

# **MICROBIAL POPULATIONS DURING LANDLOCKED FALL CHINOOK SALMON EGG INCUBATION AT McNENNY STATE FISH HATCHERY, SPEARFISH, SOUTH DAKOTA**

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## **ABSTRACT**

Microbial sampling was conducted during incubation of landlocked fall chinook salmon *Oncorhynchus tshawytscha* eyed eggs in vertically-stacked tray incubators at McNenny State Fish Hatchery, Spearfish, South Dakota. *Saprolegnia* spp. were identified from water obtained directly from the hatchery well, and also from the aeration tower and incubation headbox. Attempts to develop a fungal population census method were unsuccessful. Mean bacteria levels in the incubation tray water when the trays contained either eyed eggs or sac fry were significantly less than during hatching. Bacterial populations were also significantly elevated after removal of dead eggs and fry by hand-picking. Daily 15 minute formalin treatments of 1,667 mg/L from eye-up to hatch significantly improved embryo survival. However, there was no difference in bacterial levels in the incubation water between trays that did or did not receive formalin treatments, and bacterial levels were not reduced in the incubation water after formalin treatments.

## **Keywords**

*Saprolegnia*, chinook salmon, salmonid eggs, vertical-flow incubator, formalin, hand-picking, bacteria

## **INTRODUCTION**

*Saprolegnia* spp. fungal infections on incubating salmonid eggs are common at most hatcheries. Controlling such infections usually requires fungicidal chemical treatments such as formalin (Piper et al. 1982; Post 1987) until at least

the eyed stage of egg development. After eye-up, physical removal of the dead eggs at regular intervals can be safely performed for fungal control or chemical treatments can continue. Manual removal of dead eggs and fry is a laborious procedure (Leitritz and Lewis 1976), whereas formalin treatments are relatively easy and effective (Bailey and Jeffery 1989).

McNenny State Fish Hatchery, Spearfish, South Dakota, has utilized both methods of control during incubation of landlocked fall chinook salmon *Oncorhynchus tshawytscha* eggs obtained from feral broodstock in Lake Oahe, South Dakota. These eggs typically exhibit poor survival to eye-up, with mortality rates often in excess of 50% (Barnes et al. 2000b). An additional 15% mortality occurs from the eyed egg stage to fry swim-up (Barnes and Cordes 1992). Traditionally, these eggs have been treated daily with formalin during initial incubation. Prior to 1993, formalin treatments were discontinued at the eyed stage of development and dead eggs and fry were then manually removed until fry swim-up (Barnes and Cordes 1992). Since 1994, formalin treatments have been continued daily from eye-up until just prior to hatch, and have consistently produced a 3 to 5 % increase in embryo survival (Barnes et al. 1997). The reasons for this increase are not understood and may be due to formalin decreasing either microbial (fungal or bacterial) populations or life-cycle stages (e.g. spores) that manual egg picking is unable to remove (Barnes et al. 1997; 2000a; 2001).

The objectives of this preliminary study were to determine the impacts of daily formalin treatments on the microbial populations associated with incubating fall chinook salmon eggs and identify the presence of *Saprolegnia* spp. in the incubation source water at McNenny Hatchery.

## METHODS

### General Egg Culture

All eggs used for this study were obtained from fall chinook salmon spawned at Whitlocks Spawning and Imprint Station, Lake Oahe, South Dakota. Eggs were collected on October 21, 1996.

After spawning at Whitlocks, the eggs were transported to McNenny Hatchery (4h). Temperature of the eggs upon arrival at the hatchery was 12°C. The eggs were disinfected in a 100 mg/L buffered free-iodine solution for 10 minutes, inventoried by water displacement (Piper et al. 1982), and placed in Heath (Flex-a-lite Consolidated, Tacoma, Washington) incubator trays. Well water (11°C, total hardness as CaCO<sub>3</sub> - 360 mg/L, alkalinity as CaCO<sub>3</sub> - 210 mg/L, pH - 7.6, total dissolved solids - 390 mg/L) at a flow of 12 L/min was used for egg incubation. Formalin treatments using Parasite-S (37% formaldehyde, 6 to 14% methanol, Western Chemical Inc., Ferndale, Washington) at 1,667 mg/L for 15 minutes were administered daily until the eyed stage of egg development with a Masterflex model 7524-00 microprocessor peristaltic pump (Cole-Parmer Instrument Company, Chicago, Illinois). Formalin concentrations were not analytically verified (Rach et al. 1997).

## Experimental Design

At the eyed stage of egg development (incubation day 28), all trays were pooled and dead eggs were mechanically removed using a Van Gaalen Fish Egg Sorter (VMG Industries, Grand Junction, Colorado). This machine uses modulated infrared light to detect opaque (dead) eggs and air pressure to sort the eggs after detection. Eyed eggs were pooled, reinventoried, and retrayed at 900 mL (approximately 5,100 eyed eggs) per tray. Survival to the eyed stage was 71%.

Six trays from three incubator stacks were used during the study. The top tray of each stack served as a mixing tray and did not hold any eggs. The next two trays down the stack were included in the experiment. Each stack received a different treatment until hatch. One stack (2 trays) continued receiving daily formalin treatments (1,667 mg/L for 15 minutes) for an additional 10 days after auto-picking. A second stack (2 trays) did not receive any additional formalin treatments; fungal control was entirely due to hand-picking removing dead eggs. The third stack (2 trays) acted as a procedural control. It received daily formalin treatments and the trays were also hand-picked at the same time as the non-formalin treated trays (4, 7, and 9 days after auto-picking - incubation days 32, 35, and 37). After formalin treatments ceased (day 39), all trays were hand-picked on incubation days 42, 44, 46, 49, 52, 56, 59, 63, and 70. All mortality was recorded and percent mortality to hatch, swim-up, and total mortality was determined.

Hand-picking was performed by removing the trays from the incubation stack, floating them in a 274 x 66-cm fiberglass picking trough, and sucking out the dead eggs and fry using a pipette fitted with a hand-held rubber squeeze bulb (Leitritz and Lewis 1976). Water levels in the picking trough were maintained at 18 cm (total operating volume = 322 L) with flows set at 10 L/min.

## Microbial Sampling

Both fungal and bacterial samples were taken immediately after re-traying following auto-picking at the eyed egg stage. Samples were taken the next day from all trays in the morning 07:30 both before and after formalin treatments, and at 15:30. This sampling regime (pre- and post-formalin treatments, late afternoon sample) was repeated on the last day formalin was administered (incubation day 39). Additionally on this date, samples were also taken both before and after manual picking (11:00 to 14:00). During hatch eight days later (day 47), samples were taken at 7:30, before and after hand picking, and at 15:30. Samples were also collected on this date from one of the hatchery wells, the aeration tower, and the incubator headbox (water supply channel to all incubator stacks). Samples from pre- and post-hand picking, the incubator headbox, and the picking trough were collected on incubation day 53. Well, headbox, and 7:30 tray samples were also collected on day 57.

Both fungal and bacterial samples were taken from each of the six incubator trays in the experiment. Fungal sampling occurred by pipetting 20 mL of water from a tray into a sterile glass petri dish. Ten non-viable hemp seeds

(Carolina Biological Supply, Burlington, North Carolina) were then added to the water sample with sterile forceps to provide a substrate for fungal growth. Prior to placement in the petri dishes, the seeds were boiled for a minimum of 20 minutes for sterilization and to break their seed coat. Each seed was checked daily for any signs of fungal growth using a 10X dissecting microscope. Bacteria were sampled using the Spread Plate Method (APHA et al. 1989) by pipetting 0.1 mL of water from each tray onto a nutrient agar plate. The water sample was spread over the entire agar surface using a sterile spreader.

All microbial samples were incubated at ambient temperatures in the incubation room (10 to 13°C) in an attempt to mimic incubation water temperature at the hatchery (11°C). The number of bacterial colonies in each plate was recorded after 7 days incubation at ambient temperatures of 11°C and reported as CFU (colony forming units) per 0.1 mL of water. When ambient temperatures increased to 13°C, plates were read after only 5 days.

### Statistical Analysis

Data were analyzed by analysis of variance. Significance was predetermined at  $P < 0.05$  and pairwise mean comparisons were performed using Fisher's Protected Least Significant Difference. All embryo survival percentage data were arcsine transformed prior to analysis to stabilize the variances (Ott 1984).

## RESULTS AND DISCUSSION

Daily formalin treatments at 1,667 mg/L for 15 minutes from eye-up to just prior to swim-up produced a 5 % increase in embryo survival (Table 1). Most of the mortality in the non-formalin treated trays occurred prior to hatch. These results are nearly identical to those seen in similar experiments from 1993, 1994, and 1995 (Barnes et al. 1997).

*Saprolegnia* spp. were identified as the prevalent fungus in the hatchery water system. The fungus is ubiquitous throughout the hatchery water supply. Water samples taken from the well next to the hatchery contained *Saprolegnia* spp., as did samples from the aeration tower and incubation headbox.

**Table 1. Mean ( $\pm$  SE) percent mortality from inland chinook salmon eyed eggs either treated with formalin and no manual removal of dead eggs, treated with formalin in addition to dead egg removal, or just receiving manual picking (no formalin treatments) (N = 2). Means in the same column followed by different letters are significantly different ( $P < 0.05$ ).**

Treatment	Hatch Mortality	Swim-up Mortality	Total Mortality
Formalin	6.9 $\pm$ 0.4 z	2.6 $\pm$ 0.4	9.5 $\pm$ 0.7 z
Formalin $\pm$ Manual Picking	6.0 $\pm$ 0.5 z	3.4 $\pm$ 0.1	9.4 $\pm$ 0.1 z
No Formalin (Picking Only)	10.5 $\pm$ 0.3 y	3.9 $\pm$ 0.7	14.4 $\pm$ 0.9 y

Our attempt at developing a fungal population census method failed. *Saprolegnia* spp. first appeared in the petri dishes after 6 days of incubation at 11°C. While the first observations of infection indicated that 60 to 90% of the hemp seeds harbored fungal growth, by the next day 100% of the seeds were infected. No possibility existed to develop a population index based on the number of seeds infected.

Bacterial populations were highly variable. The four water samples taken from the well next to the hatchery averaged  $10 \pm 8$  CFU/0.1 mL and two samples taken from the aeration tower averaged  $12 \pm 8$  CFU/0.1 mL. Mean readings from five incubation headbox samples were 21 CFU/0.1 mL.

No significant differences were detected in bacterial populations between pre-formalin and post-formalin treatments ( $P = 0.2907$ ). A significant interaction ( $P = 0.0186$ ) between sampling time (pre- or post-treatment) and sampling date was observed however. This interaction is evident in Table 2, with mean CFU/0.1 mL generally increasing after the 15 minute formalin treatments on the first sampling date, but decreasing on the second sampling date.

Bacterial population levels did not appear to fluctuate diurnally during normal incubation. Samples taken at 7:30 averaged  $54 + 9$  CFU, while samples taken at 15:30 averaged  $69 + 33$  CFU. Significant differences were detected however, when the trays were removed from the incubator for hand-picking and then re-inserted in the incubator stack ( $P = 0.0001$ ). Table 3 illustrates the increase in CFU/0.1 mL observed during the second and third sampling periods, incubation days 47 and 53. These two days correlate with the initiation and near-end of hatching. Picking trough bacterial levels averaged  $34 (+ 14)$  CFU prior to egg picking, and  $400 (+ 71)$  after egg picking. No differences were observed between any of the formalin treatments (formalin only, formalin plus picking, no formalin) before or after hand-picking ( $P = 0.485$ ).

Elevated bacterial levels during hatching were also observed by comparing first of the morning samples from incubation day 29 (next day after auto-picking) through day 53 (only fry present in the trays). A peak of  $84 (+ 14)$  CFU/0.1 mL occurred during the peak of hatching activity (Table 4). The two samples taken during hatching were significantly different than the samples taken dur-

**Table 2. Mean  $\pm$  SE) number of bacterial CFU per 0.1 mL of water collected from incubator trays containing chinook salmon eggs either treated with formalin and no manual removal of dead eggs, treated with formalin in addition to dead egg removal, or just receiving manual picking (no formalin treatments). Sampling occurred both before and after any formalin treatments on two separate dates (N = 2).**

Incubation day	Sampling time	Formalin only	Formalin + picking	No formalin (picking only)
29	Before	$10 \pm 8$	$11 \pm 8$	$18 \pm 11$
29	After	$7 \pm 4$	$49 \pm 46$	$58 \pm 53$
39	Before	$70 \pm 12$	$64 \pm 12$	$64 \pm 14$
39	After	$21 \pm 5$	$9 \pm 12$	$18 \pm 13$

**Table 3. Mean ( $\pm$  SE) number of bacterial CFU per 0.1 mL of water collected from incubator trays containing chinook salmon eggs sampled before and after manual egg removal (N = 6). Means followed by different letters are significantly different ( $P < 0.05$ ).**

Incubation day	Sampling time (before/after hand picking)	CFU
39	Before	30 $\pm$ 5 z
39	After	29 $\pm$ 5 z
47	Before	67 $\pm$ 22 z
47	After	133 $\pm$ 27 y
53	Before	21 $\pm$ 8 z
53	After	313 $\pm$ 42 x

**Table 4. Mean ( $\pm$  SE) number of bacterial CFU per 0.1 mL of water collected from incubator trays containing chinook salmon eggs sampled from auto-picking (eye-up) through hatching (N = 6). Means followed by different letters are significantly different ( $P < 0.05$ ).**

Incubation Date	Embryo Development	Colony Forming Units
29	Eyed Egg	13 $\pm$ 4 z
39	Hatching	66 $\pm$ 5 y
47	Hatching	84 $\pm$ 15 y
53	Sac Fry	21 $\pm$ 8 z
57	Sac Fry	6 $\pm$ 2 z

ing the eyed-egg and fry stages ( $P = 0.0001$ ). The increase in bacterial numbers during this time is probably because of the nutrients liberated during rupture of the external egg membrane at hatching (Bell et al. 1971; Smith et al. 1985; Barker et al. 1989).

Despite our inability to determine fungal population levels, important information was obtained during this initial investigation. Fungal populations were identified as *Saprolegnia* spp., and these organisms were found to be present even in the hatchery wells. This is not surprising, since these water molds are known to survive in all types of aquatic environments and moist soils (APHA et al. 1989).

The mechanism by which formalin is producing an increase in egg survival is still unclear. Until a suitable fungal population estimation technique is developed, the impact of formalin on *Saprolegnia* spp. zoospores and microscopic hyphae will be difficult to determine. Our hypothesis that formalin is decreasing fungal levels is likely wrong however. The presence of *Saprolegnia* spp. in the incubation water supply ensures that the incubating eggs must deal with possible infection continually, except during the 10 minute formalin treatments. Even if formalin reduced incubation water fungal populations during treatment, the incoming water would quickly provide additional *Saprolegnia* spp. zoospores for possible re-infection (Rand and Munden 1993). Therefore, it is much more likely that formalin is either preventing zoospore germi-

nation or somehow removing any microscopic hyphae that may have attached to the external egg membrane (Smith et al. 1985), or possibly changing the structure of the external egg membrane.

Results from this year of the study seem to indicate that bacteria populations were unaffected by formalin treatments. However, just as with water molds, the source water just reintroduced bacteria into the incubation trays immediately after the formalin treatments ended. If bacteria are responsible for the elevated mortality associated with non-formalin treatments, then formalin could again possibly be working by possibly removing attached bacteria from the external egg membrane (Barnes et al. 2000b).

Additional information was gained beyond just the possible influence of formalin on microbial populations. Hatching, with the release of the embryo from the chorion, produced an elevated level of bacteria. This population spike was not the result of a large number of prematurely hatching embryos, since the formalin treated trays, with their minimal premature hatch, also experienced the same spike as the non-formalin trays. The large increase in CFU/0.1 mL noted during hand-picking is something that could likely be controlled by changes in culture technique. Frequent removal of the organic material (eggs/fry/shells) from the picking trough during a day of picking may be one mechanism to decrease the bacterial loads that the trays carry back into the incubators.

The results from this initial study indicate the need for additional research into fungal and bacterial levels during salmonid egg incubation, as well as investigation into the anti-microbial action of formalin.

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