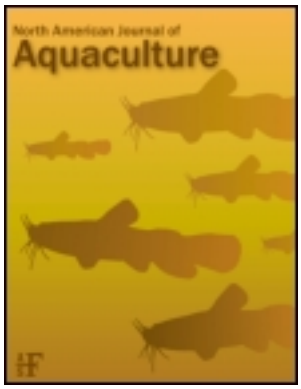

North American Journal of Aquaculture
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/unaj20

Effect of Single or Double Exposures to Hydrogen Peroxide or Iodine on Salmonid Egg Survival and Bacterial Growth

Eric J. Wagner [a] , Randall W. Oplinger [a] & Matthew Bartley [a]
[a] Utah Division of Wildlife Resources, Fisheries Experiment Station, 1465 West 200 North, Logan, Utah, 84321, USA

Available online: 01 Feb 2012

To cite this article: Eric J. Wagner, Randall W. Oplinger & Matthew Bartley (2012): Effect of Single or Double Exposures to Hydrogen Peroxide or Iodine on Salmonid Egg Survival and Bacterial Growth, North American Journal of Aquaculture, 74:1, 84-91

To link to this article: http://dx.doi.org/10.1080/15222055.2011.649887

North American Journal of Aquaculture 74:84–91, 2012

ISSN: 1522-2055 print / 1548-8454 online
DOI: 10.1080/15222055.2011.649887

ARTICLE

Effect of Single or Double Exposures to Hydrogen Peroxide or Iodine on Salmonid Egg Survival and Bacterial Growth

Eric J. Wagner,* Randall W. Oplinger, and Matthew Bartley
Utah Division of Wildlife Resources, Fisheries Experiment Station, 1465 West 200 North, Logan, Utah 84321, USA

Abstract

Four disinfection tests were conducted on the eggs of cutthroat trout *Oncorhynchus clarkii*, rainbow trout *O. mykiss*, and brown trout *Salmo trutta*. In each egg test, the effects of treating twice were determined, which simulated treatment at both the brood source and the receiving hatchery. Cutthroat trout eggs were treated at 1 h and again at 3 or 6 h after fertilization. Survival to the eyed stage was greater for eggs treated with 100 mg/L iodine for 10 min (86.9%) than with 1.5% hydrogen peroxide (H_2O_2) for 2 min (82.4%); the timing of the second disinfection had no effect on egg survival. Bacterial growth was significantly greater among H_2O_2-treated eggs. For eyed rainbow trout eggs, 2,000 mg/L iodine for 10 min was toxic (<11% hatch), but eggs treated either once or twice with hydrogen peroxide (1% H_2O_2 for 2 min) survived to hatch, as did the controls. Bacterial growth was significantly greater on eggs treated with H_2O_2 than control eggs treated with 100 mg/L iodine for 10 min. In a subsequent test with eyed rainbow trout eggs exposed to 1,000 mg/L iodine for 15 min either once or twice, hatching rates did not significantly differ from those of controls. Bacterial growth was significantly lower after 1,000-mg/L iodine treatment than in controls treated with 100 mg/L. In the fourth test, survival to hatch of eyed brown trout eggs treated with 1,000 mg/L iodine once or twice did not differ from that of controls. For bacterial control, double treatments are safe at iodine doses of no more than 1,000 mg/L or when 1% H_2O_2 is used. The bacteriological results suggest that a single or double dose of 1,000 mg/L iodine provides the best control of bacterial growth.

Fungal and bacterial growth on fish eggs can seriously compromise egg survival during incubation (Ross and Smith 1972; Barnes et al. 2003). Bacterial pathogens typically cause egg membrane degradation, which can ultimately cause egg mortality (Pavlov and Moksness 1993; Morrison et al. 1999; Barnes et al. 2009). Povidone iodine (McFadden 1969; Amend 1974) is the most common disinfectant used for the control of egg pathogens. Some egg-borne pathogens, however, are capable of surviving iodine treatment (Kumagai et al. 1998; Shaw et al. 1999; Wagner et al. 2008; Barnes et al. 2009). Consequently, alternatives such as higher iodine concentrations (Alderman 1984) and other chemicals including hydrogen peroxide (H_2O_2), glutaraldehyde, and sodium carbonate peroxyhydrate have been evaluated (Marking et al. 1994; Escaffre et al. 2001; Small 2009; Wagner et al. 2010). Iodine concentrations of 50–100 mg/L are standard for the disinfection of eggs of most salmonid species (McFadden 1969; Amend 1974; Cipriano et al. 2001). Higher iodine concentrations may be more effective for bacterial control. Povidone-iodine-based egg disinfectants are commercially available and their buffering capacity is designed for disinfection at standard concentrations for salmonids. The toxicity of iodine to salmonid eggs, however, is pH dependent (Amend 1974; Alderman 1984).

Hydrogen peroxide is one alternative to iodine that has shown considerable promise. Initial research with H_2O_2 documented the fungicidal characteristics of the chemical. For example, treatments of 0.5% to 1% for 15–60 min controlled fungal growth in rainbow trout *Oncorhynchus mykiss* eggs (Schreier et al. 1996; Barnes et al. 1998). Other experiments indicate that effective treatment levels vary among species (Yamamoto et al. 2001), and lower concentrations are needed for species such as channel catfish *Ictalurus punctatus* (Mitchell et al. 2009) or

*Corresponding author: ericwagner@utah.gov
Received March 9, 2011; accepted May 5, 2011

golden shiner *Notemigonus crysoleucas* (Bozwell et al. 2009). Differences in H_2O_2 sensitivity among rainbow trout strains have also been noted (Gaikowski et al. 1998). Some research has demonstrated that avoiding H_2O_2 treatment during certain stages of egg development can improve survival (Gaikowski et al. 1998; Arndt et al. 2001).

The bacteriological effects of egg treatment with H_2O_2 have received less scrutiny. However, recent tests have indicated that 30 g/L H_2O_2 for 5 min controlled or significantly reduced bacterial growth on 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 g/L H_2O_2 for 15 min had significantly reduced bacterial abundance, but 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 *O. clarkii* or brown trout *Salmo trutta* have not been tested. Although closely related, tests that demonstrated a higher tolerance to iodine by cutthroat trout than by rainbow trout (Fowler and Banks 1991; Pravacek and Barnes 2003) suggest that species differences warrant further investigation.

In an effort to improve control of bacterial pathogens such as *Flavobacterium psychrophilum* (causative agent of bacterial coldwater disease), we evaluated the effect of double chemical treatment with hydrogen peroxide or iodine, and that of higher iodine concentrations on the control of bacteria in salmonid eggs. A double chemical treatments may be more effective than a single treatment and could be used to disinfect eggs before that leave a hatchery and again when they arrive at a receiving hatchery. Similarly, in situations where wild fish are trapped to obtain gametes, the effect of a double treatment on water-hardened eggs is also of interest. The objectives of this study were to (1) determine whether H_2O_2 or higher doses of iodine can be safely used to improve the disinfection of rainbow trout, cutthroat trout, and brown trout eggs, and (2) determine whether treating twice alters egg survival relative to eggs treated once (simulates treating at a wild trap and at a receiving hatchery).

METHODS

Four experiments were conducted to evaluate the effect of repeated chemical treatment with iodine and H_2O_2 on eggs of cutthroat trout (test 1), rainbow trout (tests 2 and 3), and brown trout (test 4). The iodine stock solution used in all tests was povidone iodine (Argentyne; Argent Chemical Laboratories, Redmond, Washington) and a 1% active iodine concentration in the stock solution was assumed, based on the manufacturer's label. Hydrogen peroxide stock solutions were drawn from a barrel of 35% H_2O_2 (Dyce Chemical, Salt Lake City, Utah). All H_2O_2 treatment solutions were made just before each trial and were buffered with sodium bicarbonate (1.32 g/L) to maintain an approximately neutral pH (Wagner et al. 2010).

Test 1: cutthroat trout, water-hardened eggs.—Treatments in this test were (1) 1.5% H_2O_2 for 2 min, once at 1 h postfertilization (pf), (2) 1.5% H_2O_2 for 2 min, twice at 1 and 3 h pf, (3) 1.5% H_2O_2 for 2 min, twice at 1 and 6 h pf, (4) 100 mg/L iodine for 15 min, once at 1 h pf, (5) 100 mg/L iodine for 15 min, twice at 1 and 3 h pf, and (6) 100 mg/L iodine for 15 min, twice at 1 and 6 h pf. Cutthroat trout eggs were collected on 5 March 2009 at the Mantua State Fish Hatchery, Utah Division of Wildlife Resources (UDWR), Mantua, Utah, from twelve 5-year-old females and fertilized with sperm from 10 males. After 2 min the eggs were rinsed with hatchery well water and allowed to water-harden for 1 h. After water-hardening, the eggs were divided into 18 groups with 900 eggs in each group. Three replicate groups were tested for each treatment. Eggs were treated in plastic beakers containing 1.0 L of disinfectant. During exposure the eggs were held in a small net, which was moved up and down when initially placed into the beaker to ensure good mixing of the eggs with the chemical. After disinfection, eggs were transferred immediately to vertical stack incubator trays. A fresh disinfection solution was prepared for each egg group. For double-disinfection treatments, the eggs were netted from the trays and disinfected again at either 3 or 6 h after fertilization, simulating a typical range of travel times to state hatcheries from wild trap sites.

After disinfection, eggs were incubated in vertical stack tray incubators at 8.5°C. 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 of development, they were mechanically shocked ("bumped") by pouring them into water to addle infertile eggs. Two days later (31 March 2009) dead eggs were counted as they were removed, and the volumes of dead and eyed eggs in each tray were determined. These data were used to calculate the survival to the eyed egg stage (volume of live eggs / total egg volume).

The bacteria load on the treated eggs was assessed on the eggs that received disinfection either once at 1 h pf or at 3 h pf. To do this, eight eggs were removed from each group immediately after the final disinfection, placed into a sterile beaker, and rinsed three times with sterile well water. The eggs in the beaker were transferred individually with sterile forceps to a test tube containing 2 mL of a sterile peptone salt diluent solution (0.1% proteose peptone and 0.8% NaCl, Barnes et al. 2005). The tube was agitated with a vortex mixer for 2 min and 100 μL was transferred to a trypticase soy agar (TSA) petri dish. Another 100 μL was transferred to a petri dish with tryptone yeast extract salts agar with the antibiotic tobramycin (TYEST; 50 μL of 100 mg/mL stock tobramycin solution per liter of media; Buller 2004; 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. Control plates with nothing added (media control) or with 100 μL of the sterile peptone diluent (diluent control) were also made. Counts of colony-forming units (CFUs) on both media (TSA and TYEST) were made 3, 5, 7, 10, and 14 d after

inoculation. If plates had too many CFUs to count accurately, the plate was labeled as "too numerous to count" (TNC). To get an estimate of the total number of bacteria on each treated egg, the CFU plate counts were multiplied by 20 (since one-twentieth of the peptone salt diluent solution was transferred to each petri dish).

Test 2: rainbow trout, eyed eggs.—Treatments evaluated in this test were (1) 1% H_2O_2 for 2 min, eggs treated once at the receiving hatchery (1×), (2) 1% H_2O_2 for 2 min, eggs treated at both the brood hatchery and receiving hatchery (2×), (3) 2,000 mg/L iodine for 10 min, 1×, (4) 2,000 mg/L iodine for 10 min, 2×, and (5) 100 mg/L iodine for 10 min, 1×. An additional group of eggs was left untreated and sampled only for the initial bacteriology work. The untreated eggs were not followed after the initial sample owing to hatchery disinfection policies that did not permit untreated eggs to be transferred.

For the trial, eyed rainbow trout eggs were obtained from Erwin–Sand Creek strain broodstock from the J. Perry Egan State Fish Hatchery, UDWR, Bicknell, Utah. The eggs were part of a batch of eggs that had been heat-shocked for triploidy induction (26.5°C for 20 min at 20 min after fertilization). Four replicates were tested for each treatment. Each replicate consisted of 89 mL (about 1,440 eggs) of eggs placed in a bag made from mosquito netting. The bags were held in 1-L plastic beakers containing a premixed chemical for the target duration. Temperatures in the treatment containers were 10°C. After the chemical treatment, the bags of eggs were rinsed by dipping into two different buckets of fresh, hatchery well water and then transferred to coolers for transport. Three coolers were used for transport: one for eggs treated with H_2O_2, one for eggs treated with iodine, and one for untreated eggs. Each cooler was full of hatchery well water (10°C, 85 mg/L total hardness as $CaCO_3$, 85 mg/L total alkalinity, and pH 7.6). The eggs were transported to the Fisheries Experiment Station, Logan, Utah, and incubated at 14°C in vertical tray incubators, in which one replicate per tray was allocated.

The hatching success was determined by periodically counting and removing the dead and infertile eggs. The survival to hatch was expressed as the number of surviving fry divided by the total number of initial eyed eggs in each replicate. A week after hatching, fry with obvious deformities were also counted as they were removed. The prevalence of deformities was expressed as a percentage of surviving hatched fry.

Bacterial abundance was assessed on the eggs after treatment at the receiving hatchery (Fisheries Experiment Station). Twelve eggs from each replicate were agitated individually in 2 mL of sterile peptone diluent as described above and 100 μL of the solution was plated on TYEST media. Efforts were made during the process to maintain cool temperatures for eggs, tubes, and media plates. Control plates were made by inoculating with only the sterile peptone diluent ($n = 3$) and three uninoculated plates served as media controls. The test plates were incubated at 15°C and observed at 4, 8, and 10 d after inoculation. Colony-forming units were enumerated each time (each CFU counted only once). Colony descriptions were made of each type observed, noting the color, morphology, margin shape, speed of growth, and texture. Given the emphasis of the study on bacterial coldwater disease, all yellow CFUs were further isolated on another plate from which Gram stains were made. If a yellow CFU had long, thin, Gram-negative rods typical of *F. psychrophilum*, it was considered positive for statistical purposes (though this is not diagnostic for *F. psychrophilum*).

Test 3: rainbow trout, eyed eggs.—Eyed eggs of the Gunnison River–Harrison Lake strain of rainbow trout, which were heat-shocked to induce triploidy, were obtained from the J. Perry Egan Hatchery. Treatments evaluated in this trial were (1) 1,000 mg/L iodine for 10 min, 1× (treated once at receiving hatchery [Fisheries Experiment Station]), (2) 1,000 mg/L iodine for 10 min, 2×, and (3) 100 mg/L iodine for 10 min (control). For the 2× iodine treatment, the second treatment was applied 60 min after the first treatment; both treatments were done at the Fisheries Experiment Station. There were four replicates per treatment. For the exposure, about 1,000 rainbow trout eggs (108 mL volume) per replicate were placed into bags made with mosquito netting. The treatment was conducted in plastic beakers with 1.0 L of chemical solution. Eggs were rinsed by dipping the bag up and down three times within a bucket of fresh well water that was exchanged after each dip (two bags at a time). Eggs for each replicate were randomly assigned to trays within a vertical stack incubator. Hatching success and the prevalence of deformities was determined as noted for test 2. Bacteriological methods were also the same, except that the eggs were sampled the day after treatment.

Test 4: brown trout, eyed eggs.—Eyed eggs from the J. Perry Egan State Fish Hatchery were treated on arrival at the Fisheries Experiment Station with either (1) 100 mg/L iodine for 10 min (control), (2) 1,000 mg/L iodine once (1×), or (3) 1,000 mg/L iodine twice (2×). The second iodine treatment was conducted only a few minutes after the first treatment to determine, under the worst-case scenario of immediate retreatment, if double treatment compromised egg survival. No treatment occurred at the brood hatchery other than the standard iodine treatment after water-hardening (100 mg/L for 10 min) and the daily prophylactic formalin drip treatment (1,667 mg/L for 15 min) during incubation. No bacteriological analysis was conducted in this test, but egg survival to hatch and the prevalence of deformed fry were documented as in test 2.

Statistical analyses.—In test 1, two-way analysis of variance (ANOVA) was used to analyze survival at the eyed egg stage, percent deformed, and percent hatch by using chemical treatment (iodine, H_2O_2) and time (1, 3, and 6 h) as fixed factors. In the other tests, survival and deformity variables were analyzed by one-way ANOVA comparing the iodine control with the various 1× and 2× treatments. Percentage values were arcsine-transformed before analyses. Percent hatch and percent deformities in the other tests were analyzed by one-way ANOVA after arcsine transformation. Subsequent mean comparisons were made with the Sheffé's test. All analyses were

TABLE 1. Survival to the eyed egg stage (eye-up) and hatch and percentage of deformed fry (means ± SDs) of Bear Lake cutthroat trout treated once (1 h after fertilization) or twice (3 or 6 h after fertilization) with hydrogen peroxide (H_2O_2) or iodine (test 1).

Chemical	Dose (mg/L)	Duration (min)	Time after fertilization (h)	Eye-up (%)	Hatch (%)	Deformed (%)
H_2O_2	15,000	2	1	82.4 ± 2.7	71.8 ± 6.6	1.5 ± 0.9
H_2O_2	15,000	2	3	80.3 ± 0.5	67.7 ± 2.8	1.6 ± 0.9
H_2O_2	15,000	2	6	80.9 ± 1.1	72.8 ± 1.6	0.4 ± 0.1
Iodine	100	15	1	85.4 ± 3.1	80.4 ± 2.2	0.2 ± 0.2
Iodine	100	15	3	87.3 ± 2.1	83.3 ± 2.2	0.4 ± 0.2
Iodine	100	15	6	86.8 ± 0.7	81.7 ± 0.2	0.3 ± 0.1

made with SPSS version 13.0 and a $P < 0.05$ was considered significant. For test 1, the numbers of bacteria on each petri dish were placed into categories (0, 1–10, or >10) for analysis. A hierarchical log-linear analysis was performed separately for each media (TSA or TYEST) to assess differences in bacteria counts by using time (1, 3, or 6 h) and chemical type (iodine, H_2O_2) as treatment variables. In the rainbow trout tests, the CFU data were analyzed by chi-square analysis after classification of the CFU counts into one of three categories (0, 1–100 CFUs, >100 CFUs). Plates considered "too numerous to count" were classified into the >100 CFU group. The prevalence of yellow CFUs was compared among treatments by using chi-square analysis. If the chi-square test indicated a significant difference, mean separation was determined by using partial tables to compare treatment pairs (Fienberg 1989).

RESULTS

Test 1: Cutthroat Trout, Water-Hardened Eggs

Survival to the eyed egg stage in cutthroat trout varied significantly between the disinfectants tested (Table 1; $F_{1, 17} = 24.28$, $P < 0.01$). Percent survival to the eyed egg stage was greater for eggs treated with iodine (86.5 ± 2.1%; mean ± SD) than for eggs treated with 1.5% H_2O_2 (81.2 ± 1.8%, data pooled across time treatments). Percent hatch ranged from 67.7% to 72.8% among the H_2O_2 treatments and from 80.4% to 83.3% among iodine-treated eggs. The second disinfection had no effect on survival to the eyed egg stage for either chemical regardless of whether the second treatment occurred at 3 or 6 h after fertilization (Table 1; $F_{2, 17} = 0.03$, $P = 0.97$). The percentage of deformed fry was significantly different between chemical treatments ($P < 0.01$; Table 1), but not among times ($P > 0.13$). Deformity percentages were less than 1.6% for all treatments, but were significantly higher for H_2O_2-treated eggs (Table 1). Interaction terms (time × chemical treatment) for both variables were not significant (e.g., hatch: $F_{2, 17} = 0.33$, $P = 0.72$).

The bacteria counts were relatively low overall and no more than two CFUs (i.e., 40 bacteria/egg; Table 2) were observed on any plate. Statistically, results varied slightly with media type. On TSA, the log-linear analysis indicated there was no time effect, but chemical type was significant. Further testing with the chi-square test (pooling data across time treatments) indicated that a higher percentage of iodine-treated eggs had no CFUs (95.8%) compared with peroxide-treated eggs (72.9%, $P < 0.01$). On TYEST, the log-linear analysis indicated that there were significant effects of both chemical type and time. Further testing with chi-square analysis for each time indicated that for eggs treated once, the percentage of eggs with no CFUs was

TABLE 2. Frequency distributions of the number of cutthroat trout eggs in one of two categories of colony-forming unit (CFU) abundance per egg using two media (TSA and TYEST) among the various egg disinfection treatments of test 1 ($n = 24$ eggs). The CFU range values are for the actual numbers of CFUs observed (1/20th of the total).

Chemical	Concentration (mg/L)	Duration (min)	Time after fertilization (h)	0 CFUs	1–10 CFUs
			TSA		
H_2O_2	15,000	2	1	15	9
H_2O_2	15,000	2	3	20	4
Iodine	100	15	1	23	1
Iodine	100	15	3	23	1
			TYEST		
H_2O_2	15,000	2	1	15	9
H_2O_2	15,000	2	3	23	1
Iodine	100	15	1	22	2
Iodine	100	15	3	23	1

TABLE 3. Comparison of survival to hatch and fry deformities (means ± SDs; $n = 4$) among rainbow trout eggs treated at the eyed stage either once or twice (5 h apart) with H_2O_2 or iodine (test 2). Within columns, means with common letters are not significantly different.

Treatment	Hatch (%)	Deformities (%)
1% H_2O_2 for 2 min, 1×	63.8 ± 7.2 y	2.2 ± 0.5 x
1% H_2O_2 for 2 min, 2×	86.2 ± 1.7 y	2.4 ± 0.6 x
2,000 mg/L iodine for 10 min, 1×	1.7 ± 1.1 z	0.1 ± 0.1 z
2,000 mg/L iodine for 10 min, 2×	10.4 ± 2.9 z	0.3 ± 0.1 zy
100 mg/L iodine for 10 min, 1×	58.2 ± 31.5 y	1.7 ± 1.1 yx

TABLE 4. Comparison of bacterial colony-forming unit (CFU) counts on TYEST media among rainbow trout eggs treated at the eyed stage either once or twice (5 h apart) with H_2O_2 or iodine (test 2). 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 of yellow CFUs (those containing long, thin, gram-negative rods) among treatments; within columns, means with common letters are not significantly different.

	CFU category			
Treatment	0 CFUs	1–100 CFUs	>100 CFUs	Yellow CFUs (%)
1% H_2O_2 for 2 min, 1×	1	19	28 z	27.1 y
1% H_2O_2 for 2 min, 2×	25	19	4 y	43.8 y
2,000 mg/L iodine for 10 min, 1×	38	6	4 x	0.0 z
2,000 mg/L iodine for 10 min, 2×	45	3	0 w	0.0 z
100 mg/L iodine for 10 min, 1×	46	1	1 w	2.1 z

higher for iodine-treated eggs than for eggs treated with H_2O_2 (91.7% versus 62.5%, $P = 0.013$). However, for eggs treated twice, both chemical treatments resulted in a high percentage of eggs with no CFUs (95.8% for both chemicals, $n = 24$).

Test 2: Rainbow Trout, Eyed Eggs

High mortality of rainbow trout eyed eggs was observed in the groups treated with 2,000 mg/L iodine for 10 min and both 1 × and 2 × treatments had significantly lower survival to hatch (<11%) than controls (58.2%, Table 3; $F_{4, 15} = 25.0$, $P < 0.001$). However, eggs treated with 1% H_2O_2, either once or twice, had a similar survival to hatch as controls. The percentage of deformities was slightly higher for fish from the H_2O_2 treatments (2.2–2.4%) than from controls (1.7%), but the differences were not significant ($P > 0.66$).

There were significant differences among treatments in both the total CFU distribution and the yellow CFU prevalence values. For total CFU data, eggs treated either once or twice with 1% H_2O_2 for 2 min had significantly more bacteria than did controls treated with 100 mg/L iodine (Table 4). The 2,000-mg/L iodine treatments significantly reduced bacterial growth relative to the peroxide treatments, but were not significantly better than the controls (Table 4). The prevalence of yellow CFUs was 27.1–43.8% in the H_2O_2 treatments, which did not significantly differ from each other. Iodine treatment significantly reduced the yellow CFU prevalence relative to H_2O_2 treatment, but prevalence (0.0–2.1%) did not significantly differ among the three iodine treatments (Table 4). Media controls and diluent control plates were all negative for any growth.

Test 3: Rainbow Trout, Eyed Eggs

Survival to hatch of rainbow trout eyed eggs ranged from 77.4% to 79.7% (Table 5) and did not significantly differ among the iodine dose treatments ($F_{2, 9} = 0.78$, $P = 0.17$). The mean percentage of deformities ranged from 5.4% to 6.0% among treatments and also did not significantly differ among treatments ($F_{2, 9} = 0.05$, $P = 0.93$; Table 5).

The bacteriological data indicated that there were significant differences among treatments in both total CFU distributions ($P < 0.001$) and the prevalence of yellow CFUs ($P < 0.001$). For total CFU data, treating eggs twice with 1,000 mg/L of iodine provided significantly fewer eggs with high numbers of bacteria (>100 CFUs) than just treating once with the same dose and duration. Treating eggs twice with 1,000 mg/L iodine also produced significantly more eggs with no CFUs (62.5%) than eggs treated once with either 100 mg/L iodine (14.6%) or 1,000 mg/L iodine (50%; Table 6). Treating eggs once with 1,000 mg/L iodine also led to a significantly higher percentage of eggs with no CFUs compared with control eggs treated with 100 mg/L iodine. The prevalence of yellow CFUs was significantly lower after treating eggs either once or twice with 1,000 mg/L iodine than in controls treated with 100 mg/L iodine (Table 6). The prevalence did not significantly differ between the 1× and 2× 1,000-mg/L iodine treatments.

Test 4: Brown Trout, Eyed Eggs

The survival data for the brown trout eggs indicated that the higher iodine concentrations tested were safe for eyed egg treatment. The survival to hatch of the brown trout eggs treated

TABLE 5. Comparison of survival to hatch and fry deformities (means ± SDs; $n = 4$) for triploid rainbow trout (Gunnison–Harrison strain) among eggs treated at the eyed stage either once or twice (1 h apart) with various doses of iodine (test 3).

Treatment	Survival to hatch (%)	Deformities (%)
1,000 mg/L iodine for 10 min, 1×	79.7 ± 0.7	5.5 ± 0.6
1,000 mg/L iodine for 10 min, 2×	78.5 ± 1.2	5.8 ± 2.3
100 mg/L iodine for 10 min, 1×	79.1 ± 2.0	5.5 ± 0.5

TABLE 6. Comparison of bacterial colony-forming unit (CFU) counts on TYEST media among eggs treated at the eyed stage either once or twice (1 h apart) with various doses of iodine (test 3). See Table 4 for additional details.

Treatment	CFU category			
	0 CFUs	1–100 CFUs	>100 CFUs	Yellow CFUs (%)
1,000 mg/L iodine for 10 min, 1×	24	12	12 y	20.8 z
1,000 mg/L iodine for 10 min, 2×	30	17	1 z	33.3 z
100 mg/L iodine for 10 min, 1×	7	35	6 x	79.2 y

with 1,000 mg/L iodine either once (90.9 ± 0.6%) or twice (92.1 ± 1.0%) did not significantly differ ($F_{2,9} = 1.34$, $P = 0.31$) from eggs treated with the 100-mg/L iodine control treatment (91.0 ± 1.8%). Similarly the prevalence of deformities was not significantly different ($F_{2,9} = 0.54$, $P = 0.60$) between the control (0.3 ± 0.3%) and either the 1× (0.6 ± 0.3%) or the 2× (0.4 ± 0.5%) iodine treatment.

DISCUSSION

A povidone iodine concentration of 100 mg/L for 10 min is the current recommended protocol for salmonid egg disinfection (McFadden 1969; Amend 1974). In the U.S. federal hatchery system, a modification of this protocol is used (30-min treatment with 50 mg/L iodine during water hardening, and 100 mg/L for 10 min afterwards; Cipriano et al. 2001), which is based in part on research by Fowler and Banks (1991). Recent data has shown that egg disinfection is incomplete at iodine doses that are currently used (Kumagai et al. 1998; Cipriano et al. 2001; Wagner et al. 2008; Barnes et al. 2009).

Most previous research on the use of hydrogen peroxide for egg disinfection has focused on testing the efficacy of using daily, low-concentration (<1,000 mg/L) exposures for the control of bacteria and fungus (Dawson et al. 1994; Marking et al. 1994; Gaikowski et al. 1998; Barnes and Gaikowski 2004). Recent evaluations using single exposures at higher concentrations and shorter exposure durations have also been effective at controlling bacterial growth (Peck et al. 2004; Wagner et al. 2010). However, subsequent problems within production-scale tests indicated that 30 g H_2O_2/L for 1 min was too close to the toxic level for rainbow trout eggs (E. Wagner, unpublished data). So, single treatments at concentrations less than 30 g/L need to be explored further. In this study, laboratory-scale tests of 15 g/L H_2O_2 for 2 min indicated that this dose was slightly toxic for eggs of cutthroat trout, regardless of whether eggs were exposed to the dose once or twice. For rainbow trout, a lower dose of 10 g/L for 2 min was safe. However, for both species, bacteria growth was significantly higher in the hydrogen peroxide treatments than in the iodine controls. Slightly higher deformity rates were also a concern, although the percentages observed were within the range typically observed in routine fish culture operations in Utah (Wagner 1996). Since the current protocol leads to fewer bacteria than the H_2O_2 alternative, and going to higher concentrations of H_2O_2 is not an option owing to increased egg mortality, iodine disinfection appears to be preferential to H_2O_2.

Data indicate that iodine has a greater margin of safety than does H_2O_2. Iodine toxicity is pH dependent and decreases with increasing pH (Amend 1974; Alderman 1984). In this study, iodine concentrations of 2,000 mg/L were highly toxic to rainbow trout eggs, but egg survival after treatment with 1,000 mg/L did not significantly differ from controls. The pH level for the 2,000-mg/L treatment was about 5.1 (total hardness, 85 mg/L), whereas at 1,000 mg/L it was about 6.4 (total hardness, 205 mg/L). The lower pH of the 2,000-mg/L treatments possibly contributed to the higher observed mortality. The pH effects of higher iodine doses can be ameliorated with sodium bicarbonate buffer, but the effects of this on bactericidal activity needs further exploration. A 1,000-mg/L iodine concentration significantly reduced bacterial abundance relative to controls treated with 100 mg/L. Consequently, the higher concentration is recommended because of superior bacterial control.

Triploid rainbow trout eggs (induced by heat shock, 20 min at 26.5°C at 20 min after fertilization) were used in two of the experiments (tests 2 and 3). The triploidy process typically produces higher percentages of dead eggs (Happe et al. 1988; Guo et al. 1990), which can contribute to higher bacteria loads. The effect of triploidy per se on egg sensitivity could not be statistically compared in this study, but it could potentially affect egg sensitivity to higher doses of iodine and may have contributed to the high variability observed in the 100-mg/L iodine control. Additional controlled studies are needed to determine whether triploidy affects iodine toxicity. Hydrogen peroxide sensitivity appeared unaffected by triploidy given the high hatch rates in those treatments, but concentrations closer to the threshold level may prove otherwise.

The survival data indicated that treatment of the eggs twice within a short period of time was not harmful to the eggs of cutthroat trout, rainbow trout, or brown trout if iodine concentrations were no more than 1,000 mg/L. Treating twice led to higher numbers of eggs with no recoverable bacteria in tests with cutthroat trout, especially for H_2O_2. For iodine, however, treating twice did not significantly improve bacteria reduction, since the first treatment sufficiently depleted bacteria numbers. Since the eggs were freshly fertilized, there were few bacteria in the cutthroat trout test. Since overall bacteria numbers were very low, the effect of double exposure to chemical was less pronounced. For eyed rainbow trout eggs, which had more bacterial development, treating twice led to significantly fewer overall bacteria when treated with either H_2O_2 or iodine. In our studies, we enumerated the number of colonies that were yellow and consisted of long, thin, Gram-negative rods. We enumerated these colonies because bacteria with these characteristics were possibly *F. psychrophilum*, a species that has caused significant

mortality among salmonids in aquaculture facilities (Cipriano and Holt 2005). Treating twice did not necessarily lead to significant reductions in yellow CFUs in tests with rainbow trout. It is possible that *F. psychrophilum* has a greater resistance to chemical treatment than do other egg-colonizing bacteria. Like other gliding bacteria (Pate and Ordal 1967), *F. psychrophilum* have a thin slime layer that could help protect them in addition to aiding movement and adhesion (Dalsgaard 1993).

The data in this study indicated that treating salmonid eggs twice with either iodine (≤1,000 mg/L) or H_2O_2 (≤10 g/L for 2 min) did not compromise egg survival. Changes in pH with water hardness must be taken into consideration when using higher iodine concentrations, but these can be mitigated by adding buffers, when it is determined that buffering does not affect efficacy. Treating eggs twice significantly reduced overall bacterial abundance and may be helpful in controlling bacterial diseases that may be transmitted by egg transfers. Further testing on a production scale using 1,000 mg/L iodine for treatment at both the brood hatchery (or wild trap) and the receiving hatchery is recommended. These production lots should be monitored to determine whether the egg treatments lead to fewer clinical outbreaks of bacterial diseases. While bacterial coldwater disease may not be controlled directly owing to sequestration within the egg (Brown et al. 1997; Cipriano 2005), the data presented document significant improvements in overall bacterial disinfection of salmonid eggs with higher doses of iodine.

ACKNOWLEDGMENTS

We thank Don Bone and the staff at the J. Perry Egan State Fish Hatchery, Bicknell, Utah, for providing eggs for the study and assisting with the egg disinfection work. Similarly, for the cutthroat trout test, we thank Quentin Bradwisch and his staff at Mantua State Fish Hatchery for providing eggs, space for egg incubation, and assistance during the experiment. We thank Wade Cavender for use of his laboratory for the bacteriology work and Chris Heck for use of his laboratory hood for pouring plates. The work was funded by the Federal Aid in Sportfish Restoration Program (Grant F74-R) and the Utah Division of Wildlife Resources.

REFERENCES

Alderman, D. J. 1984. The toxicity of iodophors to salmonid eggs. Aquaculture 40:7–16.

Amend, D. F. 1974. Comparative toxicity of two iodophors to rainbow trout eggs. Transactions of the American Fisheries Society 103:73–78.

Arndt, R. E., E. J. Wagner, and M. D. Routledge. 2001. Reducing or withholding hydrogen peroxide treatment during a critical stage of rainbow trout development: effects on eyed eggs, hatch, deformities, and fungal control. North American Journal of Aquaculture 63:161–166.

Barnes, M. E., D. Bergmann, J. Jacobs, and M. Gabel. 2009. Effect of *Flavobacterium columare* inoculation, antibiotic treatments, and resident bacteria on rainbow trout *Oncorhynchus mykiss* eyed egg survival and external membrane structure. Journal of Fish Biology 74:576–590.

Barnes, M. E., D. Bergmann, H. Stephenson, M. Gabel, and R. J. Cordes. 2005. Bacterial numbers from landlocked fall Chinook salmon eyed eggs subjected to various formalin treatments as determined by scanning electron microscopy and bacteriological culture methods. North American Journal of Aquaculture 67:23–33.

Barnes, M. E., R. J. Cordes, W. A. Sayler, and R. P. Hanten. 2003. Soft-egg disease in landlocked fall Chinook salmon eggs: possible causes and therapeutic treatments. North American Journal of Aquaculture 65:126–133.

Barnes, M. E., D. E. Ewing, R. J. Cordes, and G. L. Young. 1998. Observations on hydrogen peroxide control of *Saprolegnia* spp. during rainbow trout egg incubation. Progressive Fish-Culturist 60:67–70.

Barnes, M. E., and M. P. Gaikowski. 2004. Use of hydrogen peroxide during incubation of landlocked Chinook salmon eggs in vertical-flow incubators. North American Journal of Aquaculture 66:29–34.

Bozwell, J. L., R. D. Clayton, and J. E. Morris. 2009. Use of hydrogen peroxide to improve golden shiner egg hatchability. North American Journal of Aquaculture 71:238–241.

Brown, L. L., W. T. Cox, and R. P. Levine. 1997. Evidence that the causal agent of bacterial coldwater disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs. Diseases of Aquatic Organisms 29:213–218.

Buller, N. B. 2004. Bacteria from fish and other aquatic animals: a practical identification manual. CABI Publishing, Cambridge, Massachusetts.

Cipriano, R. C. 2005. Intraovum infection caused by *Flavobacterium psychrophilum* among eggs from captive Atlantic salmon broodfish. Journal of Aquatic Animal Health 17:275–283.

Cipriano, R. C., and R. A. Holt. 2005. *Flavobacterium psychrophilum*, cause of bacterial cold-water disease and rainbow trout fry syndrome. U.S. Geological Survey, Fish Disease Leaflet 86, Kearneysville, West Virginia.

Cipriano, R. C., B. M. Novak, D. E. Flint, and D. C. Cutting. 2001. Reappraisal of the federal fish health recommendation for disinfecting eggs of Atlantic salmon in iodophor. Journal of Aquatic Animal Health 13:320–327.

Dalsgaard, I. 1993. Virulence mechanisms in *Cytophaga psychrophila* and other *Cytophaga*-like bacteria pathogenic for fish. Annual Review of Fish Diseases 3:127–144.

Dawson, V. K., J. J. Rach, and T. M. Schreier. 1994. Hydrogen peroxide as a fungicide for fish culture. Bulletin of the Aquaculture Association of Canada 94:54–56.

Douillet, P. A., and G. J. Holt. 1994. Surface disinfection of red drum (*Sciaenops ocellatus* Linnaeus) eggs leading to bacteria-free larvae. Journal of Experimental Marine Biology and Ecology 179:253–266.

Escaffre, A. M., D. Bazin, and P. Bergot. 2001. Disinfection of *Sparus aurata* eggs with glutaraldehyde. Aquaculture International 9:451–458.

Fienberg, S. E. 1989. The analysis of cross-classified categorical data, 2nd edition. MIT Press, Cambridge, Massachusetts.

Fowler, L. G., and J. L. Banks. 1991. A safe level of iodophor for treating eggs of fall Chinook salmon during water hardening. Progressive Fish-Culturist 53:250–251.

Gaikowski, M. P., J. J. Rach, J. J. Olson, R. T. Ramsay, and M. Wolgamood. 1998. Toxicity of hydrogen peroxide treatments to rainbow trout eggs. Journal of Aquatic Animal Health 10:241–251.

Guo, X., W. K. Hershberger, and J. M. Meyers. 1990. Growth and survival of intrastrain and interstrain rainbow trout (*Oncorhynchus mykiss*) triploids. Journal of the World Aquaculture Society 21:250–256.

Happe, A., E. Quillet, and B. Chevassus. 1988. Early life history of triploid rainbow trout (*Salmo gairdneri* Richardson). Aquaculture 71:107–118.

Kumagai, A., C. Hakayasu, and N. Oseko. 2004. Effect of tobramycin supplementation to medium on isolation of *Flavobacterium psychrophilum* from ayu *Plecoglossus altivelis*. Fish Pathology 39:75–78.

Kumagai, A., K. Takahashi, S. Yamaoka, and H. Wakabayashi. 1998. Ineffectiveness of iodophore treatment in disinfecting salmonid eggs carrying *Cytophaga psychrophila*. Fish Pathology 33:123–128.

Marking, L. L., J. J. Rach, and T. M. Schreier. 1994. Evaluation of antifungal agents for fish culture. Progressive Fish-Culturist 56:225–231.

McFadden, T. W. 1969. Effective disinfection of trout eggs to prevent egg transmission of *Aeromonas liquefaciens*. Journal of the Fisheries Research Board of Canada 26:2311–2318.

Mitchell, A. J., A. A. Radomski, D. L. Straus, and R. Carter. 2009. The effect of hydrogen peroxide on the hatch rate and *Saprolegnia* spp. infestation of channel catfish eggs. North American Journal of Aquaculture 71:276–280.

Morrison, C., C. Bird, D. O'Neil, C. Leggiadro, D. Martin-Robichaud, M. Rommens, and K. Waiwood. 1999. Structure of the egg envelope of the haddock, *Melanogrammus aeglefinus*, and effects of microbial colonization during incubation. Canadian Journal of Zoology 77:890–901.

Pate, J. L., and E. J. Ordal. 1967. The fine structure of *Chondrococcus columnaris*: III. the surface layers of *Chondrococcus columnaris*. Journal of Cell Biology 35:37–51.

Pavlov, D. A., and E. Moksness. 1993. Bacterial destruction of the egg shell of common wolffish during incubation. Aquaculture International 1:178–186.

Peck, M. A., L. J. Buckley, L. M. O'Bryan, E. J. Davies, and A. E. Lapolla. 2004. Efficacy of egg surface disinfectants in captive spawning Atlantic cod *Gaddus morhua* L. and haddock *Melanogrammus aeglefinus* L. Aquaculture Research 35:992–996.

Pravacek, J., and M. E. Barnes. 2003. Lack of effect of iodophor on survival of westslope cutthroat trout eggs during water hardening. North American Journal of Aquaculture 65:266–268.

Ross, A. J., and C. A. Smith. 1972. Effect of two iodophors on bacterial and fungal fish pathogens. Journal of the Fisheries Research Board of Canada 29:1359–1361.

Schreier, T. M., J. J. Rach, and G. E. Howe. 1996. Efficacy of formalin, hydrogen peroxide, and sodium chloride on fungal-infected rainbow trout eggs. Aquaculture 140:323–331.

Shaw, R. W., M. L. Kent, and M. L. Adamson. 1999. Iodophor treatment is not completely efficacious in preventing *Loma salmonae* (Microsporidia) transmission in experimentally challenged Chinook salmon *Oncorhynchus tshawytscha* (Walbaum). Journal of Fish Diseases 22:311–313.

Small, B. C. 2009. Evaluation of sodium carbonate peroxyhydrate as a potential catfish egg disinfectant. Journal of Aquatic Animal Health 21:117–123.

Wagner, E. J. 1996. History and fluctuating asymmetry of Utah salmonid broodstocks. Progressive Fish-Culturist 58:92–103.

Wagner, E. J., R. E. Arndt, E. J. Billman, A. Forest, and W. Cavender. 2008. Comparison of the efficacy of iodine, formalin, salt, and hydrogen peroxide for control of external bacteria on rainbow trout eggs. North American Journal of Aquaculture 70:118–127.

Wagner, E. J., R. W. Oplinger, R. E. Arndt, A. M. Forest, and M. Bartley. 2010. The safety and effectiveness of various hydrogen peroxide and iodine treatment regimens for rainbow trout egg disinfection. North American Journal of Aquaculture 72:34–42.

Yamamoto, A., S. Toyomura, M. Saneyoshi, and K. Hatai. 2001. Control of fungal infection of salmonid eggs by hydrogen peroxide. Fish Pathology 36:241–246.