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[Article]

Comparison of the Efficacy of Iodine, Formalin, Salt, and Hydrogen Peroxide for Control of External Bacteria on Rainbow Trout Eggs

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Abstract.—Two experiments were conducted in vivo with eggs of rainbow trout *Oncorhynchus mykiss* to compare the bactericidal ability of four common disinfectants. A third test compared bacterial abundance estimation methods for fish eggs (use of a vortex mixer for agitating an egg versus rolling the egg across a petri dish). In the first test, the number of colony forming units (CFU) counted on enriched Ordahl's agar with tobramycin (EOT) or trypticase soy agar (TSA) was compared among eggs treated with various doses of iodine, hydrogen peroxide, formalin, or rock salt. A treatment of 1,667 mg of formalin/L of water and all iodine, salt, and hydrogen peroxide treatments had significantly fewer bacteria on EOT than did controls, but CFU counts for a formalin treatment of 500 or 1,000 mg/L did not. All chemical treatments significantly reduced CFU counts on TSA relative to controls except salt at 0.030 mg/L and formalin at 500 mg/L. The least growth was observed on iodine-treated eggs. In the second experiment, we evaluated the effect of keeping the eggs suspended (i.e., constantly tumbling) during disinfection to increase chemical contact. Treatments were (1) static application of iodine at 100 mg/L, (2) suspension in iodine at 100 mg/L, (3) static application of iodine at 500 mg/L, (4) suspension in iodine at 500 mg/L, (5) suspension in formalin at 2,000 mg/L, (6) suspension in hydrogen peroxide at 2,000 mg/L, (7) static control, and (8) suspended control. Bacterial abundance was significantly reduced in suspended eggs in some cases but not others. Formalin and hydrogen peroxide reduced bacterial abundance but were inferior to iodine in some cases. Each chemical treatment resulted in the survival of bacteria despite attempts to attain better chemical contact by egg suspension. Comparison of the methods used to estimate total CFU per egg indicated that agitation recovered 71–100% of the bacteria on the outside of the egg. Hatchery managers should be aware that not all bacteria are killed by chemical treatment of eggs, and therefore a significant number of pathogens could still enter a hatchery via the importation of treated eggs.

Fish egg loss due to fungal and bacterial infections has led to egg disinfection research that has been ongoing for decades. Blake (1930) found that phenol and brilliant green were toxic to eggs at concentrations needed to kill the bacterium that causes furunculosis, but found that acriflavine (1:2,000), potassium permanganate, and chlorine were effective if extraneous organic matter was eliminated. Atkinson (1932) used acriflavine (1:2,000 dilution for 25 min) for disinfection of trout eggs and fry. Foster and Woodbury (1936) and Burrows (1949) advocated the use of malachite green as a fish fungicide and antiseptic, but its use was later discontinued due to teratological effects (Meyer and Jorgenson 1983). Watanabe (1940) introduced formalin as an egg disinfectant. Gee and Sarles (1942) noted that chemical concentrations of various compounds required for egg disinfection were often 20 times the concentrations that killed bacteria in vitro. These authors evaluated 13 different disinfectants, including formalin; the lethal concentration to *Aeromonas salmonicida* in the presence of trout eggs was 5,300 mg of formalin/L of water. Bailey and Jeffrey (1989) evaluated 215 candidate fungicides. Other research has indicated that treatment with 2–3% sea salt improved survival of salmonid eggs (Edgell et al. 1993; Marking et al. 1994; Lilley and Inglis 1997).

Although hydrogen peroxide has long been used for external treatment of fish (Mitchell and Collins 1997), its use for treatment of fish eggs has only recently been examined. For eggs of rainbow trout *Oncorhynchus mykiss*, Dawson et al. (1994) noted that application of hydrogen peroxide at 250–500 mg/L for 15 min inhibited fungal growth. Marking et al. (1994), Schreier et al. (1996), and Barnes et al. (1998, 2003b) have also evaluated hydrogen peroxide for salmonid egg disinfection at concentrations ranging from 0.5% to 1.0% for 15–60 min.

Kimura et al. (1976) noted that ultraviolet light treatment of hatchery water supplies reduced bacterial pathogen abundance by 99.99%. More recently, research has been conducted into the effects of

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common disinfectants on a wider range of species (e.g., Salvesen and Vadstein 1995; Bergh and Jelmert 1996; Tendencia 2001).

Tests with viruses indicated that infectious hematopoietic necrosis virus was still recovered from the surface of green or eyed rainbow trout eggs treated with iodine at 100 mg/L for 10 or 60 min, but 99.98% of the virus was destroyed (Goldes and Mead 1995). These authors cited Eskildsen and Vestergaard-Jorgensen (1974), who found similar mortality rates for infectious pancreatic necrosis virus (90%) and viral hemorrhagic septicemia virus (99.9%) after iodine treatment of eggs at 80–100 mg/L.

The standard protocol for salmonid egg disinfection at state hatcheries within Utah is a 10–15-min bath in free iodine at 100 mg/L (1% povidone iodine). The establishment of this protocol was based on work by McFadden (1969), who showed that povidone-iodine at 0.50–1.20% (free iodine: 50–120 mg/L) was superior to acriflavine or merthiolate (mercury-based compound) for controlling the growth of the fish pathogen *Aeromonas liquefaciens*. McFadden's tests were conducted both in vitro (bacteria in water and disinfectant followed by centrifugation and washing three times, then culturing) and in vivo (disinfected eggs placed in a broth culture). In vitro tests by Ross and Smith (1972), in which bacteria culture broth was mixed with a 50-mg/L iodine solution, also indicated successful suppression of many principal bacterial pathogens (*A. liquefaciens*, *A. salmonicida*, *Vibrio anguillarum*, and *Flavobacterium psychrophilum*).

Problems in Utah with coldwater disease, caused by *F. psychrophilum*, have prompted a renewed examination of disinfection efficacy. While *F. psychrophilum* and other bacterial pathogens like *Renibacterium salmoninarum* may be harbored within an egg (Evelyn et al. 1984; Brown et al. 1997; Kumagai et al. 1998; Taylor 2004), control of external bacteria is still desirable to improve egg survival and reduce the risk of introducing other fish pathogens. Recent bacteriological work on rainbow trout eggs by the authors (our unpublished data) has also shown that external bacteria persist despite disinfection. Hence, our objective was to find a disinfectant that would kill 100% of external bacteria without harming the egg.

Methods

Two experiments were conducted to determine the effectiveness of various disinfectants at killing bacteria on the outside of eyed rainbow trout eggs. A third test compared the methodology used in the first two tests for estimating total bacterial abundance on eggs.

Disinfection experiment 1.—In the first experiment, 10 different treatments were evaluated: iodine at 100 and 500 mg/L; formalin at 500, 1,000, and 1,667 mg/L; hydrogen peroxide at 500, 1,000, and 2,000 mg/L; 3.0% NaCl; and an untreated control.

The iodine used was Argentyne (10% povidone-iodine or 1% free iodine). The formalin used was Paracide F (37% formaldehyde, 6–13% methanol; Argent Chemical Laboratories, Redmond, Washington). The concentrations of the stock solutions were not tested, so calculations are based on label concentrations. Hydrogen peroxide test solutions were prepared from a stock solution of 34% hydrogen peroxide. A dilution of the stock solution was tested with a commercial test kit (Hach Company, Loveland, Colorado) to determine actual concentrations. Test stock solutions (2 L) were prepared the day before the experiment. These were cooled to 11°C before use. Eyed eggs of the TenSleep strain of rainbow trout were tested just prior to hatch.

Three replicates were conducted for each treatment; replicates were drawn from successive trays in a vertical incubator (e.g., the top tray [tray 1] = replicate 1, tray 2 = replicate 2, etc.). For each replicate, 30 eggs were removed from the tray with a bulb pipette, poured through a minnow net, and transferred to 500 mL of the test disinfectant solution in a beaker. After 15 min, the solution was poured off and the eggs were rinsed three times with sterile water (about 10–20 mL).

Two media were used to test for bacterial growth: enhanced Ordahl's agar with the antibiotic tobramycin (EOT) and trypticase soy agar (TSA). The EOT medium selects for growth of *F. psychrophilum* (Kumagai et al. 2004). The TSA medium was used to discern prevalence and abundance for all other culturable bacteria and fungi. For each medium, six eggs were used per replicate to infect three agar plates with a divider that split the plate in two. A divided plate permitted more samples to be held within the limited incubator space. Each egg was removed from the beaker with sterile forceps, rolled three times across the petri dish, and then discarded. For each medium, three un-inoculated plates served as media controls.

Plates were incubated at 15°C and examined 2, 4, 7, 9, and 15 d after inoculation. Counts were made of all colony-forming units (CFU) when possible, but many plates were classified as too numerous to count (TNC). Colony descriptions were also recorded. A loop from representative unique colony types was smeared on a second plate to establish isolation colonies that were used for attempted identification. Analytical profile index (API; Biomérieux, Inc., Durham, North Carolina) methods were used to identify isolates from the distinct colony types observed. Gram stains were also made of each isolate.

For statistical analysis, the CFU counts were

classified into four categories: 0, 1–100, 101–300, and greater than 300. Categorical data analysis (chi-square test) was used to compare the CFU frequency in each classification among the treatments. Partial tables were used to compare (1) each treatment with the inoculated control, (2) doses within a treatment, and (3) the 500-mg/L iodine treatment with the highest salt, formalin, and peroxide treatments. In addition, rank-transformed CFU data for each media type were analyzed by analysis of variance (ANOVA) to compare among the 10 chemical dose treatments. Least-significant-difference tests were used for subsequent mean comparisons.

Disinfection experiment 2.—The results of the first study indicated that bacteria were still abundant after chemical treatment. One subsequent hypothesis was that in a static disinfection scenario, the points of contact between eggs are less likely to be affected by the chemical. Therefore, eggs need to be kept in suspension (i.e., a constant, tumbling movement) during chemical treatment to ensure that exposure is consistent across the egg's outer surface. Eggs were suspended within an egg jar during the second experiment to see whether this would reduce CFU relative to static controls. Treatments were (1) static application of iodine at 100 mg/L, (2) suspension in iodine at 100 mg/L, (3) static application of iodine at 500 mg/L, (4) suspension in iodine at 500 mg/L, (5) suspension in formalin at 2,000 mg/L, (6) suspension in hydrogen peroxide at 2,000 mg/L, (7) static control, and (8) suspended control.

The tests were conducted in McDonald-type, clear-sided acrylic egg jars. A recirculation system was devised in which a submersible pump delivered solution to the center inflow pipe of the egg jar; overflow from the jar returned to the bucket in which the pump was located. A valve controlled the flow to the jars (3–4 L/min), and flow was adjusted to gently move the eggs. Total volume of the chemical treatment solution was 12 L and temperature was 13°C. Fresh chemical was added for each of the three replicates. For each replicate, 2,227 eggs (180 mL) were transferred by net to the jar. The duration of exposure was 15 min for each treatment. The eggs were subsequently poured into a new net (separate nets were used for each chemical). About 15 eggs were collected into sterile glass vials and covered with aluminum foil; the remaining eggs were transferred to an egg tray for further monitoring of hatch and crippling rates. Survival to hatch was calculated as a percentage of the initial number of eyed eggs (2,227 eggs), and percent deformity was calculated as a percentage of the number surviving at hatch.

The methodology used in experiment 1 (i.e., rolling the egg across the media) made it difficult to get good estimates of bacterial abundance. In experiment 2, a different approach was taken. Following the methodology of Barnes et al. (2005), we used a vortex mixer to vigorously agitate the eggs for 2 min in 2 mL of sterile peptone–salt diluent (0.1% proteose peptone and 0.8% NaCl); 100 μL of this solution was plated on TSA or EOT. In addition, 100 μL of a 10-fold dilution was also plated on both media. Sterile forceps were used to transfer each egg to a test tube with the diluent. Sterile pipette tips were used for the dilutions and the transfer to plates. Parafilm was used to wrap each petri dish after a sterile spreader distributed the liquid across the plate as it spun on a dish turntable. Plates were incubated at 15°C, and CFU were counted at 3, 5, 7, and 11 d after inoculation. Plates with too many bacteria to count or that were overgrown were designated TNC. Observations of colony morphology and size were recorded, and gram stains were made of representative colony types.

For statistical analysis, the TNC plates were assigned a value of 10,000 CFU. Since this was an arbitrary value, total CFU were rank transformed for analysis. Separate analyses were conducted for each level of dilution and each media type. Since the formalin and peroxide treatments lacked a static control and the suspension effects were best tested within a given treatment, comparison of chemical treatment effects were tested separately (one-way ANOVA of rank-transformed CFU) for static and suspended groups. The effects of egg suspension were analyzed by a chi-square analysis (maximum likelihood statistic) comparing the frequency distribution of total CFU per egg within four categories: 0, 1–100, 101–300, and greater than 300 CFU; these tests were conducted separately for each combination of dilution, medium, and treatment dose. The percent hatch and deformity data were nonnormally distributed based on Kolmogorov–Smirnov and Shapiro–Wilk normality tests; these data were therefore analyzed with the Kruskal–Wallis test in which combinations of chemical, dose, and suspension were combined into the eight treatments listed above (e.g., suspension in iodine at 100 mg/L, static application of iodine at 500 mg/L, etc.).

Disinfection experiment 3.—For the third experiment, the objective was to understand the relationship between the different bacteria enumeration techniques used in experiments 1 and 2 (i.e., rolling of the egg across the media or agitation of egg samples). Experiment 3 was conducted using rainbow trout eggs in the eyed stage of development, 9 d prior to hatch. The following treatments were evaluated: (1) agitated only, (2) agitated and rolled, and (3) rolled only. For the agitated sample, each egg was agitated by a laboratory vortex mixer in 2 mL of sterile peptone–salt

TABLE 1.—Number of rainbow trout eggs in each of four total colony-forming-unit (CFU) categories after a 15-min exposure to various chemical treatments and rolling on either enriched Ordahl's agar with tobramycin (EOT) or trypticase soy agar (TSA) medium. Mean ranks that are significantly different among chemical treatment–dose combinations within a media type are followed by different letters.

		CFU category				
Medium and treatment	Dose (mg/L)	0	1–100	101–300	>300	Mean rank-transformed CFU
EOT						
Iodine	100	0	7	1	10	74.2 xwv
	500	3	5	0	10	67.9 xwv
Salt	30,000	0	8	2	7	62.3 v
Formalin	500	0	0	0	18	115.0 z
	1,000	0	0	0	18	115.0 z
	1,667	0	0	4	14	99.7 zy
Hydrogen peroxide	500	0	1	5	12	90.3 yx
	1,000	0	3	3	12	87.5 yw
	2,000	1	6	1	10	71.4 xw
Control	0	0	0	0	18	115.0 z
TSA						
Iodine	100	2	15	1	0	27.9 u
	500	2	5	3	8	74.7 xwv
Salt	30,000	0	0	0	18	128.5 z
Formalin	500	0	0	0	18	128.5 z
	1,000	0	5	2	11	100.6 y
	1,667	0	10	2	6	78.3 yv
Hydrogen peroxide	500	0	10	0	8	80.0 yw
	1,000	0	10	0	8	75.8 xwv
	2,000	0	9	0	9	82.1 yx
Control	0	0	0	0	18	128.5 z

diluent as noted in experiment 2. Rolled eggs were rolled across the media plate three times as in experiment 1. Agitated-and-rolled samples were the same eggs mixed in treatment 1, but were transferred by sterile forceps to a petri dish and rolled as in treatment 3. For each treatment, 10 eggs were sampled from an egg jar that received a prophylactic formalin treatment of 1,667 mg/L daily for 15 min. The eggs were first treated with iodine at 100 mg/L for 10 min and rinsed well with sterile water. The media plates (TSA and EOT) were inoculated with 100 μL of the agitated sample and then spread on a petri dish with the aid of dish turntable and a sterile plastic spreader. Media plates without inoculum ($n = 3$ plates/media type) or with diluent only ($n = 2$ plates/media type) served as controls. Plates were examined 2, 4, 6, 9, and 13 d after inoculation, and CFU and colony morphology were recorded.

For statistical analysis, the total number of bacterial colonies was analyzed. For the agitated samples, counts were adjusted values obtained by multiplying CFU by 20 (to account for the dilution). For the rolled samples, total bacteria were unadjusted counts of CFU. For plates that were TNC, an arbitrary value of 10,000 was assigned. The CFU data were rank transformed within each media type. Since the undiluted, agitated samples had few CFU, thus allowing estimation of total bacteria, these data were used for comparison with the other techniques. The ANOVA of rank-transformed data was followed by mean comparisons using the least-significant-difference test. A significance level of 0.05 was used for all tests.

Results

Disinfection Experiment 1

The chi-square and ranked CFU analyses both indicated significant differences between treatments for both media in experiment 1 ($P < 0.001$; Table 1). On the EOT plates, samples from all the iodine, salt, and hydrogen peroxide treatments had significantly fewer bacteria than the control sample. Formalin at 1,667 mg/L significantly reduced bacterial loads, but the CFU counts for eggs treated with 500 and 1,000 mg/L did not significantly differ from those of control eggs. On the TSA plates, all chemical treatments (except salt and formalin at 500 mg/L) significantly reduced CFU relative to the control sample.

Iodine proved to be among the best disinfectants tested; however, on EOT, 10 of 18 eggs had plates that were TNC and only 3 eggs had 0 CFU (Table 1). On EOT, iodine at 500 mg/L iodine was significantly better than salt ($P = 0.043$) and formalin at 1,667 mg/L ($P = 0.003$) but did not significantly differ from either iodine at 100 mg/L ($P = 0.118$) and hydrogen peroxide at 2,000 mg/L ($P = 0.471$).

On TSA, only 2 eggs had 0 CFU, and 8 of 18 had plates that were TNC (Table 1). Eggs treated with iodine at 500 mg/L had significantly fewer CFU on TSA than

TABLE 2.—Mean ranks of colony-forming units on two media types (enriched Ordahl's agar with tobramycin [EOT] or trypticase soy agar [TSA]) at two dilutions (0 or 10-fold) for rainbow trout eggs statically treated with iodine in egg jars for 15 min. Within rows, means followed by the same letter are not significantly different.

Dilution and medium	Control	Iodine at 100 mg/L	Iodine at 500 mg/L
0 dilution			
EOT	528.0 z	161.3 y	186.4 y
TSA	488.6 z	151.4 y	188.6 y
10-fold dilution			
EOT	483.4 z	178.6 y	170.3 y
TSA	505.6 z	237.0 y	151.4 x

those treated with either salt ($P < 0.001$) or hydrogen peroxide at 2,000 mg/L ($P = 0.043$) but did not differ from those treated with formalin at 1,667 mg/L.

Gram staining of eight different isolates representing the diversity of bacteria that grew on either TSA or EOT indicated that most were gram-negative rods or cocci. However, a gram-positive diplococcus (2×1 μm) was found in a slow-growing, shiny, pink colony with a smooth, round margin isolated on EOT media from eggs treated with iodine at 500 mg/L. The API results were inconclusive, so the data are not presented here.

Differences among doses within a treatment were significant for formalin (EOT: $P = 0.009$; TSA: $P = 0.003$) but not hydrogen peroxide (EOT: $P = 0.154$; TSA: $P = 0.928$). The number of CFU significantly differed between the two iodine doses on TSA plates but not on EOT plates.

The data showed that plenty of bacteria are still present after disinfection using the standard protocol of iodine at 100 mg/L. Unfortunately, the other treatments evaluated also failed to completely disinfect the eggs.

Disinfection Experiment 2

Chemical treatment in experiment 2 significantly reduced bacterial numbers, but differences among chemicals were few. Comparisons among chemicals for each dilution, medium, and suspension treatment indicated that each treatment significantly reduced CFU relative to those of controls (Tables 2, 3). For iodine, formalin, and hydrogen peroxide, bacterial abundance was reduced by 99% relative to controls. For eggs treated statically, the differences between the two iodine doses were not significant in undiluted samples for both media, but the 10-fold dilutions on TSA resulted in significantly fewer CFU for the 500-mg/L treatment than for the 100-mg/L treatment (Table 2). For suspended eggs, iodine dose differences were not significant for any dilution or medium. On EOT plates without dilution, CFU were significantly lower for the 100-mg/L treatment with iodine than for the 2,000-mg/L treatments with formalin or hydrogen peroxide. However, after 10-fold dilution, the four chemical treatments did not differ. For undiluted samples on TSA plates, formalin-treated eggs had significantly more CFU than eggs treated with iodine at 500 mg/L or hydrogen peroxide at 2,000 mg/L (Table 4). After dilution, however, no significant differences were observed among the chemical treatments. Untreated eggs had an average of 31,744 bacteria (median = 18,200) based on extrapolation of EOT cultures and an average of 41,267 bacteria (median = 33,200) on TSA.

Gram staining of seven representative colony types indicated that five were gram-negative rods and one tangerine-colored colony on TSA was a gram-positive diplococcus measuring 0.8–1.0 μm across the long axis. In addition, a yellow colony isolated from suspended eggs treated with iodine at 100 mg/L had both gram-positive and gram-negative cocci. The gram-negative colonies were predominately shiny, cream-colored, and either (1) thinly spreading with irregular margins (0.5–0.8-μm cocci) or (2) slower growing with smooth margins (3–4-μm-long rods). Other gram-negative rods (1–2 μm long × 0.2–0.3 μm wide) were found in yellow, thin-spreading colonies. Because the API methods did not provide conclusive identification, those data are not shown.

Bacterial abundance was significantly, but inconsistently, reduced in suspended eggs. On EOT with or without dilution, significantly fewer CFU were present for control eggs treated in suspension than for control eggs treated statically (Table 4). For controls on TSA,

TABLE 3.—Mean ranks of colony-forming units on two media types (enriched Ordahl's agar with tobramycin [EOT] or trypticase soy agar [TSA]) at two dilutions (0 or 10-fold) for rainbow trout eggs that were suspended in egg jars during chemical treatment for 15 min. Within rows, means followed by the same letter are not significantly different.

Dilution and medium	Control	Iodine at 100 mg/L	Iodine at 500 mg/L	Formalin at 2,000 mg/L	Hydrogen peroxide at 2,000 mg/L
0 dilution					
EOT	492.0 z	196.2 x	236.7 yx	288.4 y	261.0 y
TSA	480.9 z	259.9 yx	213.5 x	318.3 y	241.8 x
10-fold dilution					
EOT	435.9 z	183.8 y	197.0 y	239.2 y	223.4 y
TSA	478.9 z	196.1 y	193.7 y	228.5 y	231.5 y

TABLE 4.—Frequency distribution of colony-forming units (CFU) for rainbow trout eggs that were suspended in egg jars or statically treated with 100 or 500 mg/L iodine or no chemical (control). Eggs were agitated in a diluent that was plated on two media types (enriched Ordahl's agar with tobramycin [EOT] or trypticase soy agar [TSA]) without dilution or after 10-fold dilution. Probability values for chi-square maximum likelihood tests are shown for the comparison between static and suspended treatments.

Dilution, medium, and treatment	0 CFU	1–100 CFU	101–300 CFU	>300 CFU	χ^2 probability
0 dilution					
EOT					
Control					
Suspended	0	4	7	7	0.002
Static	0	0	2	16	
Iodine, 100 mg/L					
Suspended	13	5	0	0	0.200
Static	16	2	0	0	
Iodine, 500 mg/L					
Suspended	9	9	0	0	0.080
Static	14	4	0	0	
TSA					
Control					
Suspended	0	7	2	9	0.226
Static	0	7	0	11	
Iodine, 100 mg/L					
Suspended	10	7	0	1	0.016
Static	17	1	0	0	
Iodine, 500 mg/L					
Suspended	11	7	0	0	0.275
Static	14	4	0	0	
10-fold dilution					
EOT					
Control					
Suspended	0	16	2	0	<0.001
Static	0	3	8	7	
Iodine, 100 mg/L					
Suspended	14	4	0	0	0.178
Static	15	3	0	0	
Iodine, 500 mg/L					
Suspended	13	5	0	0	0.200
Static	16	2	0	0	
TSA					
Control					
Suspended	0	6	7	5	0.026
Static	0	1	5	12	
Iodine, 100 mg/L					
Suspended	13	5	0	0	0.296
Static	10	8	0	0	
Iodine, 500 mg/L					
Suspended	13	5	0	0	0.063
Static	17	1	0	0	

egg suspension had no effect for undiluted samples or 10-fold dilutions. Suspension had no significant effect for eggs treated with iodine at either 100 or 500 mg/L and plated on EOT. On TSA, CFU were lower for suspended eggs treated with iodine at 100 mg/L (no dilution) than for static-treated eggs; however, after sample dilution, the CFU did not differ. For eggs treated with iodine at 500 mg/L and plated on TSA, the CFU did not differ between suspended and static eggs at either dilution.

Chemical treatment had no deleterious effects on the eggs: we found no significant treatment effect on eyed egg survival to hatch (91.9–95.9% among treatments; $P = 0.422$) or deformity percentage (0.26–0.66% among treatments; $P = 0.595$).

Disinfection Experiment 3

In experiment 3, there were significant differences in bacterial counts among treatments for both EOT and TSA samples. On EOT, there were significantly more bacteria in the rolled samples than in the agitated and agitated–rolled treatments, which did not differ from each other (Table 5). On TSA, all three treatments differed; the highest bacterial counts were in the rolled treatments and the lowest counts were in the agitated–rolled treatments (Table 5). Bacterial abundance per egg was generally low in all treatments on EOT (0–20 bacteria/egg) and highly variable on TSA (0–12,160 bacteria/egg). The proportion of bacteria released from an egg after agitation was determined for each egg by dividing the agitated sample abundance by the sum of

TABLE 5.—Mean ± SE rank and frequency distribution of total bacteria counts on two media types (enriched Ordahl's agar with tobramycin [EOT] or trypticase soy agar [TSA]) from sampled rainbow trout eggs that were agitated in a vortex mixer, rolled across a petri dish, or both. Within a medium, means followed by the same letter are not significantly different.

		Total bacteria frequency				
Medium and method	Mean rank of total bacteria	0	1–10	11–100	101–9,999	≥10,000
EOT						
Agitation	14.65 ± 3.13 y	7	0	3	0	0
Rolling	22.35 ± 1.00 z	0	10	0	0	0
Agitation–rolling	9.50 ± 1.00 y	9	1	0	0	0
TSA						
Agitation	16.45 ± 2.13 y	1	0	8	0	1
Rolling	23.70 ± 1.30 z	0	0	1	0	9
Agitation–rolling	6.35 ± 0.99 x	3	7	0	0	0

agitated and agitated–rolled estimates. The percentages derived from that calculation indicated that use of a vortex mixer releases a high proportion of the bacteria attached to the egg; on EOT plates with bacterial growth, 95–100% of the bacteria were in the agitated portion. On TSA, 71–95% of the bacteria were in the agitated portion.

Discussion

Egg disinfection has two purposes. One is to reduce the overall abundance of fungi and bacteria that may affect egg respiration and survival (Barker et al. 1989; Barnes et al. 1999, 2003a, 2005). The other is to reduce or eliminate pathogens that may affect egg and fry survival and compromise the health certification of a hatchery. The results of this study indicate that the current practices of formalin prophylactic treatment and iodine treatment achieve the first objective but do not result in complete elimination of external bacteria. This is important for situations in which hatcheries receive eggs from wild sources or from other hatcheries.

Comparisons among the chemicals tested in this study indicated that iodine was superior to salt, formalin, and hydrogen peroxide for bacterial disinfection. However, little benefit was obtained by increasing the iodine concentration from 100 to 500 mg/L. In contrast, Wright and Snow (1975) noted that *A. liquefaciens* was still recovered from eggs of largemouth bass *Micropterus salmoides* after treatment with iodine at 100 mg/L but not after treatment with 200 mg/L. Gee and Sarles (1942) noted that iodine was lethal to *A. salmonicida* at 56 mg/L in a 10-min treatment of trout eggs. Cipriano et al. (2001) reported that *A. salmonicida* were not found on eggs of Atlantic salmon *Salmo salar* treated with iodine at 30 mg/L during water hardening followed by a secondary treatment with 100 mg/L. Tests with *Staphylococcus aureus* indicated that iodine at 50 mg/L stopped bacterial growth (Salle and Lazarus 1935). Safety tests have indicated that during water hardening, an iodine dose of 100 mg/L or more may compromise egg survival (Leary and Pederson 1988; Fowler and Banks 1991), although research with cutthroat trout *O. clarkii* has indicated that treatments with up to 125 mg/L for 30 min during water hardening is not harmful (Pravecek and Barnes 2003). After water hardening, iodine was safe for eggs of rainbow trout (Amend 1974) and muskellunge *Esox masquinongy* (Schachte 1979) at 100 mg/L.

Use of higher iodine doses appears to be feasible based on previous studies. Toxicity tests by Amend (1974) noted that the lethal dose depends on pH and the stage of egg development; for example, at pH 6.9 the active iodine concentration that was lethal to 50% of test eggs (LC50) was 1,480 mg/L in a 15-min exposure, whereas at pH 7.0 or 8.0 the LC50 was more than 2,000 mg/L. For eyed rainbow trout eggs exposed to iodine for 10 min, Alderman (1984) found that the LC50 was about 800 mg/L at pH 6.0 and over 3,000 mg/L at pH 7.0. For eggs of red drum *Sciaenops ocellatus*, the no-observable-effect (NOE) concentration of povidone iodine was 3,000 mg/L (Douillet and Holt 1994).

In our study, hydrogen peroxide concentrations up to 0.2% significantly reduced bacterial abundance without significantly reducing egg survival. However, Gaikowski et al. (1998) found that rainbow trout eggs exposed for 15 min to 1% or 3% hydrogen peroxide had higher mortality than those exposed to 0.5%. Gaikowski et al. (1998) and Arndt et al. (2001) noted that rainbow trout eggs treated prophylactically with hydrogen peroxide survived best when the chemical was withheld during a critical development period (70–140 temperature units [°C]). Douillet and Holt (1994) successfully used 3% hydrogen peroxide for 5 min to disinfect red drum eggs, but longer exposure durations significantly reduced survival. Other species that they tested had lower NOE concentrations: 1% hydrogen peroxide for yellowtail snapper *Ocyurus chrysurus* and 2% hydro-

gen peroxide for spotted seatrout *Cynoscion nebulosus* (Douillet and Holt 1994). Rach et al. (1998) evaluated the effect of hydrogen peroxide on eggs of northern pike *E. lucius*, walleyes *Sander vitreus*, yellow perch *Perca flavescens*, white suckers *Catostomus commersonii*, lake sturgeon *Acipenser fulvescens*, paddlefish *Polyodon spathula*, common carp *Cyprinus carpio*, and channel catfish *Ictalurus punctatus*. For all species, survival was greatest for eggs treated with 1% hydrogen peroxide for 5 min, whereas a concentration of 3% or 6% reduced hatching success. The above studies indicate that concentrations of hydrogen peroxide greater than 2% may result in increased egg mortality. Although we found that hydrogen peroxide treatment significantly reduced bacterial abundance, other chemicals or combinations of chemicals may be needed to completely eliminate bacteria from egg surfaces. Application of higher doses for shorter durations should also be evaluated.

In this study, formalin concentrations up to 2,000 mg/L reduced bacterial abundance but was inferior to iodine treatment in some cases. Bacteria survived after formalin treatment despite our attempts to improve chemical contact by suspending the eggs. Subasinghe and Sommerville (1985) noted that formalin concentrations of 500–2,000 mg/L improved egg survival in tilapia *Oreochromis mossambicus*, but even higher survival was provided by iodine and acriflavine. Formalin has been effective in controlling fungal growth on eggs in several studies (Schreier et al. 1996; Barnes et al. 2000; Rach et al. 2005) at concentrations as low as 250 mg/L (Cline and Post 1972; Marking et al. 1994). However, based on a fungus mortality curve generated by Oláh and Farkas (1978), a formalin treatment of about 20 min at 2,000 mg/L was required to obtain 100% fungal mortality. In our study, formalin concentrations of 500–1,000 mg/L were ineffective for reducing bacterial abundance. In a study of Chinook salmon *O. tshawytscha*, Barnes et al. (1999) similarly noted that despite prophylactic treatment with formalin at 1,667 mg/L, bacterial abundance increased from the top to the bottom incubation trays and from fertilization to the eyed egg stage. Higher doses may be possible, but further testing is needed. Rach et al. (1997) noted that the toxicity of formalin to eggs varied with species: walleyes tolerated 7,500 mg/L, whereas lake sturgeon all died at 4,500 mg/L or higher concentrations.

We found that salt concentrations of 3.0% failed to significantly reduce bacterial abundance in certain cases. However, Edgell et al. (1993) noted that 2.0–2.5% sea salt significantly improved survival of Chinook salmon eggs relative to untreated controls. Marking et al. (1994) found 3.0% salt to be effective at controlling fungal growth on rainbow trout eggs. In a study by Lilley and Inglis (1997), 3.0% salt was effective at controlling or inhibiting fungal growth of 54 isolates. For bacterial control, Suomalainen et al. (2005) found that 2.0–4.0% salt significantly reduced *F. columnare* abundance in vitro but failed to reduce rainbow trout mortality in vivo. The data indicate that salt is helpful for controlling fungi but not as useful for control of bacteria.

Comparison of bacterial enumeration methods indicated that egg agitation with a vortex mixer removed most, but not all, of the surface bacteria. Given the percentages of bacteria removed from eggs, a correction factor of 1.06 could be applied to vortex method counts to estimate actual bacterial abundance (i.e., $1.06 \times$ vortex estimate = true estimate). When a high bacterial number is expected, the agitation method would be preferred to rolling the egg across the plate. The drawback of the latter method is that too many bacteria can adhere in one location on the plate, making counts difficult. Barnes et al. (2005) used scanning electron microscopy (SEM) to count bacteria and found that the bacterial abundance estimate was 40–120 times greater than estimates based on plate culture. The SEM method allows for direct counts of bacteria, but both live and dead bacteria would be included, making interpretation of any disinfection treatment difficult.

Suspension was most effective on untreated eggs sampled on EOT plates, indicating that it works better for some bacteria species than for other, more adhesive species. After chemical treatment, bacterial abundance was low enough that the effect of egg suspension was not detectable. The only exception was for eggs treated with iodine at 100 mg/L, sampled without dilution, and plated on TSA; enough bacteria survived this treatment that suspension contributed an additional reduction in CFU. Given that egg suspension only occurred for 15 min, follow-up studies are needed to evaluate the bacteriological aspects of maintaining continuous suspension from the eyed egg stage to hatch. Sloan (1996) observed that the suspension of eyed salmonid eggs reduced fungal infections and yielded better survival, but bacteriology was not studied. The suspension effects that we observed suggest the potential for physically reducing bacterial numbers, if not completely removing bacteria, on egg surfaces. Removal could employ tiny beads or other small particles to knock off or destroy bacteria during recirculation and upwelling flow in the interstitial spaces between eggs.

Opportunities for further research include the identification of more effective doses, mechanical strategies, or novel approaches, such as use of nonpathogenic bacteria that can compete with patho-

genic species. Until the results of such evaluations become available, hatchery personnel should (1) take care in the disposal of possibly infected water used to transport eggs originating from outside the hatchery, (2) use proper treatment ratios of chemical volume to egg volume (e.g., 4:1 for iodophors; Chapman and Rogers 1992), (3) mix new solutions when treating multiple batches, and (4) circulate the chemical during treatment.

The data suggest that eggs transferred into a hatchery from wild sources bring whatever bacteria are present in the wild, albeit at lower concentrations. Therefore, it might be wise to dedicate certain hatcheries to particular wild brood programs rather than scattering the egg take among several locations or transferring the eggs to a hatchery with broodstock. Managers will need to consider the risks and benefits of bringing in eggs with bacteria from other sources on a case-by-case basis. We recommend continued use of iodine at 100–500 mg/L for disinfection and formalin for prophylactic treatment and fungus control until future research suggests a better protocol. Continued monitoring of prohibited pathogens in the wild population via annual disease inspections is also recommended.

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