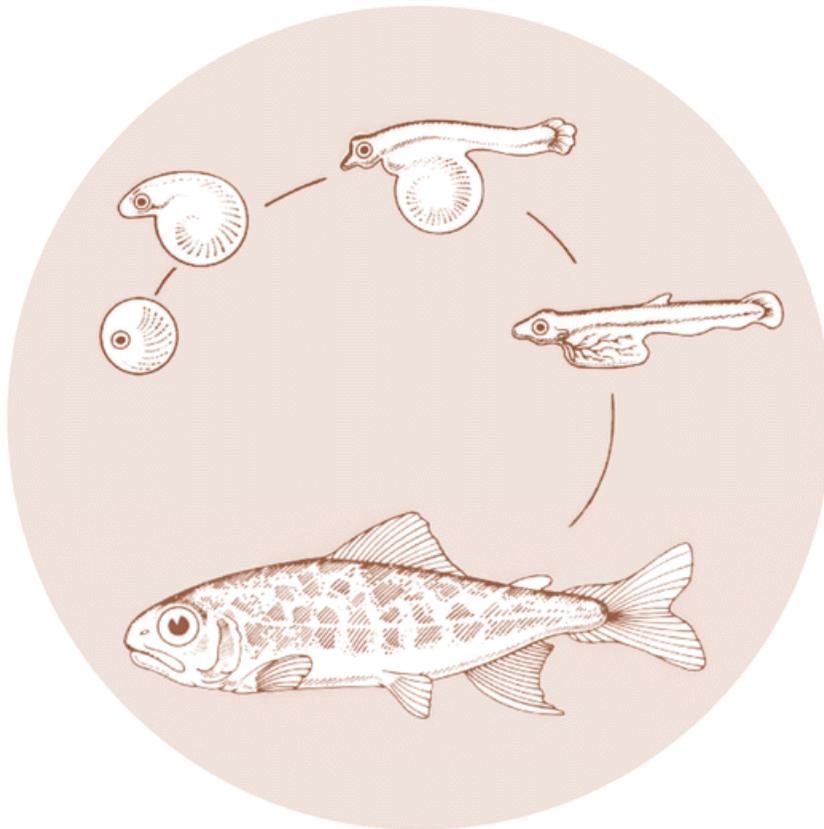


October 1993

RESEARCH TO IDENTIFY EFFECTIVE ANTIFUNGAL AGENTS

Annual Report 1993



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Abstract

Use of malachite green as a fungicide in fish culture was terminated by the Food and Drug Administration (FDA) on August 27, 1991. Formalin has been the replacement antifungal agent, but the present registration restricts its use only to eggs of salmonids and esocids and there are concerns for safety to users and effluents in the environment. Selected candidate antifungal chemicals were tested on cultured fungus for growth inhibition or on infected eggs of rainbow trout to evaluate their antifungal activity. VigorOx, peracetic acid, and clotrimazole were all too toxic to the eggs to be useful for treating fungal infections. Potassium permanganate, iodine, and Diquat were effective for preventing fungal infections in uninfected eggs, but none were effective for control of fungus on infected eggs. Formalin proved to be effective for preventing fungal infections on eggs at concentrations as low as 250 ppm. The 1000 ppm treatment of formalin was also effective for preventing infection, decreasing existing infection, and increasing hatch rates at 15, 30, or 60 minute exposures. Hydrogen peroxide, a common disinfectant, was as or more effective than formalin for control of fungus on incubating eggs; treatments of 500 ppm were effective for preventing or decreasing fungal infections and for increasing hatch rates of treated eggs. Common salt (sodium chloride) was found to be effective for preventing fungal infections at 1.5% and for decreasing infection rates at 3.0%. Hydrogen peroxide was toxic to adult Spring Chinook salmon at 250 ppm, but not at 100 ppm. Hydrogen peroxide at 100 ppm was not effective at preventing pre-maturational mortality; however, none of the mortalities had associated fungal infection. Hydrogen peroxide at 25 ppm was effective at preventing pre-maturational mortality, especially in one replicate and incidences of fungal infection were very low. Formalin at 167 ppm was effective at preventing pre-spawning mortality and controlling fungal infections.

Introduction

Aquatic fungi (Saprolegniales) are ubiquitous in natural water supplies of fish hatcheries often causing serious disease problems. Malachite green is effective in control of fungus on fish and fish eggs, but due to suspected teratogenicity (Meyer and Jorgenson 1984) its use was limited to the treatment of non-food fish (i.e., eggs or adult salmon held for spawning) under an Investigational New Animal Drug Application (INAD) held by the U.S. Fish and Wildlife Service. That INAD was canceled on August 27, 1991 and uses were to be discontinued within 45 days. Heretofore special exemptions are required by the FDA for any uses. Presently, there is one registered aquatic fungicide, formalin, but it is not completely satisfactory for control of fungus on fish or their eggs. Furthermore, formalin is registered only for use on salmonids and esocids. Consequently, the search for safe and effective aquatic fungicides must continue.

Bailey (1984) and Bailey and Jeffrey (1989) reported results of tests with over 200 compounds that were chosen for fungicidal activity. More than half were found to be unsuitable as aquatic fungicides in preliminary tests because of their lack of activity against fungi, toxicity to fish or their eggs, insolubility in water, or potential carcinogenicity. However, several of the better candidates showed potential for control of fungus on fish eggs and were chosen for further evaluation.

This study is a continuation of "Research to Identify Effective Antifungal Agents" sponsored by Bonneville Power Administration (Schreck et al. 1990, 1991, and 1992). The objectives of the present study were to select and evaluate candidate fungicides. Evaluations involve laboratory studies on efficacy of candidate compounds on cultured Saprolegniales, on eggs of rainbow trout and chinook salmon that were previously infected with the fungus, and hatchery studies on adult spring chinook salmon.

Materials and Methods

Goal I. Eggs and Juveniles

In Vitro Tests

Pure strains of aquatic fungi were obtained from the American Type Culture Collection (ATCC). *Saprolegnia parasitica* (ATCC 22284) was used for both range finding and minimum inhibitory concentration tests. Test procedures used were those developed by Bailey (1983a,b). The method involved an in vitro screening technique modified from that of Golden and Oster (1947) and a minimum inhibitory concentration determination based on the percent inhibition of growth in diameter of colonies (Bailey and Jeffrey 1989).

Chemicals in solid or liquid form were weighed or measured and dissolved in aqueous solutions; concentrations were corrected for purity but not for specific gravity. For example, malachite green was prepared from a 50% active solution twice the volume of stock was added to the dilution media to account for purity. For continuity, concentrations are reported in ppm for solid and liquid chemicals in these tests.

Range Finding

Standard petri dishes were filled with 20 ml of corn meal agar and inoculated with agar plugs augmented with fungi (5 mm in diameter). The fungi was allowed to incubate at 20-C for approximately % hours. Agar plugs were removed from the edge of the colonies with a #1 cork borer. Stock solutions of chemicals were prepared to achieve final concentrations of 1, 10, and 100 ppm. The depressions of Coors porcelain spot plates were filled with three replicates of each of the candidate chemicals and the solvent, positive, and negative controls. Agar plugs were then added to the depressions of the spot plates for exposures of 15 or 60 min. Agar plugs were removed from the spot plates, rinsed three times with sterile

distilled water, and placed on tri-petri dishes containing 30 ml of-corn meal agar. Cultures were incubated inside an environmental control chamber maintained at 20°C ($\pm 2^\circ\text{C}$). The plates were examined for mycelial growth after 48,96, and 168 hours of incubation.

Minimum Inhibitory Concentration

Inoculations, incubation, and stock solution preparation were as stated above. Agar plugs were removed from the edge of the fungal colonies as previously described and exposed in triplicate to five delineative concentrations between 0.1 and 1.0 ppm, 1.0 and 10.0 ppm, or 10.0 and 100.0 ppm, or at higher levels, depending on the activity observed in the range-finding test.

The agar plugs were exposed to the test chemicals in triplicate for 15 or 60 min. They were rinsed with sterile distilled water and placed on standard petri dishes containing 10 ml of corn meal agar. Cultures were incubated in an environmental control chamber at 20°C for 48 hours, and the colony diameters were measured with a vernier caliper.

In Vivo Tests

Unfertilized rainbow trout (*Oncorhynchus mykiss*) eggs were obtained monthly from Trout Lodge, Sumner, Washington and after fertilization, were placed in Heath incubation trays. A modified egg counting board was used to place 500 eggs in each tray. The eggs were confined within a 6-inch diameter acrylic ring that was 1 inch in height and attached with silicone to the screen of each incubator tray. Two trays of 500 eggs each were used as replicates for each treatment level. Eggs were inoculated with 12 *S. ferax* or *S. parasitica* infected hemp seeds suspended by a tea ball in the upper tray of each replicate treatment for exposures of approximately 7 days or until the initial infection rate was about 10% or 20%.

Infection rates were equalized prior to exposure by exchanging infected eggs in trays with a high infection rate with uninfected eggs in trays with a low infection rate. Eggs were exposed to the fungicides for 15,30, or 60 min.

The chemicals were delivered to the water inflow of a separate mixing tray above the egg hatching trays with the use of a peristaltic pump to achieve specific desired concentrations. The mixing tray contained a maze of baffles to ensure complete mixing. Concentrations were calculated on the basis of amount of material added to a specific volume of water flow. Treatments were administered every other day for a period of 2 weeks or until eggs began hatching. Mortality and fungal infection were assessed prior to the first treatment (pretreatment) and after the last treatment (post-treatment). Infection rates (% increase) were calculated by subtracting pretreatment infection rates from post-treatment rates. Fry hatch was assessed after eggs hatched (post hatch). The percent hatch was corrected for initial mortality by subtracting the pretreatment mortality from the total number hatched according to the following formula.

$$\text{Percent hatch} = \frac{\text{number hatched}}{\text{total} - \text{initial mortality}} \times 100$$

Characteristics of the well water used for incubation was a total hardness of 138 ppm as CaCO₃, alkalinity of 105 ppm as CaCO₃, pH of 8.0, and temperature of 12-C. Concentrations of dissolved oxygen remained at 9.0 ppm or above during the exposures. Water flow was about 1 L/min during incubation and treatments. During infection water flow was discontinued for 2 h periods in morning and afternoon to promote infection of the eggs.

Toxicity Testing

Toxicity tests of the candidate fungicides were performed in the egg incubators simultaneously with the efficacy treatments. The setup was the same as

stated above for the in vivo testing however, the eggs were uninfected. The dilution series was generally based on a use pattern of 1,3, and 5X. The 1X concentration was the concentration we felt would be effective for control of fungus. Mortality observations were taken daily and egg hatching success was recorded at the end of each test.

Exposures of 15,30, or 60 minutes

Hatchery procedures generally and traditionally suggest 15 minute exposures to therapeutants. This short exposure time requires higher treatment levels for the therapeutant to arrest the fungus than for longer exposures. The most effective antifungal agents were chosen for testing at 15,30, or 60 minute exposures at the 0 and 10% infection rates. The candidates were formalin, hydrogen peroxide, and salt

Goal II. Adults

Based on results from Goal I, hydrogen peroxide was selected as the candidate compound for testing on adult spring chinook salmon (*O. tshawytscha*). Hydrogen peroxide (50% technical grade solution) was purchased from Van Water and Rogers. On 9 July 1993,200 adult spring chinook salmon were delivered to Smith Farm Experimental Hatchery (Corvallis, OR) from Oregon Department of Fish and Wildlife's Dexter Holding Facility (Willamette River) and distributed among eight 10-foot circular tanks (flow = 45 L/min; water temperature = 13-14°C). All adults received erythromycin and oxytetracycline before transportation. A total of 15 fish were distributed (n = 5 each) among three 6-foot circular tanks for toxicity testing. These fish were exposed to 250,100, and 25 ppm hydrogen peroxide every other day for one week beginning 11 July.

To test the efficacy of hydrogen peroxide as a fungicide, fish in replicate 10-foot tanks were treated in the following manner: controls (no treatment), formalin

067 ppm), hydrogen peroxide (100 ppm) and hydrogen peroxide (25 ppm). Treatment consisted of adding the appropriate volume of stock chemical to a bucket, diluting to 20 L with water, and then adding the contents to the tank at the inflow over 1 min. The tanks were checked daily for mortalities and any mortalities that did occur were scored for the presence of fungus on the body or gills, abrasions on the body, and obvious abnormalities in the organs. Gender and maturational status were also noted. A total of 7 fish died within 2 days of transportation to the hatchery and their mortalities were attributed to transportation stress (i.e. these fish were not included in the study). A total of 179 fish were included in the study of fungicidal activity.

Results

Goal I. Eggs and Juveniles

Two coded candidate fungicides were submitted by Abbott Laboratories, North Chicago, IL for evaluation in our in vitro program. AN437 and A-85858 were not toxic to *S. parasitica* cultured on corn meal agar at 100 mg/l in range finding testings and the minimum inhibitory concentration was 500 mg/l or greater for either candidate. because of the low activity, these compounds will not be evaluated further. Two other suggested candidate fungicides, calcium propionate and propionic acid, were tested and the antifungal activity was >1,000 pm. They were not tested further.

Selected candidate fungicides in these studies were VigorOx, peracetic acid, clotrimazole, hydrogen peroxide, potassium permanganate, iodine, Diquat, and sodium chloride. Their fungicidal activity at fungal infection rates of 0, 10, or 20% were compared to that of malachite green and formalin (Table 1). Toxicity values are also reported to evaluate their relative safety. All the values generated for these summary results are found in Appendices 1 through 6.

VigorOx, peracetic acid, and clotrimizole were evaluated only on 20% fungal infection rates, and the fungus was *S. ferax* as in previous work on this project' (Table 1). VigorOx is an effective sanitizing agent that is formulated to contain 5% peracetic acid and 20% hydrogen peroxide. It is approved by the EPA and accepted as safe by the FDA as a sanitizer for food surfaces. The formulation was effective for control of fungus on the eggs, but it was toxic to the eggs at slightly higher treatment rates. Peracetic acid, tested individually, was also effective for control of fungus on the eggs, but again it was too toxic to the eggs to be used safely. Clotrimizole, tested in a similar manner, was also too toxic eggs to be used safely. None of these three compounds was considered further as candidate fungicides because of their toxicity. Hydrogen peroxide, also a component of the VigorOx formulation, demonstrated antifungal activity at 250 ppm and safety to the eggs as toxicity wasn't evident at concentrations below 1,500 ppm (Table 1).

Potassium permanganate and iodine have been suggested and used to control fungal infections on salmonid eggs (Chapman and Rogers 1992 and Piper et al. 1982). The EPA has allowed the use of permanganate as an oxidant and a detoxifier in water treatment processes and the FDA has allowed the use of iodine following the water hardening process of newly spawned salmonid eggs. In fact, the FDA concluded that povidone iodine compounds were low regulatory priority (LRP) when used at a level of 100 ppm for 10 minutes as egg disinfectants after water hardening. Although both compounds showed some prophylactic value at 50 and 100 ppm, neither one controlled fungal infections or improved the hatch rate (Table 1 and Appendix 1).

Diquat has been suggested and used as a fungicide to treat eggs and fish, but no reliable data are available to demonstrate the efficacy. Recently, the FDA granted the Illinois Department of Conservation a restricted authorization to treat fish for columnaris disease with up to 18 ppm for 1 to 4 hours per day. Concentrations of

250 and 500 ppm for 60 minute exposures were effective for preventing fungal infections in uninfected eggs, but ineffective for control of infections at the 10 and 20% rates (Table 1 and Appendix 2). These high levels of Diquat required to control fungus could never be registered; Diquat is no longer considered to be a candidate fungicide.

Common salt (sodium chloride), sea salt, and mixtures of sodium and calcium chloride have been suggested for control of fungus on incubating eggs (Edgell et al. 1993). Concentrations of 1.5 and 3.0% salt for 60 minute exposures were effective for preventing fungal infection and for increasing the hatch rate of uninfected eggs (Table 1 and Appendix 3). Once the eggs were infected with fungus, the 3.0% salt treatment was effective for decreasing the infection rate and increasing the hatch rate. The hatch rate was 82% for the eggs infected at the 10% rate as compared to 69% in the uninfected control and 25% in the infected control. Salt is a good candidate fungicide, but the volumes required preclude its practical use at many hatcheries.

Exposures of 15,30, or 60 minutes

Formalin was found to be effective for preventing fungal infections on eggs at concentrations as low as 250 ppm. Exposure of only 15 minutes was effective for control of infection and improving hatch rate. Hatch rate of treated eggs was 61%; that of untreated and infected eggs was 34%; that of untreated and uninfected eggs was only 18% (Table 2). Exposures of 30 and 60 minutes were also effective for control of fungus on the uninfected eggs. However, the 250 ppm treatment was ineffective for control of fungus at the 10% level of infection at all the exposures. The 1000 ppm treatment of formalin was also effective for preventing infection and increasing hatch rates at 15,30 and 60 minute exposures (Table 2). This treatment also progressively improved the hatch rate of eggs infected at the 10% rate in 15

minute exposures (50%), in 30 minute exposures (64%), and in 60 minute exposures (83%). In fact, the 60 minute exposure actually decreased (-1.6) the initial infection rate. Toxicity to eggs was not apparent at 5,000 ppm for 15 or 30 minute exposures.

Hydrogen peroxide is a relatively safe compound and used as an antimicrobial agent in cheese production, in the treatment of drinking water, and as a bleaching agent in the textile industry. Treatments of 500 ppm were effective for preventing or decreasing infection and increasing hatch rates in all exposures for the uninfected (0% infected) groups (Table 3). The 60 minute exposure was required to significantly improve the hatch rate in eggs infected at the 10% rate. In reality, the 10% infection rate is perhaps a “worst case” situation in hatcheries where treatments are normally done when the fungus first appears or is suspected. The 1000 ppm treatment rate seems to be effective for ‘decreasing infection rate and increasing hatch rate at all the exposure conditions and toxicity is not apparent in those exposures. The 1000 ppm treatment rate may be excessive, especially for the longer exposure.

Goal II. Adults

In the trials to establish toxicity of hydrogen peroxide, adult spring chinook salmon exposed to 250 ppm hydrogen peroxide all died within 24 hr of initial treatment (Table 4). No other treatments suffered any mortalities during the one week exposure period (three treatments).

Of the 179 fish that were included in the assessment of fungicidal activity, 27 (15.1%) died before the detection of ovulation in any group of fish and a total of 78 (43.6%) died without undergoing maturation. Fish from replicated controls suffered mortality at 57.1 and 85.096, respectively, and most had associated fungal infections (Tables 5 and 6). The duplicate groups of fish treated with formalin at 167 ppm had 4.5 and 17.4% mortality, respectively, and none of the mortalities had associated

fungal infections. The fish treated with hydrogen peroxide at 100.ppm suffered 65.2 and 68.2% mortality per replicate, respectively, but no mortalities had associated fungal infections. The fish treated with hydrogen peroxide at 25 ppm had 4.5 and 46.2% mortality per replicate, respectively, and one mortality from each replicate had evidence of fungal infection. For all groups combined, final maturation was completed by 72 (56.3%) of the surviving females and most of the males.

Discussion

Eggs were acquired and tested every month during the contract year. Condition of the eggs varied with the season; those acquired in July and August were in poor condition and those acquired in winter months were in good condition. Those in good condition generally took longer to infect with the fungus. Infection rates in eggs artificially infected (+ controls) were always greater than uninfected controls that were exposed only to ubiquitous fungi in the water system. This is good evidence that the artificial infection was successful. Since the condition of eggs varies with season, it is important to use the same group of eggs to evaluate efficacy at different exposure regimes concurrently with toxicity as we did in these experiments.

Willoughby and Roberts (1992) reported that 0.25 ppm of malachite green oxalate killed zoospores and zoospore cysts of *Saprolegnia parasitica* in the water column and that exposure for 15 minutes should control fungal growth and protect the fish. We found that up to 5.0 ppm were required to control infections that already existed on the eggs, but that 1.0 ppm was effective for preventing infection. Our data with other candidates suggest that prophylactic treatments at lower concentrations are more practical. Although iodine and potassium permanganate were not fungicidal, they and other chemicals provided some protection to eggs that were not infected with the fungus.

Formalin is routinely used in hatcheries for control of fungus on eggs at 1,667 and sometimes 2,000 ppm in 15 minute exposures. Lower concentrations were effective in our test system . A concentration of 250 ppm seemed to be fungistatic as it prevented infections in uninfected eggs. A concentration of 1000 ppm was fungicidal and the efficacy progressively increased in exposures of 15,30 and 60 minutes. Fish culturists should be aware that lower treatment rates may be effective and use of lower concentrations would be safer to the user and the environment Although formalin is effective for control of fungus on cultured eggs, concerns are expressed regarding safety in the work place. Applications should be done in closed systems to reduce the potential hazards. Also, there are growing concerns associated with the discharge of this chemical in the environment; hatchery managers report they cannot meet effluent requirements at some stations. There is consensus that a better antifungal agent is needed.

Concentrations of 250 to 1000 ppm of hydrogen peroxide were as or more effective than formalin for the control of fungus on incubating eggs. Hydrogen peroxide is active against a wide variety of organisms – bacteria, yeasts, fungi, viruses, and spores. It is listed as generally recognized as safe when used as a bleaching agent in manufacturing or feeding practices or as an antimicrobial agent in cheese production. It is also certified by NSF International for use in the treatment of drinking water. Hydrogen peroxide has been used as an antiseptic and a treatment for skin parasites, protozoans, and monogenetic trematodes on fish and is proposed as a treatment for sea lice on salmon. Hydrogen peroxide and its primary decomposition products, oxygen and water, are not systemic poisons and are considered environmentally compatible. A request to classify hydrogen peroxide as a “low regulatory priority (LRP)” substance has been submitted to the Food and Drug Administration. This classification would allow the use of hydrogen peroxide as an antifungal agent and avoid extensive registration costs. If hydrogen peroxide

is effective for fungus control under egg production conditions, it would be the antifungal agent of choice.

Salt (NaCl) has been used widely as a therapeutant and to reduce stress to fish in the aquaculture industry. Some fish farmers use salt for routine management purposes without proper knowledge of how it functions (Chinabut et al. 1992). They list the purposes as follows:

1. To prevent and treat bacterial diseases.
- 2 To eliminate external parasites.
3. To reduce stress conditions during fish transport
4. To reduce toxicity of ammonia and nitrate nitrogen in fish ponds.

Edgell et al. (1993) reported that 2.0 percent salt solutions compared favorably with 1.0 ppm of malachite green for control of fungus on eggs of chinook salmon (*Oncorhynchus tshawytscha*). Taylor and Baily (1979) reported that daily treatments of 2-3 hours with sea water were effective for control of *Suprolegnia declina* on eggs of pink salmon (*O. gorbusha*). We found that a concentration of 1.5% was effective for preventing fungal infections but did not inhibit infections once they were evident. A concentration of 3% was effective for decreasing fungal infections and increasing hatch rates. Salt is readily available and listed as a low regulatory priority (LRP) fishery chemical. Disadvantages are the large quantities that would be needed to transport, store, and administer to static or flow through aquatic culture systems. Also, effluents with these large quantities could pose problems. However, salt is a viable antifungal agent where it can be used in a practical manner.

Adult spring chinook salmon are more sensitive than rainbow trout eggs to the toxic effects of hydrogen peroxide. Adults exposed to 250 ppm (6-fold lower than the minimum toxic effect on eggs) died within 24 hr of treatment; however, fish exposed to 100 ppm showed no ill-effects over the short term. However, the groups treated with 100 ppm hydrogen peroxide throughout the period of maturation

suffered *slightly* more than 50% mortality, but had no obvious signs of fungal infection. Therefore, this exposure level may be reaching the toxic range for adult spring chinook salmon, but is also effective at controlling fungal growth. The lower exposure level (25 ppm) of hydrogen peroxide offers promise because only two fish from the 13 that died in the replicates had fungal infection and overall mortality, while varying between the two replicates, was low. These results indicate that hydrogen peroxide at either 100 or 25 ppm resulted in fewer deaths with associated fungal infections than untreated controls, and that overall survival of fish was greater in groups treated with hydrogen peroxide at 25 ppm than in controls. Further tests should be conducted on the efficacy of hydrogen peroxide at exposure levels below 100 ppm.

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Table 1. Efficacy and toxicity of candidate fungicides on different rates of fungus (*S. parasitica*) infected eggs of rainbow trout for an exposure time of 60 minutes at 12°C.

Candidate Fungicides	Minimum Applied Effective Concentration (ppm) at initial infection rates of			Minimum Applied Toxic Effect (ppm)
	0%	10%	20%	
Malachite green	25	5.0	5.0	>5.0
Formalin	250	1,000	1,000	<5,000
VigorOx ^a	--	--	100	250
Peracetic acid ^a	--	--	>5.0	15.0
Clotrimazole ^a	--	--	>10<20	<30
Hydrogen peroxide	250	250	250	1,500
Potassium permanganate	50	>100	>100	150
Iodine	50	>100	>100	<300
Diquat	250	>500	>500	>500
Sodium chloride	15,000	30,000	30,000	>30,000

^a Fungal infections were with *S. ferax*

Table 2 Efficacy of formalin on different rates of fungus (*S.parasitica*) infected eggs of rainbow trout for exposures of 15,30, and 60 minutes at 12°C and toxicity to uninfected eggs.

Treatment (ppm)	Exposure Time (min)	Infection (%)		Change Hatch (%)	Hatch (%)
		Initial	Final		
Efficacy					
(-) Control1	--	0.4	33.6	33.2	18.4
(+) Control2	--	5.7	57.1	51.4	34.4
250	15	0.0	30.1	30.1	61.1
		10.0	78.7	68.7	14.3
	30	0.0	0.1	0.1	80.9
		10.3	68.0	57.7	26.9
	60	1.0	15.9	14.9	60.5
		9.8	66.8	57.0	34.8
1000	15	0.0	0.7	0.7	78.7
		10.0	44.6	34.6	49.9
	30	0.0	0.0	0.0	82.1
		10.0	24.3	14.3	64.0
	60	0.0	0.0	0.0	88.3
		10.2	8.6	-1.6	83.3
Toxicity					
5000	15	--	--	--	91.7
5000	30	--	--	--	93.7

1 untreated and uninfected

2 untreated and infected

Table 3. Efficacy of hydrogen peroxide on different rates of fungus (*S. parasitica*) infected eggs of rainbow trout for exposures of 15,30, and 60 minutes at 12°C and toxicity to uninfected eggs.

Treatment (ppm)	Exposure Time (min)	Infection (%)		Change Hatch (%) (%)	Hatch (%)
		Initial	Final		
<u>Efficacy</u>					
(-) Control ¹	--	0.0	72.0	72.0	39.1
(+) Control ²	--	7.2	98.5	91.3	21
500	15	0.0	2.9	2.9	67.4
		10.1	84.6	74.5	14.6
	30	0.4	18.2	17.8	64.7
		10.3	78.9	68.6	27.3
	60	0.0	0.0	0.0	74.8
		10.2	54.2	44.0	61.3
1000	15	0.0	0.0	0.0	69.0
		10.8	53.1	42.3	55.0
	30	0.0	0.0	0.0	70.5
		10.2	31.4	21.2	60.8
	60	0.0	0.7	0.7	65.2
		10.3	32.7	22.4	50.2
<u>Toxicity</u>					
2500	15	--	--	--	74.0
2500	30	--	--	--	62.1

1 untreated and uninfected

2 untreated and infected

Table 4. Mortality in adult spring chinook salmon within 24 hours of initial treatment of hydrogen peroxide on 11 July 1993.

Treatment (ppm)	Number of fish	Mortalities	
		Number	Percentage
250	5	5	100
100	5	0	0
25	5	0	0

Table 5. Mortality and incidence of fungal growth in adult spring chinook salmon from 11 July through 6 September 1993 (before any fish matured) and from 7 through 29 September 1993 (after fish began to mature). Numbers indicate the percentage of starting number that suffered mortality and percentage of mortalities that had fungal infection.

Treatment (ppm)	Number of fish	Mortalities 11 July-6 Sept		Mortalities 7 Sept-29 Sept	
		Total	with fungus	Total	with fungus
Control	21	23.8	100.0	33.3	100.0
	20	20.0	100.0	65.0	38.5
Formalin (167)	23	0.0	0.0	17.4	0.0
	22	4.5	0.0	4.5	0.0
Hydrogen peroxide (100)	23	43.5	0.0	21.7	0.0
	22	18.2	0.0	45.5	0.0
Hydrogen peroxide (25)	26	11.5	33.3	34.6	0.0
	22	0.0	0.0	4.5	100.0

Table 6. Maturation, mortality and incidence of fungal growth in adult spring chinook salmon from 11 July 29 September 1993. Numbers indicate females and males that reached maturity or suffered mortality (and number mortalities that had fungal infection).

Treatment (ppm)	Number of fish	Maturation 11 July-29 Sept		Mortalities 11 July-29Sept	
		Females	Males	Females	Males
Control	21	5	4	10 (10)	2 (2)
	20	2	1	12 (5)	5 (4)
Formalin (167)	23	16	3	1 (0)	3 (0)
	22	12	8	1 (0)	1 (0)
Hydrogen peroxide (100)	23	5	3	14 (0)	1 (0)
	22	4	3	8 (0)	7 (0)
Hydrogen peroxide (25)	26	10	4	9 (1)	3 (0)
	22	18	3	1 (1)	0 (0)

Appendix

Data are presented from individual monthly experiments that show efficacy of candidate antifungal agents as compared to control treatments that were untreated and uninfected or untreated and infected. Appendix 6 contains information on toxicity of candidate agents from previous experiments.

Appendix 1. Efficacy of potassium permanganate and iodine on different rates of fungus (*S. parasitica*) infected eggs of rainbow trout for an exposure time of 60 minutes at 12°C.

Treatment (ppm)	Exposure Time (min)	Infection (%)		Change Hatch (%)	Hatch (%)
		Initial	Final		
(-) Control	--	0.0	2.6	26	84.9
(+) Control	--	18.9	68.3	49.4	15.4
Potassium permanganate	50	0.0	0.0	0.0	66.0
	100	0.0	0.0	0.0	55.9
	50	10.4	43.5	33.1	19.7
	100	10.8	37.9	27.1	19.4
	50	20.1	42.3	22.2	21.3
	100	20.8	43.1	22.3	18.4
Iodine	50	0.7	6.1	5.4	70.0
	100	0.0	2.8	2.8	63.3
	50	10.5	37.8	27.3	34.6
	100	10.6	46.3	35.7	26.7
	50	20.7	56.7	36.0	17.0
	100	20.8	45.7	24.9	21.3

Appendix 2. Efficacy of formalin and diquat on different rates-of fungus (*s. parasitica*) infected eggs of rainbow trout for an exposure time of 60 minutes at 12°C.

Treatment (ppm)	Exposure Time (min)	Infection (%)		Change Hatch (%)	Hatch (%)
		Initial	Final		
(-) Control	--	0.0	27.0	27.0	79.2
(+) Control	--	17.4	81.8	64.4	28.7
Diquat	250	0.0	3.6	3.6	78.7
	500	0.0	0.7	0.7	88.8
	250	10.7	65.9	55.2	64.3
	500	10.2	55.2	45.0	67.6
	250	20.5	60.1	39.6	68.8
	500	19.7	67.2	47.5	57.5
Formalin	250	0.0	0.0	0.0	89.4
	1000	0.0	0.0	0.0	87.9
	250	10.7	68.7	58.0	55.5
	1000	10.4	10.5	0.1	86.1
	250	20.1	64.9	44.8	57.4
	1000	19.9	13.5	-6.4	88.0

Appendix 3. Efficacy of sodium chloride and hydrogen peroxide on different rates of fungus (*S. parasitica*) infected eggs of rainbow trout for an exposure time of 60 minutes at 12°C.

Treatment (ppm)	Exposure Time (min)	Infection (%)		Change Hatch (%)	Hatch (%)
		Initial	Final		
(-) Control	--	0.0	19.1	19.1	68.8
(+) Control	--	15.7	64.9	49.2	25.2
Sodium chloride	15,000	0.0	1.1	1.1	85.6
	30,000	0.0	2.2	2.2	81.5
	15,000	10.3	47.0	36.7	55.4
	30,000	10.6	14.9	4.3	82.3
	15,000	20.6	46.2	25.6	51.0
	30,000	21.4	29.8	8.4	71.2
Hydrogen peroxide	250	0.0	0.7	0.7	91.4
	500	0.0	0.5	0.5	92.4
	250	10.7	22.8	12.1	93.9
	500	10.3	22.8	12.5	80.0
	250	19.0	30.4	11.4	76.7
	500	20.0	33.8	13.8	71.9

Appendix 4. Effectiveness of peracetic acid, hydrogen peroxide, and clotrimazole on fungus (*S. ferax*) infected eggs of rainbow trout for an exposure time of 60 minutes at 12°C.

Treatment Chemical	Treatment (ppm)	Infection (%)		Hatch (%)
		Initial	Increase	
(-) Control	--	0.0	34.5	46.0
(+) Control	--	23.9	49.9	29.3
Peracetic acid	1.0	24.0	49.5	38.0
	3.0	22.4	52.7	27.5
	5.0	24.9	42.5	42.5
Hydrogen peroxide	100	21.4	33.3	44.5
	200	24.8	15.5	64.7
	400	21.1	2.8	81.9
(-) Control	--	0.0	0.9	69.4
(+) Control	--	22.8	43.2	24.4
Clotrimazole	5	24.8	22.6	38.7
	10	24.7	26.1	38.1
	20	25.1	10.6	12.8

Appendix 5. Efficacy of malachite green and diquat on different rates of fungus (*S. parasitica*) infected eggs of rainbow trout and toxicity of diquat at 1,3, and 5X concentrations to uninfected eggs for an exposure time of 60 minutes at 12°C.

Treatment Chemical	Treatment (ppm)	Infection (%)		Hatch (%)
		Initial	Increase	
(-) Control	--	0.0	46.3	36.3
(+) Control	--	18.9	69.3	13.8
Malachite green	2.5	0.0	0.0	78.3
	5.0	0.0	0.8	77.4
	2.5	11.2	34.4	70.5
	5.0	9.3	14.7	77.6
	2.5	20.4	27.8	67.6
	5.0	19.3	11.9	74.2
Diquat	50	21.3	46.7	32.9
	100	18.6	53.2	39.1
	200	20.5	47.0	50.6

Appendix 6. Toxicity results of 1,3, and 5X concentrations of candidate fungicides to uninfected rainbow trout eggs for an exposure time of 60 minutes at 12°C.

Chemical name	Treatment Concentration (ppm)	Hatch (%)
Control (mean)	89.9	
Malachite green	1.0	94.5
	3.0	94.8
	5.0	95.0
Formalin	1,667	86.3
	5,001	40.9
	8,335	1.4
Sodium chloride	10,000	92.7
	30,000	93.0
	50,000	91.2
Hydrogen peroxide	500	86.0
	1,500	63.8
	2,500	16.6
Peracetic acid	3.0	85.1
	9.0	84.7
	15.0	53.3
VigorOx	50	97.9
	150	97.5
	250	60.9
Potassium permanganate	50	96.4
	150	75.9
	250	5.9
Iodine	100	82.9
	300	4.4
	500	0.0
Diquat	100	88.5
	300	88.2
	500	86.7
Clotrimazole	10	79.8
	30	11.2
	50	11.0