THE EFFECT OF SIX CHEMICALS FOR DISINFECTION OF LARGEMOUTH BASS EGGS

Lloyd D. Wright and J. R. Snow

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Abstract

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Introduction

A technique for the controlled incubation of eggs from the largemouth bass, Micropterus salmoides, Lac. has been developed and tested at the Warm-water Fish Cultural Development Center, Marion, Alabama (9). During the course of the search for an acceptible incubation method, occasional heavy mortality of embryos was encountered which was attributed to disease. Since the brood fish from which the eggs were obtained had a history of chronic hemorrhagic septicemia caused by Aeromonas liquefaciens, (8, 10) prophylaxis of the eggs by disinfection seemed applicable. This is a common practice in cold-water fish culture (3, 5, 6), pioneered by Gee and Sarles (4) who studied disinfection of trout eggs to combat furunculosis disease caused by Aeromonas salmonicida. Later work on this approach to the control of furunculosis has resulted in egg disinfection being a standard control measure for furunculosis disease in trout culture (3, 5).

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Materials and Methods

The object of this study was to determine which of the readily available chemicals[3], previously used in trout-egg disinfection would be best for protecting bass eggs against infection by Aeromonas liquefaciens. Chemicals tested included: formalin, Merthiolate®, acriflavine, Roccal®, and two povidone iodine compounds, Betadine®, and Wescodyne®.

The A. liquefacien strains used to infect the bass eggs were of two sources; Auburn Strain, Auburn, Alabama and Marion Strain, Marion, Alabama. Both were initially isolated from largemouth bass. The bacterial cultures were maintained on trypticase soy agar (TSA) or nutrient broth (NB). The test bacteria were periodically passed through living largemouth bass and re-isolated to insure a highly virulent organism. Identification of all recovered organisms was based on the presumptive scheme of Bullock (2).

3
acriflavine - neutral, 100% active powder - Allied Chemical Co. P. O. Box 431, Morristown, New Jersey.

formalin - 100% active, formaldehyde solution, 40% by volume, W. H. Curtin Co., Atlanta, Georgia.

Merthiolate® - 100% active powder - Eli Lily and Co., Toronto, Ontario.

Roccal® - 10% active solution - Winthrop Laboratories, New York, New York.

Betadine® - 1% active I_2 - Purdue Fredrick Co., Yonkers, New York.

Wescodyne® - 1% active I_2 - G.A.F. New York, New York.

Establishment of Chemical Concentrations

In preliminary studies it was established that the following concentrations of the chemicals would kill 99.9 per cent of the test bacteria[4]; Roccal® 200 ppm, formalin 1000 ppm, Merthiolate® 600 ppm, acriflavine 750 ppm, Betadine® and Wescodyne® 150 ppm (Active I_2).

To determine if the concentration of disinfectant required to kill the bacteria would be detrimental to the eggs, and to determine a margin of safety, groups of eggs were treated at concentrations equal to, higher, and lower than the effective bactericidal concentration. (Table 1)

Spawning mats containing eggs were removed from the pond, the eggs were removed from the mats and washed in well water to remove any silt and debris; then washed in sterile well water and divided into groups of 20, with one group set aside as controls. There were three test groups for each chemical and 3 control groups. Test groups were placed in cheese-cloth baskets and immersed in their respective disinfectants for 15 minutes. After the treatment, the eggs were washed in sterile water and placed in 250 ml. Erlenmeyer flasks containing 125 ml of 72° F. aerated, sterile well water where they were allowed to hatch. Hatching success was determined by counting fry.

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[4]Wright, L. D., Unpublished Data

Concurrent with the above tests, eggs were infected with A. liquefaciens bacteria by placing them in a nutrient broth (NB) culture of A. liquefaciens, adjusted to a No. 3 McFarland standard, for 30 minutes. The eggs were then washed 5 times in well water, and placed in different concentrations of chemicals to determine the amount required to kill bacteria on them. (Table 2). Eggs were placed in disinfectants for 15 minutes, washed 5 times in sterile well water and placed in NB media to check for viable bacteria.

The data obtained from the above tests in conjunction with information concerning the use of disinfectants in trout culture, (3, 5) established the disinfectant concentrations to be studied as follows: Merthiolate® 200 and 400 ppm, formalin 300 and 500 ppm, acriflavine 300, 500, and 750 ppm, Betadine® and Wescodyne® 100 and 200 ppm.

Hatching Success of Eggs Following Chemical Treatment

Bass eggs were removed from spawning mats as previously described and washed in well water. The eggs were then brought into the laboratory where 50 eggs were set aside to serve as negative controls. The remaining eggs were infected by placing half of them in a NB culture of Auburn Strain A. liquefaciens and half in a NB culture of the Marion Strain, A. liquefaciens. Strength of these cultures was_____. The eggs were then removed, washed, and 50 eggs set aside as positive controls. Each group of eggs was divided into lots of 50, placed in cheesecloth baskets and immersed into each concentration of the respective disinfecting treatments, 6 lots per treatment. After 15 minutes the eggs from each lot were washed in sterile well water and

transferred to hatching flasks. Hatching success as measured by a count of the fry present was determined (Table 3). Included were five disinfectants, four at two concentration levels and the fifth at three. These were applied to two strains of A. liquefaciens (Auburn and Marion) with six replications. Also included were the negative and positive controls.

Results and Discussion

Early in the investigation it became evident that Roccal®, at a concentration high enough to be effective as a disinfectant (167 ppm) was lethal to the eggs and hatching fry. Eggs treated with Roccal® at a level high enough to kill bacteria or higher, turned cloudy white shortly after first exposure to the treatment. Because of this, Roccal® was not tested further.

Eggs treated with the other disinfectants had varying degrees of hatching success. Most treatments showed a higher percentage of egg hatch than the positive controls and in several cases a higher hatching percentage than the negative controls. One exception was when one Merthiolate® replication at 200 and 400 ppm was inadvertently left for 30 minutes instead of 15, resulting in a complete mortality of the treated eggs. A low formalin concentration and both Merthiolate® treatments in the Marion Strain of A. liquefaciens showed a lower hatching percentage. Apparently in these instances enough viable bacteria survived the disinfection to affect the hatching percentage. The iodine compounds at 200 ppm appeared to be toxic to bass eggs, with survival of embryos in the Wescodyne® being only three percent.

All the chemicals tested except Roccal® and the iodine compounds (Betadine® and Wescodyne®) had a high margin of safety, and could be used at a concentration of 1000 ppm in the standard 15 minute treatment and not

adversely affect hatching success.

Acriflavine gave the best results as a disinfectant, with little difference in efficacy between the high and low concentrations. It had the disadvantage of being very hard to wash from the eggs after treatment. McFadden (6) reported that up to 15 washings were needed to remove acriflavine from treated trout eggs. After five washings in this study, enough residue remained on the bass eggs to bring the static water in the hatching flasks to an estimated 3-5 ppm concentration of acriflavine, but this did not adversely affect the hatch. This agrees with unpublished data by Beck (1) where bass fry were held in acriflavine at concentrations as high as 10 ppm. for 48 hours without any abnormal behavior. Acriflavine controlled fungus in the hatching flasks much better than the other chemicals tested. Snow (9) routinely exposed largemouth bass eggs to a concentration of 50 ppm for 15 minutes as a prophylactic measure with beneficial results. This treatment concentration was later increased to 100 ppm with no rinsing and no observed harmful effects.

Concentrations of formalin (Table 2) up to 2000 ppm were not effective in eliminating A. liquefaciens from the eggs. Merthiolate® at 750 ppm, acriflavine at 1000 ppm, and I_2 compounds at 150-200 ppm, were 100 percent effective in killing the bacteria however. The concentration of chemicals which successfully controlled bacteria on the eggs was below the lethal levels established earlier (Table 1). Merthiolate® and acriflavine were well below the safety margin and Betadine® and Wescodyne® at the extreme upper limits of safety. If these higher concentrations were used in the tests, hatching success had been

may have been improved. It is, however, evident that complete elimination of pathogenic organisms is not essential to have a high percent of hatching success and survival. The best chemical to use is one that has a high margin of safety and should be used at the lowest concentration to give the desired results.

Data in Table 3 were analyzed statistically by the analysis of variance technique of Snedecor (7) converting the hatching percentage to arcsin percentage. Replicates given double time exposure were excluded. Results of the comparisons are shown in Table 4.

All of the disinfectants tested except Roccal® and formalin appeared beneficial in improving the hatching percentage of largemouth bass eggs where the concentration was within the optimum range. All factors considered, however, acriflavine appears to be the treatment of choice at a rate of 500-700 ppm for 15 minutes. The iodine compounds performed well at 100 ppm but lacked the margin of safety which acriflavine demonstrated. Considering cost of treatment, use of iodine might be justified in spite of the narrow margin of safety. While Merthiolate® was effective in killing the test bacteria at 750 ppm, it would be fourth choice behind acriflavine and the two iodine compounds.

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Table 1. Percentages of hatch of largemouth bass eggs following a 15-minute disinfectant treatment with various chemicals at selected concentrations.

Disinfectant	Percent Hatch			
	Trial 1	Trial 2	Trial 3	Avg.
Control	90	90	95	92
Roccal®				
100 ppm.	25	15	30	23
200 ppm.	0	0	0	0
500 ppm.	0	0	0	0
Formalin				
500 ppm.	80	90	90	87
1000 ppm.	80	95	100	92
1500 ppm.	70	80	75	75
Merthiolate®				
300 ppm.	80	90	100	90
600 ppm.	90	90	90	90
1000 ppm.	80	85	90	85
Acriflavine				
500 ppm.	85	90	90	88
750 ppm.	90	95	90	92
1000 ppm.	90	85	75	83
Betadine®[a]				
100 ppm.	85	90	90	88
150 ppm.	90	95	90	92
200 ppm.	50	45	65	53
Wescodyne®[a]				
100 ppm.	90	90	90	90
150 ppm.	90	80	95	88
200 ppm.	0	0	0	0

[a] ppm. active I_2

Table 2. Concentrations of disinfectant needed to sterilize the bass eggs infected with test bacteria.

Disinfectant	Isolation and identification of A. liquefaciens from bass eggs following treatment					
	Auburn Strain			Marion Strain		
Trial No. -	1	2	3	1	2	3
Formalin						
500 ppm.	+	+	+	+	+	+
750 ppm.	+	+	+	+	+	+
1000 ppm.	+	+	+	+	+	+
1500 ppm.	+	+	+	+	+	+
2000 ppm.	+	+	+	+	+	+
Merthiolate®						
400 ppm.	+	+	+	+	+	+
500 ppm.	-	+	-	+	+	+
750 ppm.	-	-	-	-	-	-
Acriflavine						
500 ppm.	+	+	+	+	+	+
750 ppm.	-	+	+	-	-	+
1000 ppm.	-	-	-	-	-	-
Betadine®						
100 ppm.	+	+	-	+	+	+
150 ppm.	-	+	-	-	-	+
200 ppm.	-	-	-	-	-	-
Wescodyne®						
100 ppm.	+	+	+	+	+	+
150 ppm.	-	-	-	+	-	-
200 ppm.	-	-	-	-	-	-

Test bacteria recovered +
Test bacteria not recovered -

Table 3. Percentage of hatch of bass eggs infected with A. liquefaciens, following disinfection treatment.

Disinfectant	Percent Hatch												
50 eggs/trial	Auburn Strain							Marion Strain					
Trial Number	1	2	3	4	5	6	Avg.	1	2	3	4	5	6	Avg.
Neg. Control	94	82	72	78	80	92	83	20	24	90	78	80	90	64
Pos. Control	70[a]	50	48[a]	62	58	52	56	0	10	54[a]	22	18	20	21
Formalin														
300 ppm.	100	80	78	82	92	88	86	10	64	14[a]	70	24	42	37
500 ppm.	84	84	74	62	88	78	78	32	80	50	90	80	88	70
Merthiolate®														
200 ppm.	94	90	0[b]	92	82	88	89/74	28	10	36	14	72	80	40
400 ppm.	96	86	0[b]	88	80	90	87/73	50	20	22	32	64	78	44
Acriflavine														
300 ppm.	92	70	92	94	86	90	87	30	62	44	48	80	78	57
500 ppm.	78	74	92	96	82	90	85	44	58	56	54	86	94	65
750 ppm.	88	92	96	96	84	92	91	50	62	66	58	90	98	71
Betadine®														
100 ppm.	86	90	98	94	88	90	91	50	60	70	68	90	88	71
200 ppm.	26	14	30	28	6	26	22	10	30	14	22	42	38	26
Wescodyne®														
100 ppm.	88	82	78	88	90	94	87	44	52	70	58	88	92	67
200 ppm.	0	4	0	0	12	0	3	0	0	2	0	16	0	3

[a] All fry that hatched were dead after 24 hours, presumably killed by the test bacteria on the egg and in the hatching water.

[b] Eggs were inadvertently left in treatment for 30 minutes.

Table 4. Analysis of variance comparisons of [illegible] for largemouth bass eggs

Comparison	Probabilities of a larger value of F: Auburn strain	Probabilities of a larger value of F: Marion strain
Controls vs disinfectants	0.2252	0.0175
Negative control vs positive control	0.0001 xx	0.0004
Auburn strain vs Marion strain	0.0001 xx	
Iodine compounds vs other disinfectants	0.0001 xx	0.01[illegible]
Betadine® vs Wescodyne®	0.0001 xx	0.120[illegible]
Acriflavine vs formalin and Merthiolate	[illegible]	0.02[illegible]
Low formalin vs high formalin	0.0440 x	0.03[illegible]
Low Merthiolate vs high Merthiolate	0.6370	0.77[illegible]
Low Betadine® vs high Betadine®	0.0001 xx	0.00[illegible]
Low Wescodyne® vs high Wescodyne®	0.0001 xx	0.000[illegible]
Low acriflavine vs medium and high acriflavine	0.7760	0.[illegible]
Medium acriflavine vs high acriflavine	0.1042	0.[illegible]
Formalin vs Merthiolate	0.1098	0.123

x Difference statistically significant

xx Difference statistically highly significant