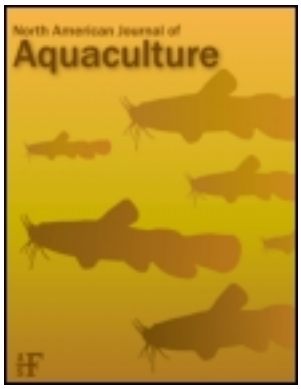


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ARTICLE

# Laboratory and Production Scale Disinfection of Salmonid Eggs with Hydrogen Peroxide

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***Abstract***

**Disinfection tests were conducted on eggs from rainbow trout *Oncorhynchus mykiss*, cutthroat trout *O. clarkii*, and brown trout *Salmo trutta* to evaluate hydrogen peroxide ($H_2O_2$) as an egg disinfectant. A daily drip of 500 mg/L hydrogen peroxide for 35 min on eyed brown trout eggs safely led to significantly reduced bacterial abundance relative to untreated controls, but abundance did not differ significantly from that in a formalin treatment (2,000 mg/L for 15 min). Using water-hardened cutthroat trout eggs, hydrogen peroxide concentrations of (1) 10 g/L for 2 min, (2) 10 g/L for 3 min, (3) 15 g/L for 2 min, and (4) 1,000 mg/L for 15 min were compared with two controls (untreated; 100 mg/L iodine). Bacteria were significantly more abundant in the untreated eggs, but abundance did not significantly differ among chemical treatments. In another test, rainbow trout eggs treated the day before hatch with 15 g/L $H_2O_2$ for 2 min had significantly higher mortality than the controls. In production-scale tests, 10–15 g/L $H_2O_2$ for 2 min was safe for rainbow and cutthroat trout eggs and significantly reduced bacterial abundance relative to that in untreated eggs. Complete disinfection was not achievable with either iodine or hydrogen peroxide. Daily drip treatment with either formalin or hydrogen peroxide significantly reduced bacterial growth and is recommended.**

Fungal and bacterial growth on fish eggs can seriously compromise egg survival during incubation (Ross and Smith 1972; Barnes et al. 2003), owing in part to egg membrane degradation (Pavlov and Moksness 1993; Morrison et al. 1999; Barnes et al. 2009). Since the use of malachite green was discontinued because of human health concerns, formalin and iodine have been used routinely in the last few decades to control fungal and bacterial growth on fish eggs (Amend 1974; Barnes et al. 1997; Barnes et al. 1998). However, current problems with vertically transmitted bacteria such as *Flavobacterium psychrophilum* (the causative agent of bacterial coldwater disease, also known as rainbow trout fry syndrome) and with microsporidiosis (e.g., *Loma salmonae*) indicate that current disinfection protocols need revision (Kumagai et al. 1998; Shaw et al. 1999; Wagner et al. 2008; Barnes et al. 2009). Furthermore, Presterl et al. (2007) observed that bacterial biofilms treated with stock concentrations of povidone iodine (1% active iodine) were still viable after a 30 min exposure.

There has been some research evaluating alternatives to the traditional iodine- and formalin-based disinfection (Bailey and Jeffrey 1989; Schrader 2008; Copur et al. 2010). Hydrogen peroxide ($H_2O_2$) has emerged as an alternative that is both effective and environmentally benign, breaking down into hydrogen, oxygen, and water (Marking et al. 1994). Hydrogen peroxide has controlled fungus when applied in daily prophylactic treatments (Gaikowski et al. 1998; Arndt et al. 2001). Treatments of 0.5% to 1.0% for 15–60 min controlled fungal growth in the eggs of rainbow trout *Oncorhynchus mykiss* (Schreier et al. 1996; Barnes et al. 1998).

The bacteriological effects of treating eggs with hydrogen peroxide are less studied. Recent data, however, indicate that 30 g/L $H_2O_2$ for 5 min controls or significantly reduces bacterial growth on the eggs of red drum *Sciaenops ocellatus* (Douillet and Holt 1994), Atlantic cod *Gadus morhua* (Peck et al. 2004), and haddock *Melanogrammus aeglefinus* (Peck et al. 2004). Rainbow trout eggs treated with 0.5–2.0 g/L $H_2O_2$ for 15 min

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had significantly reduced bacterial abundance, but a 100 mg/L iodine treatment provided better control of bacteria (Wagner et al. 2008). Wagner et al. (2010) found that 30 g/L $H_2O_2$ for 1 min or 15 g/L for 2 min significantly reduced bacterial loads on eggs without compromising rainbow trout egg survival. Effects of these concentrations on cutthroat trout *Oncorhynchus clarkii* or brown trout *Salmo trutta* have not been tested. Production-scale tests are also needed.

Research on formalin has demonstrated that it has improved survival of eggs and controls fungal growth (Watanabe 1940; Barnes et al. 1997, 2000; Rach et al. 2005; Small and Chatakondi 2006), but the bactericidal ability of formalin has not been studied adequately. Wright and Snow (1975) observed that formalin concentrations of up to 2,000 mg/L for 15 min were insufficient to kill *Aeromonas liquefaciens.* Wagner et al. (2008) found that eggs exposed to concentrations of 1,000 mg/L formalin or less had similar bacterial abundance as untreated eggs; however, 1,667–2,000 mg/L formalin led to significant reductions in bacteria numbers.

In this study, the effects of hydrogen peroxide are summarized for several different experiments. These evaluated the effects of various hydrogen peroxide concentrations on cutthroat trout eggs, the bactericidal effects of hydrogen peroxide daily drip treatments relative to those of formalin, and the effects of production-scale egg treatment with hydrogen peroxide. Problems with treating eggs just prior to hatch with hydrogen peroxide are also documented in this article.

## METHODS

The hydrogen peroxide for all tests was from the same source (35% $H_2O_2$; Dyce Chemical, Salt Lake City, Utah) and all solutions were buffered with sodium bicarbonate (1.32 g/L; Wagner et al. 2010) to maintain circumneutral pH. Iodine was in the form of povidone iodine (1% free iodine; Argentyne, Argent Laboratories, Redmond, Washington), and 1% active iodine in the stock solution was assumed from the manufacturer's label.

### Prophylactic Formalin or Hydrogen Peroxide Drip

This test evaluated the effect of a daily prophylactic drip of either formalin or hydrogen peroxide on eyed eggs of brown trout. Treatments were (1) 2,000 mg/L formalin for 15 min daily, (2) 500 mg/L hydrogen peroxide for 35 min daily, or (3) negative control (no chemical treatment). The test began at the eyed egg stage (34 d after fertilization), simulating the timing for all Utah state hatcheries that receive eggs from the brood hatchery. The eggs were shipped from the J. Perry Egan State Fish Hatchery in a cooler in which all the eggs were treated on arrival at the Fisheries Experiment Station with 100 mg/L iodine for 10 min. To each of 12 jars, 84 mL (about 1,442 eggs) of eggs were added. There were four jars per treatment. The drip treatment was applied for a total of 6 d and was then discontinued 2 d prior to hatch. The drip treatment was applied via a peristaltic pump that delivered a premixed chemical dose upstream of a manifold that distributed water to each jar. The source concentration was designed to provide the correct dose given the water flow rate and peristaltic pump flow rate. Flows were controlled by a valve to each jar and set at 3.6 L/min for the duration of the study. Water temperature was 13.2°C and total alkalinity was 222 mg/L. Dead eggs were removed periodically, and their numbers were used to calculate the percent survival to hatch as [(total eggs – dead eggs)/total eggs] × 100. Any deformed fry were enumerated to determine the proportion of deformed fry as a percentage of total fry that hatched.

To determine the effect of the chemical drips on bacterial growth, eggs were sampled on the morning of the 6th day of treatment, taking samples before the day's treatment was given. Eggs from each replicate were collected in sterile beakers and transferred to the laboratory. Twelve eggs per replicate were transferred with sterile forceps individually into test tubes with 2.0 mL of sterile peptone diluent (Barnes et al. 2005). The tubes were wrapped in laboratory film, agitated with a vortex mixer for 2 min, and 100 μL of the solution was spread on a Petri dish with tryptone yeast extract agar (TYES; 0.40% tryptone, 0.04% yeast extract, 0.05% $CaCl_2$, 0.05% $MgSO_4$, and 1.00% agar; Holt et al. 1993) by means of a sterile plastic spreader and a spin table. The plates were wrapped with a strip of laboratory film and stored in an incubator at 15°C. Plates were examined for colonies at 2, 6, 10, and 14 d after inoculation and colony-forming units (CFUs) were enumerated. Descriptions of colony types were noted on the last day. Total CFUs summed across all dates were used for statistical analysis. The prevalence of yellow CFUs (indicative of a possible infection with *Flavobacterium psychrophilum*) was noted as well.

### Hydrogen Peroxide Effects on "Green" Cutthroat Trout Eggs

On 16 April 2009, freshly fertilized eggs from the broodstock of Bear Lake strain cutthroat trout (*O. clarkii utah*) at Mantua State Fish Hatchery, Mantua, Utah, were appropriated for this test. Treatments consisted of exposure to $H_2O_2$ concentrations of (1) 10 g/L for 2 min, (2) 10 g/L for 3 min, (3) 15 g/L for 2 min, and (4) 1 g/L for 15 min. For controls, untreated eggs and eggs treated with 100 mg/L iodine for 15 min were also included as treatments. Three replicate groups of 900 eggs were tested for each treatment. Chemical exposures were done in 1 L plastic beakers at 1 h after fertilization. The eggs were held in a small net, which was moved up and down in the solution to ensure good mixing of the eggs with the chemical. A fresh disinfection solution was prepared for each replicate egg group. After disinfection, eggs were rinsed with fresh hatchery well water (hosed a few seconds in the net) before transfer to individual incubation trays supplied with 8.5°C water flowing at 15 L/min. Total hardness as $CaCO_3$ of the Mantua Hatchery well water was 171 mg/L. For evaluation of bacteria abundance after treatment, eight eggs per replicate were processed immediately after disinfection as noted above. Colony counts were made 4, 6, and 12 d after streaking on TYES media.

A prophylactic treatment with 1,667 mg/L formalin was administered via a peristaltic pump drip system to each tray stack once a day until the eggs hatched. When the eggs reached the eyed stage, the eggs were poured into water to shock infertile eggs. Dead eggs were removed the following day and percent survival to the eyed stage was calculated as [(initial eggs – dead eggs)/initial eggs] × 100. Percent survival to hatch and the percentage of deformed fry were calculated as noted above.

### Hydrogen Peroxide Effects on Rainbow Trout Eggs Near Hatching

This test used rainbow trout eggs of the Kamloops strain that were obtained from the Whiterocks State Fish Hatchery (Utah Division of Wildlife Resources), Whiterocks, Utah. The eggs were treated with either 1.5% $H_2O_2$ for 2 min or 100 mg/L iodine for 10 min. The solutions were made with Whiterocks Hatchery water (10°C, total hardness 222 mg/L). There were three replicates for each treatment, each of which had 60 mL of eggs (about 550 eggs). The experiment was conducted 1–2 d prior to the hatch of the eggs. The eggs were held in mosquito-mesh bags for treatment, rinsing, and transfer to a transport cooler. After arrival at the Fisheries Experiment Station, the eggs from both treatments were treated with 100 mg/L iodine for 10 min. The eggs were transferred to one tray per replicate in a stack of tray incubators and incubated at 13.2°C. Water flow to each incubator was 15 L/min. The percent hatch was determined by counting dead eggs and calculating survival as follows: [(initial eggs – dead eggs)/initial eggs] × 100. Deformed fry were also counted in each replicate and expressed as a percentage of the number of frypresent at hatch.

After the chemical treatment at Whiterocks Hatchery, 10 eggs per replicate were transferred to individual test tubes with 2 mL sterile peptone diluent and capped with laboratory film. Similarly, an additional 30 eggs were sampled from untreated eggs. The tubes were transported in coolers to the Fisheries Experiment Station, transferred to a refrigerator, and processed the following day using the protocol described above. Total CFU counts were classified into one of three categories (0, 1–100, or >100 CFUs) for statistical analysis.

### Production Scale Evaluation of Hydrogen Peroxide

*Cutthroat trout.*—Eggs collected on 23 June 2009 from Bear Lake cutthroat trout (Mantua State Fish Hatchery broodstock) were split into eight equal-sized groups of about 24,360 eggs each (2,480 mL at 9.8 eggs/mL). At 1 h after fertilization, three of the groups were treated for 2 min in a 10,000 mg/L $H_2O_2$ solution. An additional three groups were treated with a 100 mg/L iodine solution for 15 min. The remaining two replicates were left untreated. The $H_2O_2$ and iodine treated eggs were held in a net during treatment. At the end of treatment, the net containing the eggs was quickly transferred into a bucket containing hatchery water. The eggs were dipped up and down twice in this wash water and then rinsed a second time in another bucket of hatchery water. After rinsing, each group of eggs was split into three equal-sized groups and each was placed into a tray in a vertical stack incubator (flow set at 16 L/min) at Mantua Hatchery. The two untreated egg groups were split into three incubation trays per group.

The eggs began to hatch on 30 June 2009 and were completely hatched by 2 July 2009. At 5 d after hatch, dead eggs were hand counted and removed from each tray. The percent hatch was based on the number of dead eggs divided by the initial number of eggs in the tray, which was derived from total egg volume and eggs per unit volume estimates (Von Bayer trough method; Piper et al. 1982).

To assess the effectiveness of hydrogen peroxide and iodine at removing surface bacteria, either four ($H_2O_2$ and iodine) or six (untreated controls) eggs per replicate ($N = 36$ total) were removed after disinfection and placed into a sterile beaker. The eggs were then rinsed with sterile well water, agitated in peptone diluent, and 100 µL plated on TYES. Counts of CFUs were made 2, 5, 9, and 13 d after inoculation.

*Rainbow trout.*—Eggs of the Gunnison River–Harrison Lake strain of rainbow trout were used for this test (24 November 2009) and were treated 1 h after fertilization. Treatments were either 15 g/L $H_2O_2$ for 2 min or 100 mg/L iodine for 10 min. Previous experience showed that hydrogen peroxide treatment of large volumes of eggs (>4.0 L) led to significant mortality, which may have been a consequence of inadequate rinsing owing to the large volume (author, unpublished). Consequently, the egg lots that were disinfected with hydrogen peroxide were split into small batches for more thorough disinfection and rinsing after treatment. The control eggs treated with iodine were disinfected in a single batch, simulating current protocols. Three replicate lots of eggs were exposed to each treatment. In the first replicate, 4.02 L of eggs were treated with iodine and 3.78 L were treated with hydrogen peroxide (treated as a single batch). A larger volume of eggs (11.36 L) was exposed to each chemical for replicates 2 and 3. Eggs treated with hydrogen peroxide in replicates 2 and 3 were subdivided into three batches (of 3.78 L of eggs) for treatment. The estimated total eggs were 59,400 eggs in replicate 1 (from four-year-old females) and 256,512 eggs for each of replicates 2 and 3 (from three-year-old females). Eggs for each batch were put in a mosquito-mesh sack for ease of transfer between chemical baths and rinse containers. Two separate containers were used for rinsing, dipping a few times in the first, then doing the same in the second, before transferring the eggs to an incubation jar with well water. The rinse buckets were supplied with fresh hatchery well water for each replicate. Similarly, separate chemical solutions were prepared for each of the three replicates per treatment.

The eggs were incubated in six separate fiberglass incubation jars at 9°C at the J. Perry Egan State Fish Hatchery. A 1,667 mg/L formalin drip treatment was given daily to each of the jars during incubation, beginning the day after fertilization. At the eyed stage of development (21 December 2009), the eggs were poured into water to shock infertile eggs. The following day, dead eggs were separated from the live ones using a

commercial egg picker (Jensorter, Bend, Oregon). No attempt was made to handpick eggs from the sorted lots, though occasionally more than one pass was made through the machine to keep the sorting as accurate as possible. Survival to the eyed stage was based on the volume of live eggs as a percentage of total egg volume (dead + live).

A sample of 30 eggs was taken from each treatment (10 per replicate), each egg being transferred to a test tube with 2 mL of sterile peptone diluent. The tubes were wrapped with laboratory film and the eggs were transported on ice packs in coolers for processing at the Fisheries Experiment Station the following day. The eggs were agitated on a vortex mixer for 2 min and 100 μL of the solution was plated on TYES media. After 3 d incubation at 15°C, CFUs were enumerated and observations of colony color and morphology noted.

### Statistical Analyses

For all statistical analyses, SPSS 13.0 software was used with a significance level of 0.05. Differences in survival to the eyed egg stage, in survival to hatch, and in the percentage of cripples were compared among treatments using one-way analysis of variance. Tukey's least-significant-difference test was used for post hoc comparisons among individual means. For the rainbow trout production test, a paired *t*-test was used to compare survival to the eyed stage. For analysis of total CFUs, plates that were noted as "too numerous to count" were arbitrarily assigned a value of 10,000. The CFU values were subsequently assigned to one of three categories: 0, 1–300, or >300. For CFU analysis in the drip test, "green" cutthroat trout egg test, and the production scale cutthroat trout test, a hierarchical log-linear analysis with treatment, replicate, and CFU category as variables indicated that replicate and interaction terms were not significant. Subsequent analyses in these tests used chi-square analysis (maximum likelihood statistic). Further analysis of partial tables or collapsed frequency tables examined differences between and among means.

## RESULTS

### Prophylactic Formalin or Hydrogen Peroxide Drip

The drip treatment with either formalin or hydrogen peroxide had no significant effect on survival to hatch ($F = 1.92$, df = 11, $P = 0.20$) or on the prevalence of deformed fry ($F = 1.14$, df = 11, $P = 0.36$; Table 1). The percentage of eggs surviving to hatch was generally high, ranging from 87.1% to 92.4% among all treatments. The prevalence of deformities was low among all treatments, ranging from 0.17% to 0.37% (Table 1).

The bacteriology data indicated that drip treatment with either formalin or hydrogen peroxide significantly reduced bacterial abundance. Untreated eggs that were only given a single 100 mg/L iodine treatment on arrival had significantly more total bacterial growth than eggs treated daily with either formalin or hydrogen peroxide ($G = 57.6$, df = 4, $P < 0.01$; Table 2). The prevalence of yellow CFUs (indicative of a possible infection with *Flavobacterium psychrophilum*) was also significantly higher in untreated controls than in the two chemical treatments ($G = 75.4$, df = 2, $P < 0.01$; Table 2). There was no significant difference in either total CFU distribution or yellow CFU prevalence between the formalin and hydrogen peroxide treatments ($G = 3.70$ and 0.06, df = 2 and 1, $P = 0.16$ and 0.80, respectively). The percentage of eggs that had no bacteria recovered from them was 50% for the formalin treatment and 64.6% for the hydrogen peroxide treatment, indicating that complete disinfection is still not being achieved; yellow CFU prevalence was still 21–23% in the chemical treatments. Media controls and diluent control plates were all negative for any growth.

TABLE 1. Comparison of mean survival to hatch and fry deformities ($N = 4$; means ± SDs) for brown trout among eggs treated at the eyed stage for 6 d with daily drip treatments of either formalin or hydrogen peroxide. Means were not significantly different.

| Treatment | Treatment duration (min) | Survival to hatch (%) | Deformities (%) |
|---|---|---|---|
| Untreated control | | 87.2 ± 6.1 | 0.4 ± 0.3 |
| 500 mg/L hydrogen peroxide | 35 | 92.4 ± 0.4 | 0.3 ± 0.1 |
| 2,000 mg/L formalin | 15 | 90.6 ± 1.8 | 0.2 ± 0.2 |

### Hydrogen Peroxide Effects on "Green" Cutthroat Trout Eggs

Survival to the eyed stage ranged from 79.5% to 82.5% among the six treatments and did not significantly differ ($F = 1.86$, df = 17, $P = 0.18$) among them (Table 3). The percent hatch and percentage of deformed fry also did not significantly differ among the six treatments (Table 3; $F < 1.09$, df = 17, $P \geq 0.41$). The prevalence of deformities averaged less than a percent in each treatment, so hydrogen peroxide treatment did not induce any deformities. The survival to hatch ranged from 66.5% to 69.6%.

There were significant differences among treatments in CFU counts ($G = 50.8$, df = 10, $P < 0.01$). Analysis of partial tables

TABLE 2. Comparison of bacterial colony-forming unit (CFU) counts among eggs treated at the eyed stage with drip treatments of hydrogen peroxide or formalin. The values for the three CFU categories are the frequencies of CFU abundance ($N = 48$) for all bacteria types. The last column compares the prevalence among treatments of yellow CFUs (those with long, thin, gram-negative rods). Within columns, means with common letters are not significantly different.

| Treatment | 0 CFUs | 1–100 CFUs | >100 CFUs | Yellow CFUs (%) |
|---|---|---|---|---|
| Untreated control | 1 | 20 | 27 z | 93.8 z |
| 500 mg/L hydrogen peroxide for 35 min | 31 | 8 | 9 y | 20.8 y |
| 2,000 mg/L formalin for 15 min | 24 | 16 | 8 y | 22.9 y |

TABLE 3. Comparison of cutthroat trout egg survival to the eyed stage and hatch and fry deformities (means ± SDs; $N = 3$) for eggs treated 1 h after fertilization with various doses of hydrogen peroxide and iodine for various durations.

| Treatment | Concentration (mg/L) | Duration (min) | Eyed (%) | Hatched (%) | Fry deformities (%) |
|---|---|---|---|---|---|
| $H_2O_2$ | 1,000 | 15 | 81.7 ± 0.6 | 67.2 ± 2.0 | 0.2 ± 0.1 |
| | 10,000 | 2 | 81.4 ± 0.7 | 69.6 ± 1.8 | 0.5 ± 0.3 |
| | 10,000 | 3 | 82.0 ± 1.3 | 66.9 ± 1.2 | 0.2 ± 0.1 |
| | 15,000 | 2 | 79.5 ± 2.7 | 66.5 ± 0.9 | 0.2 ± 0.0 |
| Iodine | 100 | 15 | 82.4 ± 1.0 | 68.5 ± 2.4 | 0.1 ± 0.1 |
| Untreated | 0 | 0 | 82.5 ± 1.0 | 68.5 ± 2.9 | 0.4 ± 0.5 |

indicated that untreated eggs had significantly more CFUs than eggs treated with either chemical, but no significant difference was observed among the five chemical treatments ($G = 8.65$, df = 8, $P = 0.37$). The CFU counts were generally low; more than 70% of the treated eggs did not have CFUs (Table 4).

## Hydrogen Peroxide Effects on Rainbow Trout Eggs Near Hatching

The mean survival to hatch was significantly lower for rainbow trout eggs treated with 15 g/L $H_2O_2$ for 2 min (mean ± SD, 43.9 ± 5.8%) than for eggs treated with 100 mg/L iodine for 10 min (61.2 ± 6.5%; $t = 3.40$, df = 4, $P = 0.03$). The percentage of deformed fry was low overall and not significantly different between the hydrogen peroxide treatment (0.71 ± 0.69%) and iodine treatment (0.67 ± 0.73%; $t = –0.06$, df = 4, $P = 0.96$). The eggs began to hatch the day after treatment. Several eggs sampled for bacteria counts hatched during the agitation of the test tube with the vortex mixer (33% of iodine-treated eggs and 70% of hydrogen-peroxide-treated eggs). A high percentage of Petri plates were classified as "too numerous to count" in both the iodine treatment (56.7%) and hydrogen peroxide treatment (70%). Total CFU count analysis by a hierarchical linear model indicated that replicate effects and interaction effects were not significant and the best model from backward elimination included CFU count category and treatment as factors. Subsequent chi-square analysis indicated that there were significant differences between treatments ($G = 7.89$, df = 2, $P = 0.02$). Chi-square analysis of a collapsed frequency table, e.g., positive versus negative, indicated that iodine-treated eggs had significantly more eggs with no bacteria (33%) than eggs treated with hydrogen peroxide (6.7%;$G = 7.16$, df = 1, $P < 0.01$). As in previous tests, positive identification of the bacterial isolates was not possible.

## Production Scale Evaluation of Hydrogen Peroxide

*Cutthroat trout*.—The average hatch rate of the eggs was high and ranged little among treatments (89.7–90.4%). Survival to hatch did not significantly differ among $H_2O_2$ (mean ± SD, 90.3 ± 0.29%), iodine (90.4 ± 0.47%), and untreated egg (89.7 ± 0.08%) treatments ($F_{2,5} = 2.66$, $P = 0.16$). The disinfection treatments apparently did not provide a significant survival advantage when compared with the untreated eggs.

Colony-forming units were allocated to one of three categories: 0, 1–300, or >300 CFUs. Plates designated as 'too numerous to count" were classified into the latter category. Analysis of the CFU data with the log-linear model indicated that there were no significant interaction terms and replicate effects, but subsequent chi-square analysis of the disinfection treatment effects indicated that there were significant differences ($G = 10.86$, df = 4, $P = 0.03$) in the frequency distribution of CFUs among treatments. Analysis of partial tables indicated that there were no significant differences in CFU distributions between iodine- and hydrogen-peroxide-treated eggs, but the hydrogen-peroxide-treated eggs had significantly fewer eggs that were

TABLE 4. Frequency distribution of the number of cutthroat trout eggs in three categories of colony-forming unit (CFU) abundance per egg among various egg disinfection treatments ($N = 24$ eggs/treatment). The CFU ranges are for the actual numbers of total CFUs observed on tryptone yeast extract agar media (1/20th of total).

| | | | CFU abundance category | | |
|---|---|---|---|---|---|
| Treatment | Concentration (mg/L) | Duration (min) | 0 CFUs | 1–10 CFUs | >10 CFUs |
| Hydrogen peroxide | 1,000 | 15 | 17 | 6 | 1 |
| | 10,000 | 2 | 17 | 4 | 3 |
| | 10,000 | 3 | 18 | 6 | 0 |
| | 15,000 | 2 | 21 | 3 | 0 |
| Iodine | 100 | 15 | 18 | 5 | 1 |
| Untreated | 0 | 0 | 2 | 15 | 7 |

TABLE 5. Frequency distribution of the number of cutthroat trout eggs in three categories of colony-forming unit (CFU) abundance per egg among various egg disinfection treatments used in the production-scale test ($N = 12$ eggs/treatment). The CFU ranges are the actual numbers of CFUs observed on tryptone yeast extract agar media (1/20th of total).

| Treatment | Concentration (mg/L) | Duration (min) | CFU abundance category | | |
|---|---|---|---|---|---|
| | | | 0 CFUs | 1–300 CFUs | >300 CFUs |
| Hydrogen peroxide | 10,000 | 2 | 4 | 2 | 6 |
| Iodine | 100 | 15 | 1 | 1 | 10 |
| Untreated | 0 | 0 | 0 | 0 | 12 |

classified ">300" (50%) than untreated eggs (100%; Table 5; $G = 10.36$, df = 2, $P < 0.01$). Iodine-treated eggs did not significantly differ from untreated eggs ($G = 2.95$, df = 2, $P = 0.23$).

*Rainbow trout*.—Mean egg survival to the eyed stage did not significantly differ between the 15-g/L hydrogen peroxide treatment (mean ± SD, 72.2 ± 15.0%) and the 100-mg/L iodine-treated control (77.6 ± 14.5%; $t = 0.725$, df = 2, $P = 0.54$). Bacterial growth was high on eggs from both treatments: 29/30 iodine-treated eggs and 30/30 hydrogen-peroxide-treated eggs had too many CFUs to count. Yellow colony prevalence was high in both the iodine (90.0%) and hydrogen peroxide (96.7%) groups, and did not significantly differ between treatments ($G = 1.12$, df = 1, $P = 0.29$).

## DISCUSSION

Hydrogen peroxide was evaluated with several different species of salmonids and at different times during egg development. The treatment of eggs just after water hardening with doses of 10–15 g/L for 2 min was safe for cutthroat and rainbow trout in production-scale tests. Doses of 1 g/L $H_2O_2$ for 15 min were also safe for water-hardened cutthroat trout eggs. However, if eggs were treated the day before hatching, significant mortality occurred. Stephenson et al. (2005) observed in Chinook salmon *Oncorhynchus tshawytscha* that the internus layer of egg shells treated with hydrogen peroxide (700 mg/L daily) was significantly less differentiated than that in eggs treated with formalin or those left untreated. Iodine has also been reported to induce higher mortality and premature hatching if applied near hatching (Piper et al. 1982).

Daily drip treatment with formalin (1,667 mg/L) has been demonstrated to control fungal growth on salmonid eggs (Barnes et al. 1997, 2000, 2002; Rach et al. 2005). Every-other-day treatments with formalin concentrations of at least 1,000 mg/L also controlled fungal growth (Barnes and Soupir 2006). However, fungal growth has been observed on eggs treated every other day with 500 mg/L hydrogen peroxide for 15 min (Barnes and Soupir 2006). Daily hydrogen peroxide drip treatments have been demonstrated to control fungal growth on eggs (Gaikowski et al. 1998; Arndt et al. 2001; Barnes and Soupir 2006), though in incubator stacks with large numbers of eggs, reduced efficacy has been noted in the bottom trays (Rach et al. 2005). In this study, brown trout were evaluated for the first time and a daily treatment with 500 mg/L $H_2O_2$ for 35 min was found to be safe for eyed eggs. In our drip treatment experiment, no fungal growth was observed in any treatment group, including the untreated controls.

The bactericidal effects of hydrogen peroxide drip treatments have not been evaluated previously. However, formalin drip treatments have been investigated by Barnes et al. (2005) who found that 1,667 mg/L formalin significantly reduced bacterial growth but did not completely disinfect eggs. Similar results were found in this study. Barnes et al. (1999) noted that despite daily formalin treatment (1,667 mg/L for 15 min) bacterial abundance increased over time from fertilization until eggs were mechanically picked at the eyed stage; incubator tray location within a stack also affected bacterial abundance as lower trays had more bacteria present. The results of our production-scale rainbow trout test similarly showed that bacteria abundance is high by the time the eggs reach the eyed stage. A single initial treatment with iodine or hydrogen peroxide at water hardening failed to completely eliminate the yellow-pigmented bacteria and these bacteria populations augmented during incubation. The bactericidal ability of both iodine and hydrogen peroxide was comparable in treatment of water-hardened eggs when bacterial abundance was low, such as in the laboratory-scale and production-scale cutthroat trout tests. However, in tests on eyed eggs near hatching, better control of bacteria was noted using iodine. Drip treatment with either hydrogen peroxide or formalin significantly reduced the abundance of yellow-pigmented bacteria. This strategy would hopefully reduce the probability of fry becoming infected as well as the infective dose leading to disease.

Since bacteria are not completely eliminated with current disinfection protocols, additional research is needed to discover other chemicals or strategies for control of pathogenic bacteria. One approach may be the use of probiotic bacteria that effectively compete with pathogenic bacteria, especially as fry begin initial feeding (Verschuere et al. 2000). In the meantime, prophylactic treatments with either formalin or hydrogen peroxide should help reduce overall bacteria abundance. Given the problems observed in treating eggs near hatching with hydrogen peroxide, as well as the production-scale results of Rach

et al. (2005), which indicated formalin was better for fungus control than hydrogen peroxide, formalin is still the most effective prophylactic chemical for egg treatment. Overall results in this study also favor povidone iodine for single treatments of eggs at water hardening or at the eyed egg stage. However, hydrogen peroxide can be an effective alternative if care is taken to avoid treating eggs near hatching, if solutions are buffered, and if eggs are treated in smaller batches with quicker rinses that preclude overexposure.

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