chemical, Ogden, Utah, USA) and assayed for actual concentrations using a commercial test kit (ammonium molybdate/sodium thiosulfate titration method; Model HYP-1, accuracy ±1 mg/L, Hach Co., Loveland, Colorado, USA). Assays were also performed using a portable water test kit (Model FF-1A, Hach Co., Loveland, Colorado, USA) to see if the addition of the hydrogen peroxide had an impact on pH, total hardness, total alkalinity, or CO_2 of the hatchery water. During the course of the toxicity trials, one tank per concentration per day was randomly assayed to verify the actual concentration of the test dilutions. The five concentrations were also added to separate aquaria, provided with aeration, and left for 24 h after which they were assayed to determine the stability of the dilutions.

At the beginning of a trial, aquaria were filled to 50.5 L, hydrogen peroxide was added and allowed to diffuse for 5–10 min. Fry were removed from troughs inside the hatchery building and fingerlings from an outside raceway. Twenty fish were then placed into each of the test aquaria. Any mortalities which occurred during the test were recorded. Upon completion of a given test, fish were removed and placed into a fiberglass trough with flow-through water where additional mortalities were recorded through 96 h. Any mortalities that occurred within one hour post-treatment were considered treatment mortalities and any subsequent mortalities were considered 96-h mortalities. During the 96-h post-treatment period, fish were offered feed but were not subjected to any additional disturbances.

Histological Analysis

Following the 96-h post-treatment period, gill and pseudobranch samples were taken from five fish from each treatment and the controls for later histological analysis. Tissue samples were also taken periodically from groups which experienced 100% mortality during their treatment. For fry samples the entire head was removed immediately posterior from the opercle. A gill arch and one pseudobranch were removed from each of the fingerlings sampled. Tissue samples were immediately fixed in 10% buffered formalin and stored until histological analysis could be performed. Samples were embedded in paraffin and stained with hematoxylin and eosin. Following tissue preparation, slides of gills from all treatments from the rainbow trout fry toxicity trial and select treatments from the remaining three trials were examined for possible tissue changes caused by the treatment.

Data Analysis

Lethal concentrations (LC_{50}) were calculated from the initial treatment mortality data. Percent mortality data were converted to probit values and hydrogen peroxide concentrations were log transformed (Gad and Weil 1988; Newman 1995). A weighted linear regression was performed on the transformed data from which LC_{50} values were calculated. Confidence intervals (95%) were calculated according to the methodology of Litchfield-Wilcoxon (1949). The cutthroat fingerling 30-min series of treatments exhibited only one partial kill. This precluded direct calculation of an LC_{50}, so to overcome this problem the series of data were manipulated according to Berkson (1951). Mean cumulative mortalities were calculated across treatment duration for each of the four trials and a one-way ANOVA was run using NCSS 6.0 Statistical System for Windows (Hintze 1995). Mean differences were analyzed by the

TABLE 1. *Percent cumulative mortality (±SD, N = 3) of rainbow trout fry and fingerlings at 1- and 24-h post-treatment (hpt). Fish were treated with varying concentrations of hydrogen peroxide for 30, 60, and 120 min at 15 C.*

Concentration (ppm)	Duration (min)	Cumulative mortality (1 hpt)		Cumulative mortality (96 hpt)	
		Fry	Fingerling	Fry	Fingerling
Control	30	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	60	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	120	0 ± 0	0 ± 0	0 ± 0	0 ± 0
70 ppm	30	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	60	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	120	2 ± 2	0 ± 0	0 ± 0	0 ± 0
170 ppm	30	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	60	8 ± 12	2 ± 2	8 ± 12	2 ± 2
	120	25 ± 15	35 ± 16	70 ± 22	78 ± 6
280 ppm	30	2 ± 2	2 ± 2	2 ± 2	3 ± 2
	60	40 ± 18	23 ± 13	42 ± 19	53 ± 14
	120	85 ± 4	92 ± 5	98 ± 2	100 ± 0
420 ppm	30	23 ± 18	32 ± 20	27 ± 16	38 ± 15
	60	58 ± 6	82 ± 9	60 ± 7	92 ± 12
	120	100 ± 0	100 ± 0	100 ± 0	100 ± 0
540 ppm	30	58 ± 23	35 ± 4	58 ± 23	78 ± 6
	60	93 ± 6	100 ± 0	95 ± 7	100 ± 0
	120	100 ± 0	100 ± 0	100 ± 0	100 ± 0

Newman-Keul's Multiple Comparison Procedure.

Results

The initial-treatment mortality of rainbow trout fry was highest at 540 ppm, across all three test durations, 58%, 93%, and 100%, respectively (Table 1). As the duration of the test increased, so did the percent mortality. For a duration of 30 min, a 280-ppm concentration resulted in 2% mortality. At 60 min this increased to 40%, and at 120 min it rose to 85%. This trend was evident for all treatments except the control group. The rainbow fingerling trial exhibited the same trends with respect to mortality. Comparing the two life stages of rainbow trout, there was no clear trend of one age of fish being more sensitive to treatment than the other. The highest levels of mortality (>50%) for both stages occurred at treatment durations of 60 min and concentrations of 420 and 540 ppm and durations of 120 min and concentrations of 280 ppm and greater.

The two cutthroat life stage trials exhibited similar trends of mortality as concentration and treatment time increased compared with the rainbows. However, within the cutthroat trout trial the fry consistently had higher mortalities across all treatments compared with the fingerlings, except for one instance where both had 100% mortality (Table 2). The highest levels of mortality (>50%) for both occurred during the 60-min 540-ppm treatment, and durations of 120 min and concentrations of or greater than 280 ppm.

After the 96-h post-treatment, rainbow trout fingerlings had the highest mortality at 30-min 540-ppm (43%), 60-min 280-ppm (30%), and at 120-min 170-ppm (43%). The rainbow trout fry groups also had elevated mortalities (45%) following the 120-min 170-ppm treatment. Cutthroat trout 96-h mortalities were 48% and 90% for the 120-min 170-ppm treatment for the fry and fingerlings, respectively. Fingerling mortalities were 30, 60, and 30%, 96 h after the 60-min treatment at concentrations of 280, 420, and 540 ppm, respectively. Fin-

TABLE 2. *Percent cumulative mortality (±SD, N = 3) of cutthroat trout fry and fingerlings at 1- and 24-h post-treatment (hpt). Fish were treated with varying concentrations of hydrogen peroxide for 30, 60, and 120 min at 15 C.*

Concentration (ppm)	Duration (min)	Cumulative mortality (1 hpt)		Cumulative mortality (96 hpt)	
		Fry	Fingerling	Fry	Fingerling
Control	30	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	60	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	120	0 ± 0	0 ± 0	0 ± 0	0 ± 0
70 ppm	30	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	60	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	120	2 ± 2	0 ± 0	3 ± 5	0 ± 0
170 ppm	30	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	60	0 ± 0	0 ± 0	0 ± 0	12 ± 8
	120	23 ± 14	0 ± 0	72 ± 8	90 ± 4
280 ppm	30	0 ± 0	0 ± 0	0 ± 0	5 ± 7
	60	3 ± 2	0 ± 0	7 ± 6	30 ± 15
	120	100 ± 0	83 ± 17	100 ± 0	100 ± 0
420 ppm	30	7 ± 9	0 ± 0	12 ± 10	22 ± 24
	60	75 ± 18	10 ± 8	78 ± 13	70 ± 16
	120	100 ± 0	98 ± 2	100 ± 0	100 ± 0
540 ppm	30	28 ± 17	2 ± 2	32 ± 21	32 ± 16
	60	100 ± 0	67 ± 26	100 ± 0	97 ± 2
	120	100 ± 0	100 ± 0	100 ± 0	100 ± 0

gerlings also had a 30% mortality in the 30-min 540-ppm treatment.

Cumulative mortality (sum of initial treatment and 96-h mortality) for the rainbow trout fry was elevated (≥27%) for 30-min treatments greater than 280 ppm, 60-min treatments greater than 170 ppm, and 120-min treatments greater than 70 ppm (Table 1). Average percent mortality calculated across all treatments for the rainbow trout fry was 37%. Rainbow trout fingerling cumulative mortality was elevated (≥38%) for 30-min treatments greater than 280 ppm, 60-min treatments greater than 170 ppm, and 120-min treatments greater than 70 ppm (Table 1). Average percent mortality calculated across all treatments for the fingerlings was 41%.

Cumulative mortality for the cutthroat trout fry was elevated (≥32%) for the 30-min 540-ppm treatments, 60-min treatments greater than 280 ppm, and 120-min treatments greater than 70 ppm (Table 2). Rainbow trout fingerling cumulative mortality was elevated (≥22%) for 30-min 420- and 540-ppm treatments, 60-min treatments greater than 170 ppm, and 120-min treatments greater than 70 ppm (Table 1). Average percent mortality calculated across all treatments for the fingerlings was 37%, compared to 34% for fry.

There were no significant differences between species and fish size, however LC_{50}s calculated for each of the three durations (Table 3) showed that for a treatment of 30-min duration, rainbow fry and cutthroat fingerlings had the lowest mean LC_{50} (514 ppm) followed by the rainbow fingerlings (574 ppm) and the cutthroat fry with the highest (636 ppm). For a 60-min treatment rainbow fry had the lowest LC_{50} (322 ppm), followed by rainbow fingerling (329 ppm), cutthroat fry (377 ppm), and cutthroat fingerling (506 ppm). Rainbow fingerling had the lowest 120-min LC_{50} (189 ppm), followed by cutthroat fingerling (197), rainbow fry (207 ppm), and cutthroat fry (280 ppm).

Water quality analysis revealed the addition of hydrogen peroxide had minimal impact at any of the five test dilutions on water hardness, 216 ± 9 mg/L; alkalinity,

TABLE 3. *Mean (N = 3) LC_{50}'s (ppm hydrogen peroxide) and 95% confidence intervals for rainbow and cutthroat trout fry and fingerlings.*

Treatment duration	Rainbow trout		Cutthroat trout	
	Fry	Fingerling	Fry	Fingerling
30 min	514 (482–584)	574 (487–675)	636 (535–757)	514 (452–584)
60 min	322 (266–389)	329 (288–376)	377 (342–416)	506 (463–554)
120 min	207 (173–248)	189 (159–225)	280 (185–424)	197 (157–247)

216 ± 9 mg/L; pH, 7.4 ± 0.1, or CO_2, 21 ± 2 mg/L. Assays of the actual concentrations for the 70, 170, 280, 420, and 540 ppm treatments were: 74 ± 10; 170 ± 12; 276 ± 15; 428 ± 24; and 543 ± 30 ppm respectively. Assays from the treatments which had been sitting for 24 h were 60, 150, 260, 400, and 480 ppm for their respective dilutions.

The analysis of mounted tissue samples showed no discernable changes in gill or pseudobranch histology in fish that survived to 96-h post-treatment. However several tissue samples taken from fish that had died during their initial exposure to the 540-ppm treatments exhibited tissue damage. One fish from each of the rainbow trout fry and cutthroat fingerlings that had died during the 540-ppm 30-min and 60-min treatments, showed gill tissue damage. The tissue damage was manifested by extensive epithelial separation of the primary lamellae and tissue edema. Cellular debris was also present in the space formed by the separated epithelial layer.

Discussion

Based on this work and others, there appears to be a safe zone, with respect to water temperature and treatment duration, within which hydrogen peroxide can be used. At 12 C, Rach et al. (1995) found no mortalities with 15-min treatments until concentrations were 1,000 ppm for juvenile rainbow trout. This concentration dropped to 500 ppm when treatment time increased to 45 min. For the two life stages of rainbow trout tested in this study at 15 C, cumulative mortalities were minimal (<8%) when the following combinations of concentration and time were applied: 280 ppm or less for 30 min; 170 ppm or less for 60 min; 70 ppm for 120 min. Cutthroat trout fry and fingerlings exhibited similar mortalities (<12%) under the same treatments. Comparing average cumulative mortalities, there were no significant differences between species and age classes, indicating no divergence in sensitivity to hydrogen peroxide with respect to age and species.

Rach et al. (1995) began to see an increasing sensitivity to hydrogen peroxide as fish grew from swim-up fry to fingerlings (7.71 g) and beyond. It is possible that a lack of differential mortalities related to age found in this study may be a result of a single treatment compared to four treatments in the Rach et al. (1995) study. Fingerling (7.71 g) from their study exhibited 20% mortality during a 45-min 500-ppm treatment compared to fingerling in this study (7.3 g) which had 35% mortality from a 30-min 540-ppm treatment. There were differences in experimental design and water temperature between the two studies, but the findings of this study and those of Rach et al. (1995) generally agree. In their study they also found clear differences between species in their sensitivity to hydrogen peroxide; brown trout were much more sensitive than lake trout. Johnson et al. (1993) found chinook salmon to be more sensitive than Atlantic salmon when the two species were treated with identical concentrations

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of hydrogen peroxide under the same temperature and treatment duration regimes. Rainbow and cutthroat trout appear to be alike in their sensitivity to hydrogen peroxide. Perhaps their genetic similarity contributes to this.

With the exception of the 540-ppm treatments that experienced mortalities during the exposure to hydrogen peroxide, there were no apparent changes in branchial tissue as a result of exposure. This result is similar to that of Bruno and Raynard (1994) who found no tissue damage in Atlantic salmon treated at concentrations up to 1,260 ppm at 8, 10 or 13 C. However, the tissue damage found during this study in the 540-ppm fish, which included epithelial separation, was comparable to that described by Johnson et al. (1993). They found epithelial seperation of the lamellae in those groups that had experienced high post-treatment mortalities.

Gill tissue damage was also similar to that described by McDonald and Wood (1993) where gills of trout exposed to metals exhibited epithelial separation, tissue edema, and clubbing of the lamellae. They also discussed the absence of cellular hyperplasia and hypertrophy, indicative of cellular repair, until 4 d after the initial treatment. This may explain why evidence of cellular repair from tissue samples from our test fish was absent. Because the majority of the fish we sampled were from fish that had survived the initial treatment and the 96-h post-treatment period, the chances of finding these indicators of toxic exposure were minimal.

Because the doses of hydrogen peroxide for treating diseased fish has yet to be determined, we are only able to suggest a safe operating range for the application of hydrogen peroxide. From the results of this study, we recommend treatment ranges of 70–280 ppm for 30 min or 70–170 ppm for 60 min. However, it must be noted that healthy fish were used throughout this study and the sensitivity of a sick fish to hydrogen peroxide may be increased. Before a large lot of fish are treated it would be advisable to treat a few fish to obtain information on the treatment's safety. It should be mentioned that differences in water temperatures and water chemistry may dramatically influence toxicity levels. Bruno and Raynard (1994) found that when fish were treated at similar concentration levels, no mortalities occurred in 10 C water, but at 13.5 C, 65% mortality occurred. With each given application it must be decided whether the cost of the benefits accorded by a treatment outweighs the cost of no action. It could be argued that a loss of even 8% of one's fish would be unacceptable, but faced with possibly larger losses if left untreated, the treatment losses may be more acceptable. It remains to be determined whether or not treatment concentrations within this range will be effective therapeutically.

Hydrogen peroxide has potential as a fungicide and therapeutic drug in fish culture; however, several areas of research must be explored in order to increase the base of knowledge necessary for its inclusion as a tool to the fish-culturist. Future work should be directed towards testing the toxicity of hydrogen peroxide to different life stages of many different species at various temperatures representative of hatcheries. Work also needs to be aimed at quantifying the efficacy of hydrogen peroxide to reduce or eliminate bacterial infections and parasites and to further determine the effects of multiple treatments on sick fish. The effects of other factors such as organic load and water quality differences upon treatment efficacy should also be of interest. As with all chemicals, care must be taken when handling hydrogen peroxide. Hydrogen peroxide can be an extremely corrosive compound in its concentrated form. Care must be taken in its application including the use of proper protective clothing and the proper preparation and storage of the chemical.

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