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Brief Report

Different Iodine Concentrations Impact Walleye (*Sander vitreus*) Egg Survival and the Number of Bacteria on the Chorionic Membrane

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Abstract: Disinfection of fish eggs with iodophor is a common biosecurity procedure. This study evaluated the effects of three concentrations of iodine on walleye (*Sander vitreus*) egg survival and bacterial loadings. Approximately five hours post fertilization, eggs from ten female walleyes were disinfected in active iodine concentrations of 0, 100, 200, and 400 mg/L for 10 min. There was a significant decrease in survival in the 200 mg/L treatment group compared to the 0 mg/L (control) and 100 mg/L groups. Near-total mortality occurred in the eggs receiving the 400 mg/L disinfection regime. Bacterial Colony Forming Units (CFU) were significantly reduced with any iodine disinfection compared to the control, but there was no significant difference in CFU among any of the iodine treatment concentrations. There was no relationship between egg survival and either pre- or post-disinfection CFU levels. These results indicate that 10 min treatments of active iodine at a concentration of 100 mg/L can be safely used to reduce bacterial loadings on newly spawned walleye eggs, but complete disinfection will not occur. Higher iodine concentrations, which lead to walleye egg mortality, do not further decrease bacterial numbers.

Keywords: iodine; egg disinfection; walleye; *Sander vitreus*

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1. Introduction

The chorionic membrane surrounds fish eggs [1]. As the primary defense against biological and environmental factors that could harm the fish embryo, this external membrane can be infected with microorganisms [2]. When eggs are shipped among production fish hatcheries, bacteria, fungi, or viruses attached to the chorion can then be transferred [3]. To reduce the risk of such pathogenic microorganism transfer, fish eggs are commonly disinfected [4–6]. Several chemicals have been evaluated as potential egg disinfectants [7]. Hydrogen peroxide and formalin have been used [8–13], but iodophors (iodine with a solubilizing agent so that free iodine is released in the solution) have become the standard [14]. In comparison to other potential disinfectants, iodophor is relatively safe for both fish eggs and hatchery employees, as well as effective and insensitive to changes in water chemistry or temperature [4,12,15–17]. Research has examined iodophor egg disinfection with different concentrations, treatment durations, and treatment frequencies [9,10,12,18–20]. However, the United States Food and Drug Administration regulates iodine as a drug with Low Regulatory Priority Status in the United States, allowing fish eggs to be treated with concentrations at or below 100 mg/L of active iodine for 10 min [21]. It should be noted that, although these treatments are typically called disinfection, they do not kill all microorganisms on the chorionic surface [9,22–24].

Disinfection is an established and routine practice with salmonid eggs [5,15,18,25–29]. However, limited research has been carried out on egg disinfection with non-salmonid species of fish, such as white yellowtail (*Seriola dorsalis*), white seabass (*Atractoscion nobilis*), California halibut (*Paralichthys californicus*), and walleye (*Sander vitreus*) [11,30–33]. Both within salmonid species and among different fish species, iodophor's disinfection efficacy and impacts on egg mortality are likely species-specific [18,30,32,34]. Walleyes are part of the Percidae family. Their native range includes most of Canada and the United States south of Arkansas and Alabama [35]. Walleyes are a popular sport fish, with the artificial spawning of wild stocks and hatchery propagation routinely used to establish and maintain fishable populations [36–39]. Unlike salmonid eggs, disinfection is not a normal practice. Two studies evaluating iodophor disinfection of walleye eggs raised concerns about its efficacy in reducing or eliminating specific pathogenic microbes [31,33]. Two other studies examining iodophor disinfection's effects on walleye egg survival either used longer (non-standard) treatment durations or took place during the developmental stage of egg water-hardening [11,30]. No studies have examined the impacts of iodophor disinfection on bacterial numbers adhering to the chorionic membrane or the effects of such disinfection on post-water-hardened egg survival.

Given the importance of walleye as a sport fish, the prevalence of walleye egg incubation and hatchery rearing, and the potential for spreading pathogenic microbes, additional information is needed on the use of iodophor as a walleye egg disinfectant. Thus, the objective of this study was to evaluate the effects of different iodophor concentrations on walleye egg survival and microbial disinfection efficacy.

2. Materials and Methods

2.1. Egg Collection

Walleye eggs were obtained from spawning operations at Merritt Reservoir, rural Valentine, Nebraska, USA, on 9 April 2024. Eggs were stripped from ten female walleye, fertilized with milt from wild males, and adhesion was removed using Fuller's earth. The eggs were water-hardened for one hour and then transported (four hours) to McNenny State Fish Hatchery, rural Spearfish, South Dakota, USA. Just prior to transport, approximately 20 mL of eggs from each female was place into a 946 mL plastic bag containing 60 mL of lake water. Eggs from each female were thus maintained discretely during transportation.

2.2. Disinfection Treatments

Upon arrival at McNenny Hatchery, the eggs from each female were split into four approximately equal-sized groups and subjected to one of four povidone iodine 10 min treatments with buffered free iodine (Ovadine, Syndel USA, Ferndale, WA, USA). The treatment concentrations used were 0 (control, no disinfection), 100, 200, or 400 mg/L active iodine.

2.3. Incubation and Survival

After the 10 min treatment, 40 eggs were removed from each group, rinsed, and split equally between two 9.5 cm diameter Petri dishes (20 eggs/dish) filled with 30 mL of well water (17 °C; total hardness 360 mg/L $CaCO_3$; alkalinity as $CaCO_3$, 210 mg/L; pH 7.6; and total dissolved solids 390 mg/L) for incubation using the technique described by Voorhees et al. [40]. During incubation, the Petri dishes were placed in a refrigeration unit (Antarctic Star, model JC-90VEL-F, Chengdu, China). Water was not exchanged during initial development. However, water was exchanged daily after the eyed stage of egg development (when eye pigment could be easily observed). Dead eggs were removed and recorded every three days, while hatched fry were removed and recorded daily. Hatching

began on incubation day 8 and concluded on day 13. Percent survival to hatch was calculated using the following equation.

$$\text{Survival (\%)} = 100 \times \frac{\text{number of hatched fry}}{\text{initial number of eggs}}$$

Survival was calculated for each of the two paired Petri dishes from each female for each treatment. The mean from the two dishes was then used for subsequent statistical analysis.

2.4. Bacteriology

To collect post-disinfection CFU data, approximately 400 eggs (2 mL) were removed from each disinfection treatment group after iodophor treatment, rinsed in sterile water, and placed into a 50 mL plastic centrifuge tube containing sterilized water and 500 mg/L MS-222 (Tricaine-S, tricaine methane sulfonate, Syndel USA, Ferndale, WA, USA). All tubes were then transported for 15 min to Black Hills State University in Spearfish and stored at 4 °C overnight before bacterial sampling.

After arrival at Black Hills State University, the eggs were rinsed four times in sterile phosphate-buffered saline (PBS) solution in a sterilized sieve, added to 3.0 mL sterile PBS, and vortexed vigorously for five minutes in sterile 15 mL Falcon centrifuge tubes. The remaining vortexed fluid was transferred to two sterile 1.5 mL Eppendorf micro-centrifuge tubes and micro-centrifuged at 10,000 rpm for ten minutes. The supernatant was removed with a sterile 1.0 mL pipette tip and placed into sterile tubes. An aliquot of 100 µL of each sample was spread onto plates with 50 µg/mL cycloheximide-infused nutrient agar (two plates per sample). The cycloheximide-infused nutrient agar plates were incubated for four days at 20 °C. Bacterial CFUs were counted, and the colony morphology was recorded.

2.5. Statistical Analysis

Data analysis was conducted using the SPSS (24.0) statistical analysis program (IBM, Armonk, NY, USA). A linear mixed model was used to examine the differences in survival and bacterial numbers between treatment, with the treatment as the fixed factor and the female as the random effect. Linear regression analysis was conducted to examine a possible relationship between survival and bacterial numbers. Significance was predetermined at $p < 0.05$.

3. Results

Survival to hatch varied significantly among the different iodine treatment groups (Table 1). While survival in the control (0 mg/L) and 100 mg/L iodine treatments was nearly identical at 62.3% and 64.0%, respectively, the 42.6% survival in the 200 mg/L treatment was significantly lower. Near-total mortality occurred in the 400 mg/L treatment, with the percentage of eggs surving less than 1%. Egg percent survival by individual female and treatment is shown in Figure 1.

Table 1. Mean ± SE percent survival and Bacterial Colony Forming Units (CFUs) of walleye (*Sander vitreus*) eggs subjected to one of four iodophor treatments for ten minutes prior to incubation. Means in the same row with different letters are significantly different (N = 10, $p < 0.05$).

	Iodine (mg/L)			
	0	**100**	**200**	**400**
Survival (%)	62.3 ± 3.1 z	64.0 ± 4.5 z	42.6 ± 8.4 y	0.3 ± 0.3 x
CFU	820.0 ± 193.7 y	39.8 ± 14.8 z	128.4 ± 34.2 z	74.4 ± 20.0 z

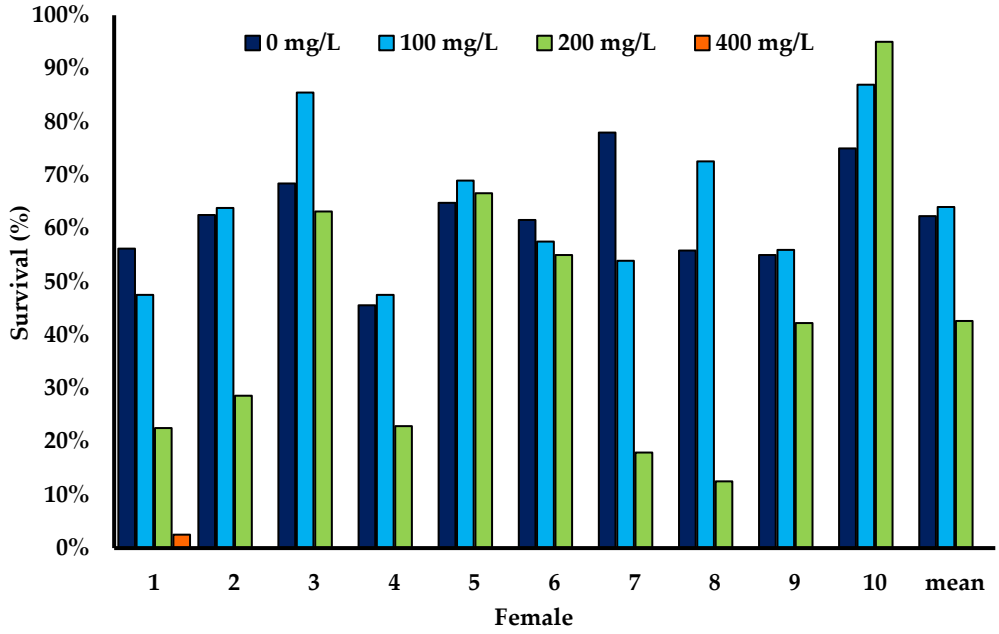


Figure 1. Percent survival of walleye (*Sander vitreus*) eggs from ten distinct females subjected to either 0, 100, 200, or 400 mg/L active iodine disinfection treatment for 10 min and mean survival by treatment.

All the iodine treatments significantly decreased the CFUs compared to the untreated control eggs (Table 1). However, the CFUs were not significantly different among the 100 mg/L, 200 mg/L, and 400 mg/L treatment groups. The highest bacterial count was approximately 2100 CFUs, observed in the untreated eggs from female nine, while the lowest bacterial loads observed were in eggs from female five at approximately 10 CFUs in both the 100 and 400 mg/L iodine treatments. The mean CFUs from the untreated control eggs of this female were approximately 820 CFUs. The 200 mg/L treatment group experienced an approximate six-time reduction in bacterial CFU, while the 100 and 400 mg/L treatments produced an approximately 16 times greater reduction in CFU. CFUs by spawn from individual females by treatment are shown in Figure 2.

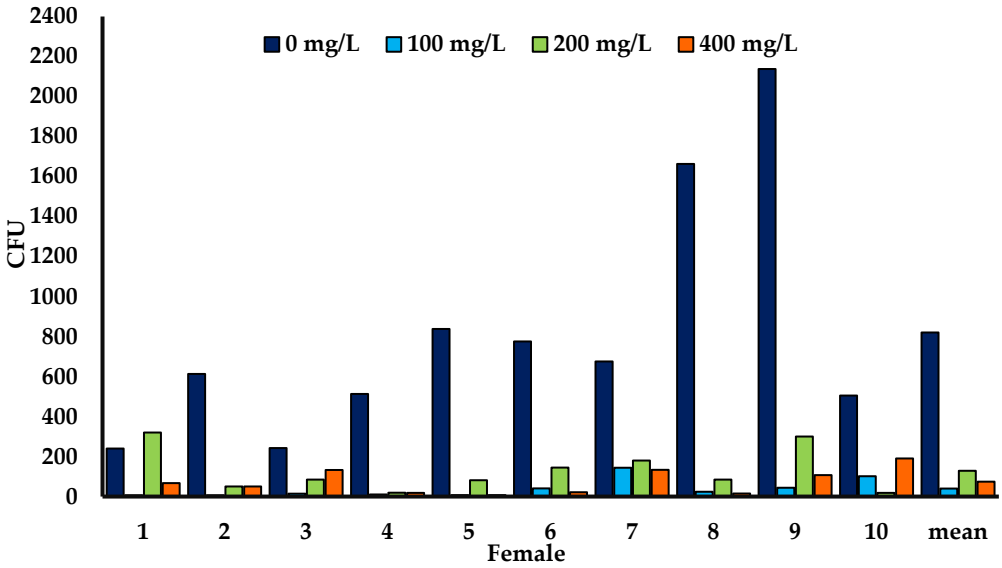


Figure 2. Bacterial Colony-Forming Units (CFUs) of walleye (*Sander vitreus*) eggs from ten distinct females subjected to either 0, 100, 200, or 400 mg/L active iodine disinfection treatment for 10 min and mean survival by treatment.

There was also no significant relationship between egg survival and the control (pre iodine treatment) CFU ($R^2 = 0.089$, $p = 0.401$; Figure 3). There was no significant relationship between egg survival and the post-disinfection bacterial numbers ($R^2 = 0.051$, $p = 0.161$; Figure 4).

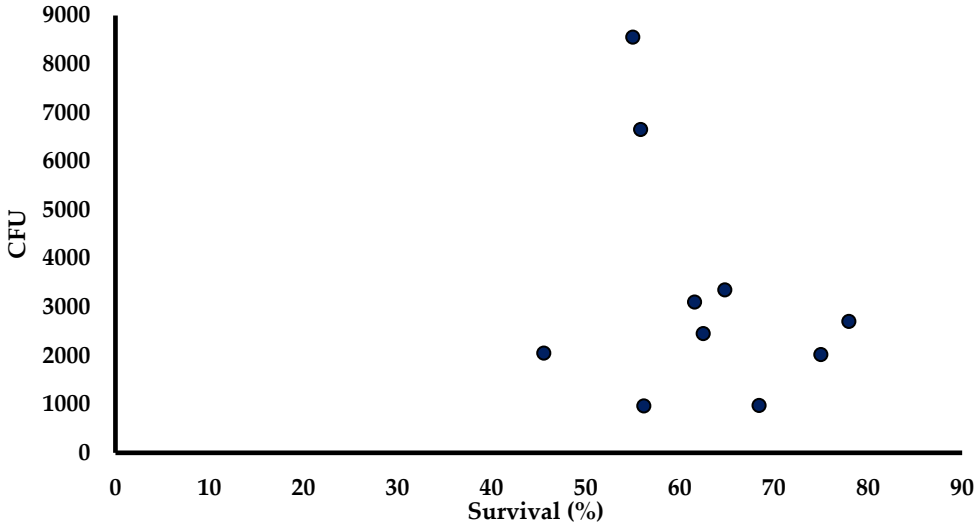


Figure 3. Scatterplot of walleye (*Sander vitreus*) egg survival and control (pre iodine) treatment Bacterial Colony-Forming Units (CFUs).

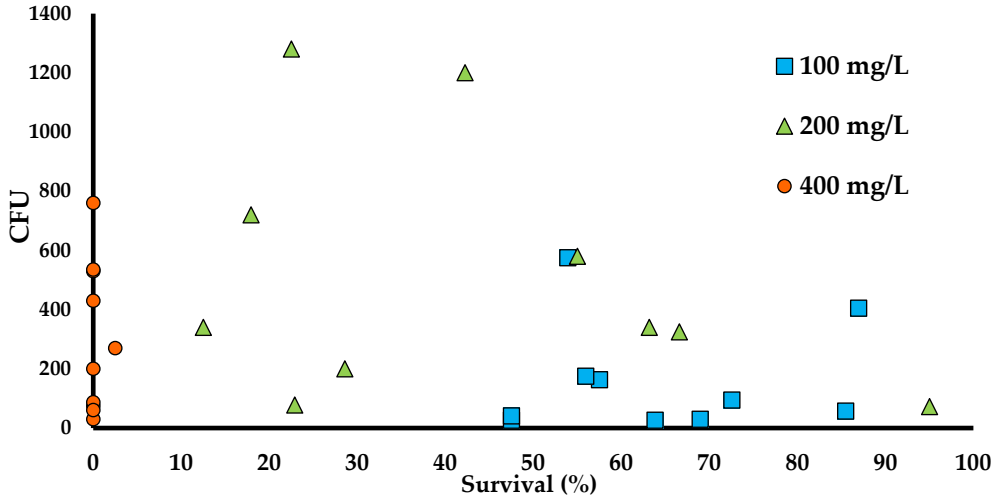


Figure 4. Scatterplot of walleye (*Sander vitreus*) egg survival and post-disinfection Bacterial Colony-Forming Units (CFUs) by treatment.

4. Discussion

While other studies have indicated the viricidal effects of iodine treatments on walleye eggs [11,31,33], this is the first study to document the bacterial levels on walleye eggs both before and after treatment with iodophor. This study showed a significant decrease in egg bacterial loadings after iodine treatment, but no difference in bacterial numbers was observed among the three iodine concentrations. In studies with other fish species describing bacterial numbers immediately after the disinfection of recently fertilized eggs, as iodine concentrations increase, egg bacterial numbers generally decrease [12,24]. In addition, just as in this study, egg disinfection with iodine at a number of different concentrations and treatment times does not completely sterilize external egg membranes [9,22–24]. It is possible that the different results among these studies could be because of differences in water chemistry or endemic bacterial populations [16,41].

The lack of a relationship between the number of bacteria on the external egg membrane and egg survival was not surprising. Huysman et al. [24] also did not observe any relationship between CFU and survival in Chinook salmon (*Oncorhynchus tshawytscha*) eggs. It is likely that most of the bacteria on eggs are non-pathogenic and non-symbiotic. The presence and number of pathogenic bacteria are likely more important than the overall bacterial numbers. Therefore, new studies that examine the number of pathogenic bacteria are needed.

The results of this study both support and contradict those reported by Dabrowski et al. [30], who evaluated iodine concentrations from 50 to 800 mg/L with treatment times of 15, 30, and 180 min on walleye egg survival. The present study, using 10 min treatment durations, noted no effect on egg survival with the 100 mg/L iodine treatment. Dabrowski et al. [30] also noted no negative effects on walleye egg survival with the 100 mg/L iodine treatment for 15 min. However, Dabrowski et al. [30] also observed no increase in mortality when iodine was increased to 200 mg/L for 15 min, whereas survival significantly decreased with the 200 mg/L iodine treatment for 10 min in the present study. While Dabrowski et al. [30] noted an approximately 40% decrease in egg survival with the 400 mg/L iodine treatment, it was not the near-complete mortality observed in this study. In another study examining walleye egg survival and iodine treatments, Bowzer et al. [11] noted that a 100 mg/L iodine treatment for 30 min during water-hardening (and immediately after tannic acid treatment) led to significantly decreased survival rates when female walleyes were kept overnight before gametes were collected. Conversely, when gametes were collected on the same day, survival was not affected by the same 100 mg/L, 30 min iodine treatment [11]. The difference in these results could have been because of the timing during the development of the iodine treatments. Our study and that of Dabrowski et al. [30] applied iodine treatments well after initial fertilization, whereas Bowzer et al. [11] applied iodine immediately after fertilization during water uptake by the egg in conjunction with membrane separation (water-hardening). The developmental stage at which iodine is used can dramatically affect egg survival [18,26,27,29,42]. The studies also differed in spawning procedures, with tannic acid used to remove egg adhesion by Dabrowski et al. [30] and Bowzer et al. [11], while our study used Fuller's earth. Although tannic acid may not appear to impact walleye egg survival [43], it impacts the anti-microbial properties of iodophors [32]. The water chemistry parameters differed among the studies, which may have impacted the results as well. For example, Rosberg et al. [44] found extremely hard water of 800 or 1600 mg/L $CaCl_2$ significantly decreased rainbow trout egg survival, compared to concentrations of 400 mg/L or less, which had no impact on survival. Iodine is frequently used for egg disinfection because, unlike other disinfection chemicals, its efficacy is neither temperature- nor pH-dependent, although organic matter can greatly decrease the efficacy [7]. However, few studies have directly examined different water quality parameters and the efficacy of iodine or other disinfection chemicals [7]. Lastly, the genetic strain of walleye used in this study was different from those used by Dabrowski et al. [30] and Bowzer et al. [11], which likely impacted the results. Even within the same strain, there appears to be considerable variation in egg sensitivity to iodophors among spawns from individual female walleyes [30].

The increase in mortality observed in this study beginning with the 200 mg/L iodine treatment was much lower than that observed with the eggs from other fish species. However, tolerance appears to be species-specific. For example, iodine concentrations up to 400 mg/L for 10 min did not impact Chinook salmon egg survival [24]. Concentrations of up to 1000 mg/L iodine had no effect on the survival of rainbow trout (*Oncorhynchus mykiss*) or brown trout (*Salmo trutta*) eggs, but 2000 mg/L was lethal to rainbow trout eggs [9,10,12]. Iodine at 100 mg/L for 10 min caused the complete mortality of California halibut eggs,

while concentrations above 50 mg/L caused mortality in the eggs of Senegalese sole (*Solea senegalensis*), red porgy (*Pagrus pagrus*), and white seabream (*Diplodus sargus*) [32,45,46]. These different results may also be due to differences in egg disinfection procedures. There is no protocol regarding the developmental stage at which iodine can be applied or how long and how many times iodine disinfection should be used [7]. Fish species develop at different rates, with disinfection occurring at any point, from water-hardening to hatching, without any examination or consideration of potentially sensitive periods for egg development [7].

5. Conclusions

In conclusion, the results of this study clearly showed that iodine disinfection at 100 mg/L for 10 min dramatically reduced bacterial numbers on walleye eggs with no negative impacts on egg survival. In addition, treatment concentrations of 200 mg/L or greater were shown to cause egg mortality. Further research could examine different treatment durations in relation to iodine concentrations. For example, would disinfection at 100 mg/L for 30 min lead to further bacterial reduction and still be safe for walleye eggs? Research is also needed to determine the effects of iodine on specific bacteria on walleye eggs, particularly focusing on pathogenic bacterial species which may be iodine-resistant [47]. Basic research on development stages and how different water chemistry parameters affect the efficacy of treatments and survival of eggs is still needed.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

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