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[Article]

The Safety and Effectiveness of Various Hydrogen Peroxide and Iodine Treatment Regimens for Rainbow Trout Egg Disinfection

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Abstract.—Four tests were conducted to evaluate iodine and hydrogen peroxide for the disinfection of rainbow trout *Oncorhynchus mykiss* eggs at higher doses for shorter durations than previously studied. In the first test, eyed eggs were exposed to (1) 2,000 mg iodine/L for 10 min, (2) 100 mg iodine/L for 15 min, (3) 30 g hydrogen peroxide/L for 1 min, (4) 6 g hydrogen peroxide/L for 5 min, or (5) no treatment. Iodine (2,000 mg/L) or hydrogen peroxide (30 g/L) significantly reduced bacterial loads on eggs but did not significantly affect egg survival or fry deformity rates. Hydrogen peroxide at 30 g/L for 1 min was generally better for bacterial control than the other treatments, but the 2,000-mg/L iodine treatment also was effective. A second test assessed the effect of hydrogen peroxide on pH at various levels of water hardness. The pH of hydrogen peroxide solutions dropped as total hardness levels decreased, but buffering with at least 1.32 g $NaHCO_3$/L returned pH to approximately neutral levels. In the third test, in which eggs were treated 30 or 60 min postfertilization, there was no significant difference in survival between those treated with 15 g of buffered hydrogen peroxide/L for 2 min and that of the controls. However, at both 30 and 60 min postfertilization, the 2,000-mg/L iodine treatment induced higher levels of egg mortality than in eggs treated with hydrogen peroxide and the controls. In the fourth test, the serial combination of both 30 g hydrogen peroxide/L and 2,000 mg iodine/L was highly lethal if hydrogen peroxide was the first of the two treatments. The survival of eggs treated in the reverse order (iodine first) did not significantly differ from that of controls. These results indicate that hydrogen peroxide was effective in safely reducing the abundance of bacteria on eggs in small-scale tests when buffered, but production-scale experiments with hydrogen peroxide are recommended before implementation of this treatment.

Fungal and bacterial agents have been implicated in reduced survival of fish eggs (Gee and Sarles 1942; Burrows 1949; Ross and Smith 1972; Barker et al. 1989; Barnes et al. 2003). Egg disinfection plays a critical role in improving the survival to hatch for many fish species reared in captivity. Despite screening of numerous candidate chemicals (Bailey and Jeffrey 1989; Marking et al. 1994; Schrader 2008), the number of compounds known to control these pathogens is limited. Based on the work of McFadden (1969) and Amend (1974), iodine at 100 mg/L for 10–15 min has been established as the standard salmonid egg treatment.

Recent data have suggested that an alternative to iodine is necessary for adequate disinfection of rainbow trout *Oncorhynchus mykiss* eggs (Kumagai et al. 1998; Shaw et al. 1999; Wagner et al. 2008; Barnes et al. 2009). For example, Kumagai et al. (1998) found iodophor treatment failed to control the bacterial fish pathogen *Flavobacterium psychrophilum*. Shaw et al. (1999) noted the failure of treatments using up to 200 mg/L iodine to control the microsporidian *Loma salmonae*. Barnes et al. (2009) noted that rainbow trout eggs treated with 100 mg/L iodine still had an average of 11,120 colony-forming units per egg. In Utah, increased mortality in hatchery stocks due to *Flavobacterium psychrophilum* has also prompted renewed interest in egg disinfection as part of a broader fish health management plan. Thus, better disinfection methods are needed.

Some research has indicated that hydrogen peroxide may be a viable egg disinfectant (Dawson et al. 1994; Barnes et al. 1998; Rach et al. 1998) at concentrations of 0.5–30 g/L for 15–60 min. The toxicity of hydrogen peroxide to eggs varies among species (Rach et al. 1998: Gaikowski et al. 1999), but can be reduced by avoiding prophylactic treatment during critical developmental stages (Gaikowski et al. 1998; Arndt et al. 2001). These studies observed control of fungal infection, but did not explore the use of hydrogen peroxide as a bacteriological control agent. Douillet and Holt (1994) examined bacterial growth on red drum *Sciaenops ocellatus* eggs, noting treatment with 30 g/L hydrogen peroxide for 5 min led to bacteria-free larvae. Peck et al. (2004) observed a reduction in bacteria on eggs from Atlantic cod *Gadus morhua* or

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haddock *Melanogrammus aeglefinus* that were treated with 30 g/L hydrogen peroxide for 5 min. Wagner et al. (2008) assessed 2 g/L of hydrogen peroxide for control of bacteria on the surface of rainbow trout eggs. Those investigators found that 100 or 500 mg/L iodine was superior to 2 g/L hydrogen peroxide in 15-min treatments, but bacteria still survived treatment with either chemical. Therefore, higher concentrations of hydrogen peroxide needed to be assessed.

Higher iodine concentrations for disinfection may be possible. With iodine, toxicity is dependent on pH and stage of egg development (Amend 1974). Therefore, the 2,000-mg/L iodine dose could be lethal to rainbow trout eggs at pH 6.9 (dose lethal to 50% of the egg specimens [LC50] = 1,480 mg/L in a 15-min treatment; Amend 1974), but safe at higher pH. Alderman (1984) found in tests with Atlantic salmon *Salmo salar* eggs that the LC50 for iodine was 800 mg/L at pH 6.0, but in excess of 3,000 mg/L at pH 7.0.

The objective of our egg disinfection research was to explore the potential for killing all external bacteria with higher concentrations of iodine and hydrogen peroxide applied for shorter durations, without killing eggs in the process. Four tests were conducted, the first of which examined the effect of higher than previously tested doses of iodine and hydrogen peroxide for shorter durations on rainbow trout eyed eggs. A second test evaluated the effect of hydrogen peroxide on pH at various water hardness levels. Another test evaluated the effects of these chemicals on eggs treated shortly after fertilization. The fourth test evaluated the effect of treating eggs serially with both iodine and hydrogen peroxide.

Methods

Test 1: short-duration tests on eyed eggs.—Rainbow trout eggs of the Ten-Sleep strain were treated with iodine or hydrogen peroxide just before hatch. Treatments were (1) 100 mg iodine/L for 15 min (control), (2) 2,000 mg iodine/L for 10 min, (3) 6 g hydrogen peroxide/L for 5 min, (4) 30 g hydrogen peroxide/L for 1 min, and (5) untreated eggs (no chemical treatment, but similarly handled). The iodine used was from a povidone-iodine complex in Argentyne (Argent Laboratories, Seattle, Washington) and a 1% concentration of active iodine was assumed from the label. Hydrogen peroxide was formulated from a stock concentration of 34% hydrogen peroxide purchased from a commercial source (Dyce Chemical, Salt Lake City, Utah). A commercial test kit (Hach Chemical Co., Loveland, Colorado) was used to verify the hydrogen peroxide concentration. Three replicate groups of eggs were disinfected for each treatment in 8.0-L containers of disinfectant solution. Each replicate had 100 mL of eggs or about 910 eggs. The estimate of number of eggs per milliliter was based on the average of three replicate samples of eggs on a Von Bayer trough (Piper et al. 1982). The eggs were observed after disinfection and percent hatch was derived from the number of fry obtained divided by the initial number of eggs, times 100. Owing to space limitations, only one replicate of untreated eggs was evaluated.

In addition, to evaluate the effectiveness of each treatment for the removal of bacteria, eggs not previously disinfected were transferred to a sterile glass beaker and subjected to the same chemical treatments. Three replicate groups of 10 eggs each were exposed to each disinfection procedure. After the duration of chemical exposure, the eggs were drained and rinsed with sterile hatchery water. The excess rinse water was poured off and each egg was transferred with sterile forceps to a test tube with 2 mL of sterile peptone salt diluent solution (Barnes et al. 2005). The tube was capped with laboratory film and agitated with a vortex mixer for 2 min. For treatments exposed to chemicals, no dilutions of this diluent solution were made. However, owing to the higher expected numbers of bacteria among untreated eggs (based on previous experiments), dilutions of 10^2, 10^4, and 10^6 in sterile phosphate-buffered saline (PBS) were used for plating. From either the undiluted solution or diluted solutions (untreated eggs), 100 μL were transferred to a trypticase soy agar (TSA) petri dish and another 100 μL were transferred to a petri dish containing enhanced Ordahl's agar with the antibiotic tobramycin (EOT, 50 μL of 100-mg/mL stock solution per liter of media; Kumagai et al. 2004). A sterile spreader and spinning-plate table were used to distribute the shaken solution onto the plate. The plate was wrapped in laboratory film and incubated at 15°C for up to 12 d. Non-inoculated plates were used as media controls.

Counts of colony-forming units (CFUs) on both media (TSA and EOT) were made 2, 4, 7, and 12 d after inoculation. If plates had too many CFUs to count accurately, the plate was labeled as too numerous to count. Occasionally plates with high CFU numbers (>about 500 CFUs) were subdivided into halves or quarters to reduce the number to count. The counts were subsequently multiplied by two or four, respectively, to get an estimate of CFUs for the entire plate. To estimate the total number of bacteria on each treated egg, the CFU plate counts were multiplied by 20 (1/20th of the peptone diluent solution was plated). These data were used for the statistical analyses.

Test 2: effect of hydrogen peroxide on pH.—After completion of the first test, concerns arose regarding the pH of hydrogen peroxide disinfection solutions. To prevent adverse effects associated with low pH, we

determined the appropriate amount of buffer needed for hydrogen peroxide solutions. Commercial baking soda ($NaHCO_3$) was dissolved in water of hardness levels ranging from 0 to 240 mg/L to create concentrations of 0.00, 0.26, 0.53, 0.79, 1.06, 1.32, and 2.64 g/L of $NaHCO_3$. Hydrogen peroxide was then added to each buffered solution to achieve a 30-g/L final concentration. The pH was measured in each buffered solution before and after hydrogen peroxide addition. The solution was considered adequately buffered when, across all water hardness levels tested, pH did not drop below 7.0 after hydrogen peroxide addition.

Once an appropriate buffer concentration was determined, another series of measurements were made using hatchery well water diluted with de-ionized water. This created seven dilutions with total hardness levels ranging from 0 (pure de-ionized water) to 240 mg/L as $CaCO_3$ (100% hatchery well water). For each dilution, total alkalinity and total hardness levels were measured before and after the addition of $NaHCO_3$ buffer (1.32 g/L) using a commercial test kit (Hach Chemical Co.). Based on the previous test, 1.32 g/L of $NaHCO_3$ was added to each dilution. The pH of the dilution mixtures, as well as the pH of the water before the addition of hydrogen peroxide (15 g/L after addition) or buffer, was measured with a digital pH meter calibrated with two bracketing pH buffers. The pH was also measured for 100-mg/L free-iodine solutions made with Argentyne using the seven total hardness levels.

Test 3: short-duration chemical tests during water hardening.—To determine whether the higher doses of chemicals were potentially harmful to freshly fertilized eggs we evaluated three treatments at either 30 or 60 min after fertilization: (1) 100 mg iodine/L for 15 min (control), (2) 2,000 mg iodine/L for 10 min, or (3) 15 g hydrogen peroxide/L for 2 min. The hydrogen peroxide was buffered with 1.32 g/L of $NaHCO_3$ to control pH. Rainbow trout eggs from three lots of 2-year-old females of the German Rainbow–Harrison Lake strain were treated on 12 November 2008 using a different lot for each of the three replicates. The eggs were exposed in 1-L containers at the Utah Division of Wildlife Resources Egan State Fish Hatchery, Bicknell, Utah. A fresh batch of the 1-L solution was made for each replicate. A net containing 90 mL of eggs was moved in the solution a few times to ensure that all eggs were exposed to the disinfectant. The eggs were subsequently rinsed in freshwater and transferred to a mesh bag for transport (6 h) in a cooler of well water to the Utah Division of Wildlife Resources Fisheries Experiment Station in Logan. Upon arrival, the eggs were treated with 100 mg/L iodine (Argentyne) for 15 min. Each bag of eggs (replicate) was transferred to an individual tray in one of three egg incubation stacks (Heath Techna Corp., Kent, Washington) that received flow-through water at 15 L/min. Tray location was randomized among treatments. During the next 15 d, a drip system delivered 1,667 mg/L formalin to the trays once a day for 15 min. After the eyed egg stage was reached, the trays were periodically examined. Counts of dead eggs, dead fry, and deformed fry were kept for each tray. Live fry surviving to 8 d after hatching were also enumerated to get exact counts on the number of eggs at the start. Percent hatch and deformity were calculated as noted previously.

Test 4: combinations of iodine and hydrogen peroxide.—Triploid Sand Creek–Erwin strain rainbow trout eggs at the eyed stage of development were treated with combinations of iodine and hydrogen peroxide or with each chemical individually. Treatments were (1) 2,000 mg iodine/L for 10 min, then 30 g buffered hydrogen peroxide/L for 1 min, (2) 30 g hydrogen peroxide/L for 1 min, then 2,000 mg iodine/L for 10 min, (3) 30 g hydrogen peroxide/L for 1 min, (4) 2,000 mg iodine/L for 10 min, (5) 15 g hydrogen peroxide/L for 2 min, and (6) untreated eggs (no chemical treatment, but similarly handled). The 12-L chemical solutions were prepared in plastic pails. For each of the hydrogen peroxide treatments, the solutions were buffered by the addition of 1.32 g/L of baking soda ($NaHCO_3$). Eggs were treated by dipping a net with 90 mL of eggs in the solution. For the combination treatments, the eggs were rinsed between the two chemical exposures by dipping the net into a bucket of clean hatchery well water.

Eggs for the trial were taken from a larger lot drawn randomly from different trays within an egg incubation tray stack. The eggs had been transported the day before the test and treated with 100 mg/L iodine for 15 min upon arrival. Given the low volume of eggs, the same solution was used for each of three replicates, but new solutions were prepared for each treatment. After the duration of chemical exposure, the eggs were rinsed with sterile hatchery water, 10 eggs were transferred to sterile beakers, and the remaining eggs were transferred to one tray of an egg incubation tray stack. Treatments and replicates were randomly assigned to trays among the four eight-tray stacks used. Dead eggs were enumerated and removed every 2–3 d. Hatching occurred 6 d after the chemical treatments. Percent hatch was derived from the number of fry obtained divided by the initial number of eggs, times 100. On 2 October 2008, 8 d after hatching, the deformed fry were removed and all surviving fry were hand-counted. The percentage of crippled fry was expressed as the number of deformed fry removed divided by the number of fry that hatched, times 100. Bacteriology

TABLE 1.—Mean ± SD ($N = 3$) percent survival to hatch and percentage of fry deformities by chemical treatment and durations. The data in test 1 are from the initial tests on eyed eggs. The data in test 3 pertain to experiments performed during water hardening. The data in test 4 are from experiments evaluating combined iodine and hydrogen peroxide treatments. In the time column, 10 and 1 represent 10 min in the iodine solution followed by 1 minute in hydrogen peroxide; similarly, 1 and 10 represent 1 minute in hydrogen peroxide followed by 10 min in iodine. Within a column and test, means followed by a common letter are not significantly different ($P > 0.05$); the untreated egg treatment had only one replicate (test 1), so these data were not included in the statistical analysis.

Test	Treatment	Egg stage[a]	Duration (min)	Hatch rate (%)	Deformity rate (%)
1	6 g/L hydrogen peroxide	Eyed	5	95.7 ± 0.8 z	0.15 ± 0.1 z
	30 g/L hydrogen peroxide		1	95.5 ± 0.8 z	0.50 ± 0.4 z
	2,000 mg/L iodine		10	94.0 ± 0.7 y	0.47 ± 0.0 z
	100 mg/L iodine (control)		15	93.7 ± 0.7 y	0.35 ± 0.3 z
3	100 mg/L iodine	30 min PF	15	91.8 ± 3.2 z	0.20 ± 0.1 z
	2,000 mg/L iodine		10	40.1 ± 18.3 y	2.76 ± 2.2 z
	15 g/L hydrogen peroxide		2	87.6 ± 5.7 z	1.28 ± 0.6 z
	100 mg/L iodine	60 min PF	15	92.4 ± 2.0 z	0.41 ± 0.4 z
	2,000 mg/L iodine		10	84.4 ± 2.9 y	1.63 ± 0.1 y
	15 g/L hydrogen peroxide		2	88.3 ± 3.1 z	1.20 ± 0.4 y
4	2,000 mg/L iodine	Eyed	10	59.6 ± 1.2 yz	7.9 ± 1.0 z
	30 g/L hydrogen peroxide		1	57.6 ± 4.9 y	8.7 ± 2.3 z
	2,000 mg/L iodine to 30 g/L hydrogen peroxide		10 and 1	61.8 ± 0.9 z	7.9 ± 0.8 z
	30 g/L hydrogen peroxide to 2,000 mg/L iodine		1 and 10	0.2 ± 0.4 x	20.0 ± 34.6 z
	15 g/L hydrogen peroxide		2	60.9 ± 2.2 yz	8.9 ± 0.8 z
	Untreated control			62.3 ± 0.8 z	7.8 ± 0.5 z

[a] PF = postfertilization.

was performed on the eggs transferred to the sterile beakers using the same methods as in test 1. Counts of CFUs were made on both TSA and EOT media for 10 eggs per replicate. However, owing to low numbers of CFUs in all treatments, including untreated controls, these data are not shown.

Statistical analysis.—All statistical analyses were performed with SPSS version 13. A probability value of 0.05 was used for all tests. One-way analysis of variance (ANOVA) was used for comparing egg hatch and deformity percentages among treatments after arcsine transformation. For comparison of CFU counts among treatments, total CFUs-per-egg values were classified into ordinal categories (0, 1–300, 301–1,000, or >1,000 CFUs) for analysis. Plates with bacteria too numerous to count were placed into the >1,000 CFU category. For test 1, we chose the data for the 10^4 dilution for EOT and 10^2 dilution for TSA plates for statistical analysis since these dilutions provided the most accurate estimates of CFU numbers (i.e., did not have too few or too many CFUs). The data were analyzed with a chi-square test with treatment and CFU category as factors. Separate tests were conducted for each media (TSA or EOT). If overall differences were significant among treatment means, partial tables were constructed to determine significant differences among pairs of treatments or other subsets. Likelihood ratio probabilities were used to determine significance of the tests. For the test with freshly fertilized eggs, time (30 or 60 min) and chemical treatment were used as fixed factors in a saturated general linear model. A significant interaction term led to separate tests for each time period and subsequent mean comparisons among treatments using the Least Significant Difference test.

Results

Test 1: Short-Duration Tests on Eyed Eggs

Survival rates were slightly, but significantly, lower for eggs treated with iodine ($P = 0.02$; test 1 in Table 1). However, hatching rates were 93–96% for all treatments. Deformity rates were less than 1% for all treatments and did not significantly differ among treatments ($P = 0.40$). The survival and deformity data indicated that there were no deleterious effects of the higher chemical concentrations used in the study.

The CFU count data (Table 2) indicated that chemical treatment with either iodine or hydrogen peroxide significantly reduced bacterial abundance on the rainbow trout eggs relative to untreated eggs for both media ($P < 0.01$), but some bacteria still persisted on the eggs. For TSA data, eggs treated with 30 g/L hydrogen peroxide had significantly fewer bacteria than did eggs treated with either 100 mg/L iodine or 6 g/L hydrogen peroxide ($P < 0.01$), but did not significantly differ from eggs treated with 2,000 mg/L iodine ($P = 0.40$). Eggs treated with 2,000 mg/L iodine did not significantly differ in CFU counts from that for controls treated with 100 mg/L iodine ($P = 0.21$). For EOT data, eggs treated with 30 g/L

TABLE 2.—Distribution of extrapolated colony-forming unit (CFU) counts on two different media (trypticase soy agar and Ordahl's agar with tobramycin) for eyed rainbow trout eggs ($N = 30$) treated with different concentrations of iodine (I) and hydrogen peroxide (H) for different durations. Significant differences among treatments within a given medium are noted in the last column by different letters.

Chemical dose (mg/L)	Duration (min)	CFUs				Maximum likelihood result
		0	1–300	300–1,000	>1,000	
Ordahl's agar with tobramycin						
I 100	15	3	4	8	15	y
I 2,000	10	4	10	3	13	y
H 6,000	5	0	8	7	15	y
H 30,000	1	6	18	6	0	x
Untreated		1	0	0	29	z
Trypticase soy agar						
I 100	15	5	15	6	4	y
I 2,000	10	10	22	3	2	yx
H 6,000	5	0	24	4	5	w
H 30,000	1	12	16	2	0	x
Untreated		1	0	0	29	z

hydrogen peroxide had significantly fewer bacteria than did eggs exposed to any of the other chemical treatments, which did not significantly differ from each other (Table 2).

Test 2: Effect of Hydrogen Peroxide on pH

Our initial tests determined the sodium bicarbonate concentration required to adequately buffer (i.e., pH $\geq$ 7.0) a 30-g/L hydrogen peroxide solution. In this test, $NaHCO_3$ buffer concentrations of 0.26, 0.53, 0.73, 1.06, 1.32, and 2.64 g/L resulted in pH values of 6.6, 6.8, 7.0, 7.1, 7.1, and 7.4 respectively, after addition of hydrogen peroxide. The pH of some of the solutions dropped below 7.0 after hydrogen peroxide addition when the $NaHCO_3$ concentration was less than 1.06 g/L. However, $NaHCO_3$ concentrations of 1.32 g/L or greater sufficiently maintained a pH above 7.0 across all hardness levels. There was little difference in pH between the 1.32- and 2.64-g/L buffer treatments after adding hydrogen peroxide, so 1.32 g/L $NaHCO_3$ was chosen as the appropriate buffer concentration in the subsequent test. As expected, the addition of buffer had no effect on water hardness.

Addition of hydrogen peroxide (15 g/L final concentration) to water of various hardness levels dropped the pH. The amount of change in pH was inversely proportional to the total hardness. At 240 mg/L total hardness, the pH drop was only 0.36 units, resulting in a pH of 7.01 (Table 3). However, at a hardness of 10 mg/L, the resulting pH decrease was 1.84 units (pH 4.5). Addition of $NaHCO_3$ restored the pH to levels that were approximately neutral. Iodine solutions held near-neutral pH values until total hardness dropped to 10 mg/L (pH 6.2) or below (Table 3).

Test 3: Short-Duration Chemical Tests during Water Hardening

The general linear model indicated that there were significant differences in survival to hatch. Both time (30 or 60 min postfertilization; $P < 0.01$) and treatment ($P < 0.01$) were significant, as was their interaction term ($P = 0.01$). Survival was less at 30 min than at 60 min after fertilization, especially for the 2,000-mg/L

TABLE 3.—Effect of hydrogen peroxide (H_2O_2 [15 g/L]) and povidone–iodine (100 mg of free iodine/L) on pH at various levels of total hardness and total alkalinity (mg/L), with and without buffering with baking soda (1.32 g/L).

Total hardness before buffering	Total alkalinity before buffering	Total hardness after buffering	Total alkalinity after buffering	pH without H_2O_2; no buffer	pH without H_2O_2; buffer	pH with H_2O_2; buffer	pH with H_2O_2; no buffer	pH of 100 mg/L iodine
0	<17.1	<17.1	633	4.9	8.2	7.5	3.8	5.0
10	<17.1	<17.1	616	6.0	8.2	7.6	4.5	6.2
86	68.4	68.4	616	6.9	8.2	7.7	6.5	7.1
120	68.4	102.6	684	7.2	8.1	7.6	6.7	7.4
154	102.6	153.9	701	7.2	8.1	7.7	6.9	7.5
188	119.7	188.1	718	7.4	8.1	7.6	7.0	7.6
240	153.9	222.3	718	7.4	8.0	7.4	7.0	7.6

iodine treatment (40.1 ± 18.3% [mean ± SD] versus 84.4 ± 2.9%, respectively). If treatments were compared separately for each time, eggs treated with 2,000 mg/L iodine had significantly lower survival than did controls or the hydrogen peroxide treatment in both time periods ($P \leq 0.03$; test 3 in Table 1). Survival to hatch for eggs exposed to 15 g/L hydrogen peroxide for 2 min did not differ significantly from controls ($P = 0.10$).

Deformity rate averages ranged from 0.20% to 2.76% among the chemical treatments (test 3 in Table 1). The percentage of deformities did not differ significantly between times (two-way ANOVA: $P = 0.47$; 30 min: 4.1 ± 6.8%; 60 min: 1.6 ± 1.0% [means pooled across chemical treatments ± SD]) and the interaction term was not significant ($P = 0.46$). However, significant differences were observed among chemical treatments ($P = 0.02$). If treatments were compared separately for each time, no significant difference was noted among the chemical treatments at 30 min after fertilization ($P = 0.13$), but at 60 min after fertilization, eggs exposed to 100 mg/L iodine had significantly lower deformity rates than did eggs exposed to the other two treatments ($P \leq 0.02$), which did not significantly differ from each other ($P = 0.15$).

Test 4: Combinations of Iodine and Hydrogen Peroxide

There were significant differences in survival of the eggs among the various chemical treatments ($P < 0.01$; test 4 in Table 1). The combination of 30 g/L hydrogen peroxide for 1 min followed by 2,000 mg/L iodine proved to be highly lethal (0.2% hatch), with only a few eggs surviving the first 48 h after treatment. In contrast, if the order of the combination treatment was reversed, the hatching success (61.8%) was not statistically different from the untreated controls (62.3%; $P = 0.78$). If eggs were exposed to 2,000 mg/L iodine alone, survival was also not significantly different from controls ($P = 0.17$). Survival to hatch was slightly, but significantly ($P = 0.03$), lower in the 30-g/L hydrogen peroxide treatment (57.6%) than in the controls. However, the percent hatch (60.9%) for eggs exposed to 15 g/L hydrogen peroxide for 2 min did not significantly differ from controls ($P = 0.47$). The percentage of deformed fry ranged from 7.8% to 20.0% and did not differ significantly among treatments ($P = 0.85$). The 20% deformity rate was based on the five fry surviving in a single replicate of the hydrogen peroxide-to-iodine treatment. No fry survived in the other replicates of that treatment.

Discussion

Bacteria perform many valuable ecological functions such as providing food for larger organisms, aiding animals in digestion, fixing nitrogen, denitrification, and decomposition (Jordan and Burrows 1945). Bacteria can also be significant pathogens of a wide variety of organisms, including fish (Austin and Austin 1987). In fish culture, sanitation is a key component of fish health management and egg disinfection is a regular practice. While recognizing that many bacteria can be beneficial, we are also interested in preventing the transfer of pathogenic bacteria on fish eggs. Since it is not known a priori whether the eggs harbor pathogens, we must assume that the eggs are contaminated and have developed protocols that lead to complete disinfection. Egg treatment with 100 mg/L iodine for 10–15 min, which is the present practice in Utah and other states, significantly reduces bacterial abundance, but does not completely kill external bacteria on rainbow trout eggs (Wagner et al. 2008; Barnes et al. 2009). Increasing the iodine dose to 500 mg/L, or using 2 g/L hydrogen peroxide, 1,667 mg/L formalin, or 3% rock salt solutions, also failed to completely disinfect the eggs (Wagner et al. 2008). Thus, better disinfection protocols were still needed.

Further tests in this study evaluated higher doses of both iodine and hydrogen peroxide than have been previously tested. With iodine, toxicity varies with pH, stage of egg development, and species (Amend 1974; Alderman 1984). In some species such as the Mozambique tilapia *Oreochromis mossambicus* (Subasinghe and Sommerville 1985) or largemouth bass *Micropterus salmoides* (Wright and Snow 1975), mortality can be severe at even 200 mg/L. Alderman (1984) found that Atlantic salmon could tolerate 3,000 mg/L iodine at pH 7.0, but lower pH values increased iodine toxicity. For rainbow trout in this study, 2,000 mg/L iodine for 10 min was toxic to eggs that were water hardening, but when used for eyed eggs, the survival was comparable to controls. Other authors have previously documented the toxicity of iodine concentrations of greater than 75 mg/L during water hardening (Amend 1974; Fowler and Banks 1991). However, Pravecek and Barnes (2003) reported that iodine concentrations of up to 125 mg/L during water hardening of westslope cutthroat trout *O. clarkii lewisii* eggs did not compromise survival.

The hydrogen peroxide dose used in this experiment was the same as that used by Douillet and Holt (1994) and Peck et al. (2004), though for a shorter duration. In the research by Douillet and Holt (1994), complete disinfection was achieved, whereas Peck et al. (2004) still observed some bacterial growth after the 5-min

treatment of Atlantic cod eggs. Douillet and Holt (1994) also noted differences in susceptibility to hydrogen peroxide treatment among species, and eggs from yellowtail snapper *Ocyurus chrysurus* and spotted seatrout *Cynoscion nebulosus* experienced higher mortality when hydrogen peroxide levels exceeded 1% and 2%, respectively. For rainbow trout eggs, Gaikowski et al. (1998) noted that in daily 15-min treatments, concentrations of 1.0 or 3.0 g/L hydrogen peroxide increased mortality. For a variety of warm-water species Rach et al. (1998) noted that 1.0 g/L hydrogen peroxide daily for 5 min provided the best survival, whereas 3.0 or 6.0 g/L reduced hatching success. These summarized results indicate that increasing the duration of hydrogen peroxide treatment will probably result in poorer egg survival, but higher concentrations at shorter durations needed further evaluation.

In this study, shorter durations of hydrogen peroxide exposure did improve hatching success. Eggs treated with 15 g/L hydrogen peroxide for 2 min that was buffered with sodium bicarbonate had hatching rates that did not significantly differ from controls. Eyed eggs treated with 30 g/L hydrogen peroxide for 1 min had similar survival. However, eggs treated with the same dose during or just after water hardening had slightly higher mortality than controls. We discovered in some field applications of 30 g/L hydrogen peroxide that low pH may have contributed to poor egg survival. The pH tests indicated that buffering is necessary when using hydrogen peroxide. To date, hydrogen peroxide has only been tested in small-scale experiments and it is not known whether it can be safely used at a production scale. We anticipate testing the effectiveness of hydrogen peroxide disinfection on larger egg groups. Egg quality or genetic differences among family groups, the amount of organic material, such as dead eggs, broken chorion shells, bacterial growth on the eggs or in the ovarian fluid, could all influence the effectiveness of hydrogen peroxide treatment at a production scale. Verner-Jeffreys et al. (2007) noted high variability among egg batches of Pacific threadfin *Polydactylus sexfilis* and almaco jack *Seriola rivoliana* exposed to hydrogen peroxide. Temperature is another variable to consider; increases in temperature have been observed to increase toxicity of hydrogen peroxide to channel catfish *Ictalurus punctatus* eggs (Small 2004).

Control of bacterial growth was slightly better with 30 g/L hydrogen peroxide for 1 min than 2,000 mg/L iodine for 10 min, but both failed to completely disinfect egg surfaces. Similarly, Presterl et al. (2007) found that 30–50 g/L hydrogen peroxide was more effective at killing *Staphylococcus epidermidis* than 10% povidone-iodine. Results with 30 g/L hydrogen peroxide were comparable (80% of eggs with <300 CFUs) to using 2 g/L (80%), based on growth on EOT media (Wagner et al. 2008). Similar conclusions can be drawn from the growth results on TSA (90–100% of eggs had <300 CFUs at both concentrations). However, 20% of eggs treated with 30 g/L hydrogen peroxide had no bacterial growth, which was an improvement (0–5% at 2 g/L hydrogen peroxide). Iodine concentrations of 2,000 mg/L also significantly reduced bacterial abundance, though not to the same degree on EOT (13% had no bacterial growth, but 43% had over 1,000 CFUs per egg, compared with 0% for eggs treated with hydrogen peroxide). On TSA media, 33% of eggs treated with 2,000 mg/L iodine had no growth compared with 40% for eggs treated with hydrogen peroxide. Verner-Jeffreys et al. (2007) found that bacterial control was dependent on bacterial abundance; when heavily colonized, hydrogen peroxide concentrations of at least 1.134% for 5 min or iodine concentrations greater than 500 mg/L for 10 min were required for effective sterilization. In batches with fewer bacteria, 550 mg/L (0.055%) hydrogen peroxide resulted in less than 1 CFU per egg.

Combinations of treatments may provide more complete disinfection. Results with eggs treated with both iodine and hydrogen peroxide indicated that good survival is possible if iodine is applied first. Further work is needed on the bacteriology of combined treatments. Attempts to do this in the present study were made, but there was poor bacterial growth in all treatments, including untreated controls. Possibly the 100-mg/L iodine treatment the day before or laboratory temperatures during the plating process had some effect on bacterial growth. We are not aware of any literature on serial disinfection treatments in aquaculture. In food science research, although not in a serial treatment, DeQueiroz (2004) noted that a mixture of hydrogen peroxide and sodium hypochlorite provided synergistic benefits for antimicrobial activity against human pathogens. Similarly, a commercially available alcohol mixture comprised of propanol, ethanol, and chlorhexidine was an effective disinfectant against *Staphylococcus epidermidis* (Presterl et al. 2007).

Further work is needed to develop protocols that lead to 100% egg disinfection. Some chemicals that have shown promise in other studies, such as n-propanol, Biotensid, glutaraldehyde, tannic acid, and alcohol ethoxylate, need further evaluation for the disinfection of eggs (Pavlov and Moksness 1993; Presterl et a. 2007; Schrader 2008). Until these options are evaluated, this study demonstrated that higher doses of hydrogen peroxide or iodine for shorter durations significantly reduced bacterial abundance

relative to the current practice of 100 mg/L iodine for 10–15 min. Tests with 15 g/L (15,000 mg/L) hydrogen peroxide for 2 min indicated it is safe for both eyed eggs and just after water hardening, if it is properly buffered and eggs are well rinsed immediately afterwards. Regardless, more production scale testing is necessary before we formally recommend the use of hydrogen peroxide as a standard disinfectant for rainbow trout eggs. We recommend trying this protocol with other species and documenting bactericidal effects.

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