Vol. 53: 133–142, 2003 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published February 13

Physical and chemical effects on viability of the *Myxobolus cerebralis* triactinomyxon

Eric J. Wagner[1,*], Mark Smith[1], Ronney Arndt[1], Donald W. Roberts[2]

[1]Fisheries Experiment Station, 1465 West 200 North, Logan, Utah 84321, USA
[2]Department of Biology, Utah State University, Logan, Utah 84322, USA

ABSTRACT: Various chemical and physical methods for destroying the triactinomyxon (TAM) stage of the myxozoan parasite *Myxobolus cerebralis* were tested. The fluorescent stains propidium iodide and fluorescein diacetate were used as indicators of viability. Physical variables tested included freezing, drying, high temperature, sonication, and pressure of 6.2×10^7 Pa (9000 psi). Chemicals evaluated included chlorine bleach, povidone-iodine, and hydrogen peroxide. Freezing or drying for 1 h was effective in killing TAMs, but pressure was not. Temperatures above 75°C for at least 5 min were also effective. Sonication with a laboratory instrument cleaner for 10 to 13 min killed and ruptured TAMs, resulting in <1.9% recovery. However, among the surviving TAMs, 39 to 58% were still viable. Chlorine concentrations of 130 ppm for 10 min were also effective at temperatures ranging from ice-water to room temperature and total hardness ranging from 10 to 500 mg l^{-1}. Lethal concentrations of hydrogen peroxide and povidone-iodine (10% solution) were quite high: 10% for 10 min, and 50% (5000 ppm active iodine) for 60 min, respectively. The stain results indicating TAM death were verified in 2 tests in which rainbow trout *Oncorhynchus mykiss* were exposed to TAMs that had been either frozen for 1 h or treated with 66 ppm chlorine as sodium hypochlorite for 1 min. None of the fish exposed to the treated TAMs became infected. These results should provide disinfection guidelines to prevent transfer of *M. cerebralis* TAMs to uninfected areas and provide information on the risks of parasite transfer under various treatment scenarios.

KEY WORDS: Myxobolus · Whirling disease · Disinfection · Chlorine · Ultrasound

INTRODUCTION

Myxobolus cerebralis, the parasite that causes whirling disease in salmonids, has a 2-host life cycle (Markiw & Wolf 1983). The actinospore stage, called a triactinomyxon (TAM), develops between epithelial cells in the digestive tract of the aquatic worm *Tubifex tubifex*. The TAM is infective to salmonids and may be the stage most vulnerable to inactivation or destruction due to its short life outside the host (<8 d; Markiw 1992, El-Matbouli et al. 1999) and its greater fragility in comparison to the resistant myxospore, which can survive in nature for 12 yr or more (Halliday 1976). Control strategies aimed at the TAM stage may be fruitful.

Disinfection of equipment is an important control measure to prevent transfer of the parasite to uninfected sites. In aquaculture and fisheries management, sodium hypochlorite (5.25% solution, i.e. bleach), povidone iodine, and hydrogen peroxide are widely used as disinfectants and for treatment of external parasites and pathogens (Ross & Smith 1972, Schäperclaus 1991, Bruno & Raynard 1994). Hydrogen peroxide and povidone iodine (1-ethyenyl-2-pyrrolidinone homopolymer and 1-vinyl-2-pyrrolidinone polymer iodine complex) have been recommended for prophylactic treatment of fish eggs (Chapman & Rogers 1992, Barnes et al. 1998). Betadine®, or PVP-iodine, is a commercial product that is 10% povidone-iodine (1% active iodine). For disinfection of salmonid eggs, 1% or 100 ppm of active iodine has been effective for controling the majority of external bacteria and viruses (Amend & Pietsch 1972, Ross & Smith 1972, Chapman & Rogers 1992, Goldes & Mead 1995). However, lethal concentrations of povidone iodine or hydrogen peroxide

*Email: ericwagner@utah.gov

have not been determined for either the myxospore or actinospore of *Myxobolus cerebralis*.

Previous research has investigated the effect of chlorine on the viability of *Myxobolus cerebralis*, though primarily the myxospore stage (Hoffman & Putz 1969, Hoffman & Hoffman 1972, Hoffman & O'Grodnick 1977). The effect of chlorine on TAMs has been examined only indirectly. For example, a hatchery water supply treated with 0.5 ppm chlorine for 2 h once a week reduced infection by 73% in one group of trout, and 63% in another (Markiw 1992). It is not known if TAMs or worms were affected by the treatment. Similarly, disinfection of a water supply with chlorine gas (maximum of 300 ppm) prevented the recurrence of the disease in a Pennsylvania hatchery (Hoffman & Dunbar 1961).

In addition to chemical disinfection, pathogen control has been achieved by using physical means such as freezing, heating, drying, and pressure (Karel et al. 1975). Sonication has also been used for destruction of harmful bacteria (Harvey & Loomis 1929, Chambers & Gaines 1932). More recently, it has been used synergistically with chlorine or ozone for disinfection (Dahi 1976, Lillard 1993, 1994). Disinfection by sonication is achieved by the cavitation of microscopic bubbles in liquids induced by ultrasonic waves (Scherba et al. 1991). The extreme temperature and pressure gradients within the bubble during cavitational collapse can create hydroxyl radicals, free oxygen atoms, and hydrogen peroxide. These can combine with organic compounds, and together with the physical cavitation effects, disrupt cellular membranes (Scherba et al. 1991). Sonication could be applied to hatchery water supplies as a means of keeping the water free of pathogens such as *Myxobolus cerebralis*.

In our study, the potential for destroying *Myxobolus cerebralis* triactinomyxons with chlorine, iodine, and hydrogen peroxide was examined. In addition, we examined the effects of freezing, desiccation, pressure, sonication, and high temperature on TAM viability. The objective of the research was to develop disease control strategies and recommendations for disinfection concentrations and treatment protocols to prevent the transfer of the TAM stage of the parasite to uninfected areas.

MATERIALS AND METHODS

Fresh TAMs were filtered 3 times a week from water drawn from infected worm cultures maintained at 12 to 15°C. These worms were collected from the Little Bear River system at a site known to harbor *Myxobolus cerebralis*-infected salmonids. TAMs were kept cold (0 to 4°C) in vials and used within 24 h of collection. Vital staining with propidium iodide (PI) and fluorescein diacetate (FDA) was conducted using the techniques of Markiw (1992), which were based on the pioneering work of Jones & Senft (1985). A stock solution of PI (52 mg ml^{-1} H_2O) was frozen in 1 ml aliquots and stored at −40°C. Vials were thawed as needed on the day of the test. A stock solution of FDA (5 mg ml^{-1} acetone) was also kept at −40°C. A working solution of FDA was made fresh daily by diluting 100 µl of FDA stock with 8.0 to 8.3 ml of well water. Minor changes in the dilution were made to adjust for changes in strength of the fluorescence over time and for the amount of organic matter in the sample. Well water had a total alkalinity of 222 mg l^{-1}, total hardness of 222 mg l^{-1} as $CaCO_3$, temperature of 13.5°C, dissolved oxygen concentration of 6.5 mg l^{-1}, and pH of 7.6. All tests other than the freezing test and the ice-water test for chlorine were conducted at room temperature (15 to 21°C).

For each of the tests described below, equal amounts of FDA and PI were added to the test or control solution on each microscope slide, and a cover-slip was placed on the mixture. An effort was made to distribute the dyes across the solution for optimal mixing. The total amount of each dye varied slightly among the tests according to the amount of sample to dye. Slides were stored in a light-proof container to prevent photodegradation of the dyes and were incubated for at least 30 to 45 min in a refrigerator (7°C). With the aid of an epi-fluorescence microscope, TAM sporoplasts (germ cells within the spore body) fluoresced either red (classified as dead, i.e. non-viable) or green (classified as live, i.e. viable). Some TAMs contained both red and green sporoplasts, so were classified as possibly viable if even a single sporoplast was green.

Chemical tests. ***Chlorine:*** For each chlorine test, equal volumes of a TAM stock solution and sodium hypochlorite were mixed in a test tube for either 1 or 10 min. After mixing with TAMs, the resulting chemical concentrations ranged from 0.005 to 0.5% bleach solution or 2.6 to 262 ppm actual chlorine as sodium hypochlorite. After the test time had elapsed, the solution was filtered through a 10 µm mesh screen and rinsed with 20 ml of well water. As the last of the rinse water filtered through, the retentate was harvested with a pipette and 100 µl put on each of 1 to 3 slides. The slides were stained with 50 µl of PI and 50 µl of FDA, and incubated and examined as noted above. This process was repeated 2 to 3 times for each treatment. Control slides consisted of TAMs that were transferred directly from the stock solution, stained, and incubated in a refrigerator.

Additional tests were conducted to evaluate the efficacy of the recommended chlorine concentration (0.25% bleach) under different water quality scenar-

ios. These included cold water (4°C) for 1 or 10 min and total hardness of either 10 or 500 ppm as $CaCO_3$ for 1 min. Hardness treatments were created by diluting well water with deionized water (10 ppm $CaCO_3$ treatment) or adding $CaCl_2$ (500 ppm treatment). Preparation of controls, mixing with chlorine, rinsing, and staining of these test TAMs were conducted using the methods described for the previous chlorine test.

Hydrogen peroxide: For each test, hydrogen peroxide was added to an equal volume of TAM stock solution and left to sit at room temperature for 10 min. This solution was filtered, rinsed, and stained as noted above for chlorine. For each of the 3 replicates, a fresh solution of the hydrogen peroxide was made. The concentration of the stock solution (33 to 34%) was verified by measuring a diluted sample with a commercial test kit (Hach Chemical). Control slides consisted of TAMs that were transferred directly from the stock solution, stained, and incubated in a refrigerator.

Iodine: To evaluate the efficacy of iodine for killing TAMs, concentrations of PVP-iodine ranging from 1 to 50% and durations of 10, 30, or 60 min were tested. For each concentration, 3 to 6 replicate tests were conducted. For each test, 2 ml of the test iodine solution were mixed with 2 ml of TAM stock solution in a test tube and left at room temperature for 10, 30 or 60 min. A minute or 2 before the time was up, the mixed solution was poured into a 10 µm mesh filter. At the allotted time, the filter retentate was rinsed with 20 ml of hatchery well water. This process took several minutes, after which 100 µl aliquots of retentate were transferred to 3 to 4 microscope slides. These slides, plus control slides made directly from TAM stock solution, were subsequently stained with 50 µl each of PI and FDA and incubated in a refrigerator. The iodine concentration of the stock solution was verified with a commercial colorimetric test (Hach Chemical). The same stock solution was used for all tests.

Physical variable tests. ***Freezing:*** For evaluation of freezing, 2 × 30 µl samples of TAM stock were placed in 1.5 ml microcentrifuge tubes and frozen for 105 min at −20°C. The suspension was thawed and stained with 30 µl PI and 30 µl FDA. The vials were refrigerated in light-proof boxes for at least 30 to 45 min. After the incubation period, each of the 90 µl samples was split equally on 2 slides. Control slides consisted of TAMs that were transferred directly from the stock solution, stained, and incubated in a refrigerator.

Desiccation: For the desiccation trial, 10 µl of the concentrated TAM stock solution was put on each of 6 slides and dried at room temperature (19 to 21°C). Upon appearing dry, the slides remained on the laboratory bench for an additional 15 min. Slides were stained with 50 µl each of PI and FDA, and examined for viability of TAMs after incubation as noted above. An additional test was conducted in which TAMs were dried for 60 min. Control slides consisted of TAMs that were transferred directly from the stock solution, stained, and incubated in a refrigerator.

Pressure: A 3 ml vial was filled with a suspension of TAMs in well water and was covered with parafilm. This was clipped to a metal support that was lowered into a hydrostatic pressure chamber (38 × 5.7 cm stainless steel tube with a 'piston' plug to which a pressure gauge was laterally attached). A pressure of 6.2×10^7 Pa (9000 psi) was achieved after about 30 s using a press. After 5 min, the pressure was immediately released. TAMs on 3 slides were examined for viability as noted above. Total TAMs per slide ranged from 126 to 148. Control slides consisted of TAMs that were transferred directly from the stock solution, stained, and incubated in a refrigerator.

Temperature: To test the effect of high temperature, 500 µl of fresh TAMs were heated in microcentrifuge tubes in a water bath at 9 temperatures (7 [as control], 21, 27, 32, 38, 49, 58, 75, and 96°C). For each temperature, 3 tubes were placed in a plastic rack and submersed for 5 min. The tubes were then transferred to a water bath at room temperature (19 to 21°C) to cool rapidly. PI and FDA were added to the tubes (250 µl of each) and incubated for at least 45 min in a refrigerator. Samples of 150 µl from each tube were examined for viability.

Sonication: Sonication was evaluated for its ability to kill TAMs using a laboratory sonicator designed for cleaning instruments (Branson model 2200). Initial tests with the TAM suspension in 12 × 75 mm glass test tubes (55 s and 10 min treatments) indicated that sonication had little effect on viability. Approximately 85 to 92% of the TAMs were still viable after exposure to either 55 s or 10 min treatments in these tubes. Subsequent tests were conducted with TAMs suspended in well water in the 2.8 l sonicator chamber. There was no mechanism for changing the intensity (130 W) or frequency (47 kHz) of the sonication, so time (10 to 13 min) was the variable tested. After sonication, the solution was poured through a 10 µm mesh screen and the retentate rinsed into a vial. On each slide, 50 µl of retentate was mixed with 25 µl each of FDA and PI. Viability was categorized as noted above. Previous testing indicated no difference in viability for TAMs stained directly from stock solutions versus those put in the sonicator (without sonication) and recovered on the filter, so control slides were made directly from TAM stock.

Verification of stain results. To verify that the data generated by the vital stains were valid (i.e. red TAMs were indeed dead), a test was conducted in which TAMs were frozen and then used to expose young rainbow trout *Oncorhynchus mykiss*. A second test

evaluated the infectivity of TAMs treated with chlorine bleach for young rainbow trout. For the frozen-TAM evaluation, fresh TAMs were harvested from worm cultures on the day of the fish exposure, put into 50 ml centrifuge tubes, and frozen. The tube was monitored and 60 min after the liquid had frozen solid, the tube was thawed on a bench at room temperature. Three slides of 50 µl each were stained with PI and FDA to verify that freezing had killed the TAMs (i.e. sporoplasts stained red). The remaining solution was used to expose rainbow trout (mean weight 12 g). Fish exposures were conducted in 2 coolers with 8 l of hatchery well water at a dose of 2000 TAMs $fish^{-1}$ for 2 h. An equal number of fish (18 total) were put in separate coolers, but not exposed to any triactinomyxons. Both exposure and control coolers were oxygenated via an airstone during the 2 h period. After exposure, fish were transferred to tanks fed by a spring water supply and fed a commercial diet daily by automatic feeders. After 43 d, fish were anesthetized with a lethal level of tricaine methane sulfonate and the heads were packaged individually and frozen for later testing using the polymerase chain reaction (PCR) assay. The saw used to remove the heads was brushed clean with a strong bleach solution and wiped off with a paper towel between individual fish to prevent cross contamination among samples. The single-round modification (Schisler et al. 2001) of the PCR assay developed by Andree et al. (1998) was used to determine whether exposed fish had been infected. PCR results were categorized as negative, weakly positive, positive, strongly positive, or very strongly positive based on a visual assessment of the diagnostic DNA band in agarose gels (Schisler et al. 2001).

For stain verification of chlorine-treated TAMs, freshly harvested TAMs were exposed to 66 ppm chlorine as sodium hypochlorite (2.5 ml of bleach in 1 l) for 1 min at room temperature (15 to 21°C). After 1 min, the 50 ml mixture was poured through several 10 µm mesh filters and each filter rinsed with 20 ml of hatchery well water. The retentate was rinsed into a vial from which 100 µl was transferred to each of 3 slides for vital staining (50 µl each of PI and FDA) and counting. Three control slides of untreated TAMs were also examined. The treated TAMs were added to 8 l of hatchery well water in a cooler for 2 h at a dose of 2000 TAMs $fish^{-1}$. Three replicates were conducted over a week's time; 8 fish per cooler were used in the first replicate, 11 in the second, and 15 in the third (mean fish weight was 2.9 g for all replicates). For each replicate, 2 additional oxygenated coolers contained matching quantities of either unexposed controls or positive controls exposed to TAMs that had not been treated with chlorine. Fish were maintained on spring water in tanks as noted for the frozen TAM evaluation. After 7 wk, heads were sampled and analyzed by single-round PCR as noted above.

Data analysis. For all tests, a significance level of 0.05 was used, and percentages were arc-sine transformed to achieve homogeneity of error variance prior to statistical analysis. Differences among the control data for the chlorine, iodine, sonication, and hydrogen peroxide tests were significant using 1-way ANOVA for both the hydrogen peroxide and iodine tests. Subsequent tests using Student's *t*-test evaluated differences between each chemical concentration and its respective control. Duncan's test was used for mean comparisons after 1-way ANOVA (SPSS 1993) for the chlorine and temperature test data; control data were pooled. Student's *t*-test was also used to compare between test and control for the pressure, drying (separate test for each duration), and freezing tests. For analysis of PCR results for the verification tests, the maximum likelihood statistic of chi-square analysis was used to compare frequencies among the treated TAMs (frozen or chlorinated), positive controls, and negative controls (SPSS 1993) using all categories of infection intensity.

RESULTS

Chemical tests

Chlorine

In a 10 min treatment, concentrations of chlorine greater than 13 ppm were effective in killing TAMs at room temperature ($p < 0.001$; Table 1). For 1 min treatments, higher concentrations were necessary to kill 100%. At room temperature, 131 ppm chlorine was adequate, but in ice water, 12% of TAMs were still viable after 1 min. In a follow-up ice-water test, increasing the duration to 10 min resulted in a 100% kill. Total hardness of 10 or 500 mg l^{-1} did not alter the effect of chlorine on TAMs (100% kill at 131 ppm at room temperature). Therefore, for quick disinfection, concentrations of about 130 ppm or greater are recommended when targeting the triactinomyxon. This is about a 0.25% solution of bleach (2.5 ml l^{-1}; 9.5 ml $gallon^{-1}$). At colder temperatures, the duration of treatment or chlorine concentration should be increased.

Hydrogen peroxide and iodine

There were significant differences in viability among the controls (1-way ANOVA; $p = 0.042$ for iodine, $p = 0.031$ for hydrogen peroxide), so these are listed separately for each concentration in Tables 2 & 3. The source of variation is unknown, but may be related to

Table 1. *Myxobolus cerebralis*. Mean percentage (±SD; n = 3 slides) of viable, non-viable, and possibly viable of triactinomyxon stage of *M. cerebralis* treated with various concentrations of sodium hypochlorite for either 1 or 10 min at room temperature (15 to 21°C). Means within a duration and column that are not significantly different from each other share a common letter (1-way ANOVA and Duncan's test; $p \geq 0.05$)

Duration	Sodium hypochlorite (ppm)	Viable (%)	Non-viable (%)	Possibly viable (%)
1 min	0	78.0 ± 8.5 z	10.8 ± 8.9 z	11.2 ± 6.9 z
	26	2.0 ± 2.9 y	88.9 ± 9.0 y	8.8 ± 5.6 z
	131	0.0 ± 0.0 y	100.0 ± 0.0 x	0.0 ± 0.0 y
10 min	0	69.8 ± 27.9 z	16.8 ± 20.4 z	13.4 ± 8.1 z
	2.6	0.5 ± 1.0 y	76.3 ± 8.9 z	23.2 ± 8.0 z
	13	0.0 ± 0.0 y	100.0 ± 0.0 y	0.0 ± 0.0 y
	26	0.0 ± 0.0 y	97.6 ± 5.1 y	2.4 ± 5.0 y
	262	0.0 ± 0.0 y	100.0 ± 0.0 y	0.0 ± 0.0 y

the quantity of liquid remaining in the worm cultures after siphoning. This aliquot would contain older TAMs that may be non-viable and the amount could vary slightly from one harvest to the next. The results of tests with iodine and hydrogen peroxide indicated that the required concentration of these chemicals for complete disinfection was quite high. Povidone-iodine concentrations of 50%, or 5000 ppm, of active iodine for an hour were required to kill greater than 99% of the TAMs (Table 2). Although all hydrogen peroxide concentrations significantly reduced viability ($p < 0.05$; Table 3), a 10% solution was the minimum effective concentration for killing 100%.

Physical variables

Freezing

Freezing resulted in a marked decrease in TAM viability ($p < 0.001$; Table 4). The unfrozen TAM stock solution was 46% non-viable, compared to 98–100% after freezing. A few TAMs had both red and green sporoplasts fluorescing, but these made up just 0 to 2% of the frozen samples. The true viability of these is unknown. None of the frozen samples contained TAMs that were classified as viable.

Desiccation

Desiccation at room temperature effectively killed TAMs ($p < 0.001$). As shown in Table 4, very few TAMs (0 to 1%) survived the 15 min drying period to which they were exposed. Partially viable TAMs made up only 0 to 7% of the 6 samples. Drying for 1 h killed 100% of the TAMs.

Pressure

The percentage of viable (green) TAMs ranged from 43 to 60% after pressure treatment. This was a significant drop from the viability of controls (70 to 74%;

Table 2. *Myxobolus cerebralis*. Mean percentage (±SD) of non-viable, viable, and possibly viable triactinomyxons (TAMs) after exposure to povidone-iodine. Iodine concentrations are given as a percentage of the commercial povidone-iodine stock solution (10% povidone-iodine) and in active iodine concentration in ppm (given in parentheses). Control means for each concentration are also shown as well as the range in the number of triactinomyxons per replicate and total number of slides (n). Significant differences between test and control (*t*-test, $p < 0.05$) are indicated by an asterisk

Povidone-iodine concentration % (ppm active iodine)	Duration (min)	Non-viable (%)	Viable (%)	Possibly viable (%)	Range of TAM numbers per replicate (n)
1.0 (100)	10	64.3 ± 17.8*	16.5 ± 21.1*	19.2 ± 19.2	82–100 (3)
0.0		0.0 ± 0.0	94.7 ± 2.5	5.3 ± 2.5	100–100 (3)
2.5 (250)	10	67.2 ± 2.9*	25.4 ± 3.6*	7.4 ± 1.1	76–104 (4)
0.0		4.2 ± 3.6	77.1 ± 3.1	18.8 ± 5.8	31–100 (3)
5.0 (500)	10	66.3 ± 6.1*	11.7 ± 3.8*	22.0 ± 3.6	100–100 (3)
0.0		3.9 ± 4.6	84.6 ± 11.4	11.5 ± 8.2	28–100 (4)
50.0 (5000)	10	76.5 ± 6.0*	9.3 ± 7.5*	14.2 ± 4.0	100–160 (3)
0.0		1.1 ± 2.6	93.6 ± 6.2	5.4 ± 5.4	10–36 (6)
50.0 (5000)	30	90.7 ± 11.0*	8.8 ± 11.2*	0.4 ± 0.7	70–102 (3)
0.0		2.7 ± 2.6	94.5 ± 5.6	2.8 ± 4.8	14–36 (3)
50.0 (5000)	60	99.3 ± 1.8*	0.5 ± 1.3*	0.2 ± 0.4	91–117 (6)
0.0		0.5 ± 1.2	95.7 ± 5.2	3.8 ± 4.3	24–51 (6)

Table 3. *Myxobolus cerebralis*. Percentage of viable, non-viable, and possibly viable (mean ± SD) triactinomyxons of *M. cerebralis* after 10 min exposure to hydrogen peroxide as determined by vital staining. The range of the number of triactinomyxons per slide among the 3 or 4 slides used to calculate the mean is also presented. A significant difference (*t*-test, $p < 0.05$) between a treatment and its respective control (shown below each treatment; each was run on a different day) is indicated with an asterisk

Hydrogen-peroxide concentration (%)	Viable (%)	Non-viable (%)	Possibly viable (%)	Sample size range
1.7	47.3 ± 14.9*	35.5 ± 10.9*	17.2 ± 4.0*	7–133
0.0	93.7 ± 7.3	2.2 ± 2.4	4.1 ± 5.0	47–91
8.5	0.5 ± 0.4	98.6 ± 1.3*	0.9 ± 1.0	95–159
0.0	51.2 ± 43.1	22.6 ± 18.0	26.0 ± 25.0	48–131
10.2	0.0 ± 0.0*	100.0 ± 0.0*	0.0 ± 0.0*	93–135
0.0	81.7 ± 5.2	11.1 ± 5.0	7.2 ± 3.2	66–100
17.0	0.0 ± 0.0*	100.0 ± 0.0*	0.0 ± 0.0	18–94
0.0	68.9 ± 17.9	24.8 ± 14.8	6.3 ± 4.6	51–140

$p = 0.004$), but was not low enough to consider pressures of up to 9000 psi effective for controlling whirling disease.

Temperature

Results of the heat treatments are presented in Table 5. Viability of control TAMs averaged 79.7% ($n = 12$). Heat treatment for 5 min killed 100% of TAMs at temperatures of 75°C or greater. Temperature treatments of 49 or 58°C resulted in some TAMs that stained both red and green. If these are not truly viable, only 1% or less of the treated TAMs were viable (all green) at these 2 temperatures. Temperatures of 38°C or lower had increasingly higher percentages of viable and possibly viable TAMs (Table 5).

Sonication

Sonication of TAMs in test tubes did not result in any significant reduction of viability in treatments up to 10 min duration. However, if the TAMs were free in the sonication chamber, both survival (number of TAMs recovered) and viability (percentage of green-stained TAMs) after durations of 5 to 13 min were significantly lower than controls ($p < 0.05$; Table 6). As the sonication duration increased, fewer TAMs were recovered. Sonication for 10, 11, or 13 min respectively resulted in 1.9, 1.2, and 0.6% recovery of TAMs relative to the stock concentration. These presumably were broken up and destroyed. A few pieces were observed on slides and the data are summarized in Table 6. However, a few TAMs were still viable even after 13 min of sonication (Table 6).

Verification of stain results

Frozen TAMs examined by vital staining were 100% red, indicating that they had been killed by freezing. TAMs examined prior to freezing were 84.3% viable (stained green). Exposure of rainbow trout fry (10 fish in one tank and 8 in another) to the frozen TAMs did not result in any infection. Control fish not exposed to TAMs also were negative for *Myxobolus cerebralis*. For positive controls, all fish exposed to fresh TAMs (60 to 80% green, positive control) in these experiments were positive to strongly positive by PCR.

TAMs treated with 66 ppm chlorine were 100% red (dead) in all 3 replicates. Control TAM viability averaged 92%. Rainbow trout fry exposed to chlorine-treated TAMs were all negative for *Myxobolus cerebralis* ($n = 32$; $p < 0.001$). The positive control fish ($n = 33$) were 100% infected in all 3 replicates and each individual was classified as 'very strong positive' based on the intensity of the DNA band. The 34 negative control fish were all negative except for a single fish that was weakly positive (this sample was repeated 5 times with the same result).

Table 4. *Myxobolus cerebralis*. Percent (±SD) of triactinomyxons (TAMs) of *M. cerebralis* classified as viable (green fluorescing), non-viable (red), or possibly viable (both red and green), after freezing or desiccation and vital staining

Treatment	TAMs per slide (range)	Number of slides	Viable (%)	Non-viable (%)	Possibly viable (%)
Freezing at –20°C	96–192	4	0.0 ± 0.0	99.0 ± 0.8	1.0 ± 0.8
Control (7°C)	45–131	3	30.7 ± 5.9	46.0 ± 5.2	23.3 ± 1.2
Drying 15 min	27–110	6	0.3 ± 0.5	96.5 ± 2.9	3.2 ± 2.8
Drying 60 min	145–211	3	0.0 ± 0.0	100 ± 0.0	0.0 ± 0.0
Control (19–21°C)	51–124	4	71.8 ± 1.7	5.0 ± 1.4	23.2 ± 2.9

DISCUSSION

The results of the vital stain verification tests indicated that TAMs with red-stained sporoplasts were indeed dead. This was true for both tests in which fish had been exposed to TAMs that had either been frozen or treated with chlorine. Similar verification has been conducted for *Giardia* cysts by Schupp & Erlandsen (1987) and for *Cryptosporidium parvum* oocysts by Campbell et al. (1992). Schupp & Erlandsen (1987) noted that *G. muris* cysts that were stained red by PI were incapable of infecting mice, whereas green, FDA-stained cysts resulted in infection. Later work (Smith & Smith 1989, Labatiuk et al. 1991) has indicated that the dyes are conservative, overestimating actual viability of *Giardia* cysts. This is apparently true also for *Myxobolus cerebralis* TAMs, since large doses of TAMs frozen with cryoprotectants, of which approximately 30% stained green, were incapable of infecting trout fry (D. Roberts, Utah State University, unpubl. data). However, freezing with cryoprotectants may be a special case for viability interpretation. Green fluorescence is due to non-specific intracellular esterases cleaving the FDA to allow fluorescence (Jones & Senft 1985). *M. cerebralis* survival (viability) is the sum of all physiological functions, and esterases are only a small part of this sum; other crucial enzymes or proteins used in the infection process apparently did not survive freezing. However, red TAMs were not infective to fish; therefore, inability to exclude PI (red) can be considered a clear indication of non-viability. In the present study, TAMs staining both red and green were considered as potentially viable for disinfection purposes, but in reality they may not be viable. Accordingly, recommendations for disinfection are conservative and based on the vital staining result of 100% dead.

Table 5. *Myxobolus cerebralis*. Mean percent viability of the triactinomxyon of *M. cerebralis* after heating to test temperature in a water bath for 5 min. Due to rounding, percentages across columns may not add up to 100%. Means within a column that are not significantly different (1-way ANOVA, Duncan's test) share a common letter

Temperature (°C)	Non-viable (%)	Viable (%)	Possibly viable (%)
7 (control)	14.2 ± 8.4 z	79.7 ± 9.1 z	6.1 ± 3.0 zy
21	17.6 ± 6.2 z	66.6 ± 1.3 z	15.8 ± 6.8 x
27	4.6 ± 1.9 z	47.2 ± 6.9 y	48.2 ± 5.2 v
32	11.2 ± 2.6 z	44.3 ± 2.5 y	44.5 ± 3.4 v
38	32.2 ± 9.9 y	5.3 ± 1.6 x	62.4 ± 9.5 u
49	73.1 ± 6.6 y	0.6 ± 5.5 x	26.3 ± 7.1 w
58	89.8 ± 6.4 y	0.6 ± 0.5 x	9.6 ± 6.3 yx
75	100.0 ± 0.0 x	0.0 ± 0.0 x	0.0 ± 0.0 z
96	100.0 ± 0.0 x	0.0 ± 0.0 x	0.0 ± 0.0 z

Table 6. *Myxobolus cerebralis*. Mean percentage (n = 3 slides of 1 to 9 triactinomyxons each) of viable (green), non-viable (red-stained), broken (pieces only), empty (no spore body present), and possibly viable (red and green stained) triactinomyxons of *M. cerebralis* after exposure to sonication (47 kHz, 130 W) for 10 to 13 min. An asterisk indicates a significant difference from the control (n = 3 slides of 24 to 518 triactinomyxons each)

	Time (min)		
	10	11	13
Recovery (%)	1.9	1.2	0.6
Viable (%)			
Treatment	58.3 ± 34.7*	38.9 ± 19.2*	44.4 ± 50.9
Control	96.3 ± 3.4	81.3 ± 10.9	80.9 ± 14.4
Non viable (%)			
Treatment	12.5 ± 17.7	0.0 ± 0.0	0.0 ± 0.0
Control	1.6 ± 2.7	0.2 ± 0.2	4.2 ± 6.1
Broken (%)			
Treatment	0.0 ± 0.0	47.2 ± 21.0	0.0 ± 0.0
Control	0.0 ± 0.0	6.9 ± 3.8	7.2 ± 2.4
Empty (%)			
Treatment	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0*
Control	1.7 ± 1.7	10.0 ± 9.6	4.0 ± 2.0
Possibly viable (%)			
Treatment	29.2 ± 5.9	13.8 ± 12.7	55.6 ± 50.9
Control	0.4 ± 0.6	1.5 ± 1.1	3.7 ± 4.4

Chlorine was the most effective of the chemicals tested, killing TAMs at concentrations of 13 ppm or greater in 10 min treatments. Cold temperatures increased the contact time required for complete disinfection, but water hardness had no effect. For shorter contact times (<10 min), concentrations of 130 to 260 ppm are recommended for disinfection. Hoffman & O'Grodnick (1977) may have treated TAMs with chlorine, as they treated water that had been mixed with contaminated muds at 10, 20, 50, and 250 ppm chlorine for 30 min; control fish were infected, but no infection was found in fish from any of the treatment groups.

In chlorine tests with the myxospore stage, higher concentrations have been needed to deactivate the parasite, e.g. 1600 ppm chlorine (as sodium hypochlorite) for 24 h (Hoffman & Putz 1969). More recent research by Elizabeth McConnell and her coauthors (US Fish and Wildlife Service, Bozeman Fish Technology Center, unpubl. data) indicated that 5000 ppm of chlorine was required to kill the myxospore (based on methylene blue staining) in 10 min exposures. Hoffman & O'Grodnick (1977) treated a 2 cm layer of infected mud in aquaria with 1200 ppm chlorine (calcium hypochlorite) for 18 h; when fish from these aquaria were sampled 5 mo later, the fish were infected.

Povidone-iodine concentrations of 50% (5000 ppm of active iodine) for 1 h were required to kill greater than

99% of the TAMs. This is 50 times the concentration typically recommended for treatment of fish eggs for bacterial and viral disinfection (McFadden 1969). Although eggs are not infected by *Myxobolus cerebralis* (O'Grodnick 1975), the parasite may be carried to a hatchery in water with eggs obtained from wild sources. To adequately disinfect incoming water for *M. cerebralis*, the question becomes, 'How high can we safely go?' Amend (1974) found the toxicity of Betadine and Wescodyne® (1.6% active iodine in the form of 9.1% polyethoxy polypropoxy polyethoxy ethanol-iodine complex, 8.74% nonylphenoxypolyethanoxyethanol iodine complex and 82% inert ingredients) to rainbow trout eggs was dependent upon pH and the stage of development of the eggs; at pH 6.9, the LC_{50} for active iodine was 1480 ppm in a 15 min treatment or 1050 ppm in a 60 min treatment of eyed eggs. If the solution was buffered, the LC_{50} of eyed eggs was increased to greater than 2000 ppm at either pH 7.0 or 8.0. However, when eggs were water-hardened in iodine, 100 ppm resulted in significant mortality. McFadden (1969) noted that up to 2.5% povidone iodine for 10 min was not toxic to eyed rainbow trout eggs, but concentrations of 3, 4, and 5% (300 to 500 ppm iodine) resulted in eggs surviving less than 24 h (no pH given). For eyed rainbow trout eggs, Alderman (1984) found that the LD_{25} was about 800 ppm at pH 6.0 and in excess of 3000 ppm at pH 7.0. Based upon the above research, achieving 5000 ppm iodine without killing the eggs might be possible for eyed eggs at pH 8, but unrealistic for freshly fertilized eggs.

For adequate egg disinfection, chemicals other than iodine may be necessary, such as glutaraldehyde (Salvesen & Vadstein 1995, Salvesen et al. 1997). For treatment of largemouth bass *Micropterus salmoides* eggs, acriflavine (500 to 700 ppm for 15 min) was the disinfectant recommended over 5 other disinfectants by Wright & Snow (1975). Exploration of alternative egg disinfectants may be necessary for prophylaxis against organisms such as *Myxobolus cerebralis* actinospores that may be carried with eggs into the hatchery. In the meantime, consideration should always be given to where water is dumped when bringing in eggs from the wild, taking care not to contaminate water supplies. For disinfection of equipment that could potentially transfer the parasite, 50% povidone-iodine solutions (or stronger) would be needed for at least 60 min.

Hydrogen peroxide concentrations of 10% or greater for 10 min were required to kill the TAMs in this study. For the salmon louse *Lepeophtheirus salmonis*, hydrogen peroxide concentrations of 1500 ppm for 20 min did not control the mobile adult and pre-adult stages, nor the chalimus stages I and II, but did kill the nauplii and most of the copepodid larvae (McAndrew et al. 1998). For equipment disinfection, breakdown of hydrogen peroxide over time and the ability of organic compounds to reduce the toxicity must be considered when applying these data. For applications where fish may be present, the recommended dose exceeds the lethal limit for largemouth bass *Micropterus salmoides* (150 ppm for 60 min; Gaikowski et al. 1999), rainbow trout and cutthroat trout *Oncorhynchus clarki* (respectively, 420 and 540 ppm in 30 min exposures; Arndt & Wagner 1997). Of the physical variables examined, high pressure was ineffective for killing TAMs. However, drying and freezing for at least 1 h were effective in killing the parasite. Thorough drying also was effective against the myxospore stage (Hoffman & O'Grodnick 1977). Research on the effect of freezing on the myxospore stage of *Myxobolus cerebralis* has indicated that this stage is more resistant to freezing, surviving at least 18 d at –20°C (Hoffman & Putz 1969, 1971). Using pathogen-free tubificids, El-Matbouli & Hoffman (1991) also demonstrated that infected fish heads frozen for 2 or 3 mo still harbored viable myxospores that could infect the worms.

High temperatures were effective in deactivating TAMs if temperatures were kept above 75°C for at least 5 min. Markiw (1992) also examined the effect of temperature (7, 12.5, 19 to 20, or 23 to 24°C) on TAM viability using PI-FDA vital stains. Survival was temperature-dependent: only 2 d at 23 to 24°C, but 7 to 8 d at 7°C. Yokoyama et al. (1993) examined the effects of temperature on the actinospore stages of the genera *Raabeia*, *Aurantiactinomyxon*, and *Echinactinomyxon*, using sporoplasm loss as an indicator of viability. The results indicated that cooler temperatures increased longevity, similar to Markiw's (1992) findings. Yokoyama et al. (1993) also noted differences in longevity among types of actinospores: *Echinactinomyxon* > *Raabeia* > *Aurantiactinomyxon*.

In studies with *Escherichia coli*, sonication at 4.6 to 74 W cm^{-2} and 205 kHz was effective for inactivation of the bacterium (Hua & Thompson 2000). Scherba et al. (1991) successfully applied sonication (26 kHz at about 5 W cm^{-2}) to the treatment of fungi, bacteria, and a herpesvirus. In this study, sonication was effective in killing most of the TAMs, but the durations were long enough that retention time is an issue for flowing water treatment. The unexpected change in viability as sonication duration increased is partially explained by the larger number of TAMs added for longer durations, increasing the likelihood of finding viable TAMs. Also, the small number of TAMs recovered after sonication resulted in highly variable percentages in the treatment groups. Despite this problem, it was evident that viable TAMs were still present after 13 min of sonication, indicating that further study is needed in which optimal frequencies and sound intensities for TAM destruction are determined.

From these trials it appears that the infective stage of *Myxobolus cerebralis* is effectively killed when frozen or dried for a relatively short period of time. This knowledge is useful for understanding the epidemiology of whirling disease and potential risks of parasite transfer by boats, humans, and other vectors. Chlorine concentrations of 130 ppm for 1 min or more, hydrogen peroxide concentrations of >10% or temperatures above 75°C for at least 5 min were also effective. Chlorine would be especially useful for disinfection of field and laboratory equipment to prevent transfer of the triactinomyxon to negative waters, including hatcheries. Higher concentrations will be needed to treat the myxospore stage. The inability of iodine to control the parasite at concentrations typically used for hatchery disinfection was important. Fish culturists should be aware of the danger for parasite transfer when dealing with water potentially infected with *M. cerebralis*. For hatchery water supplies, the treatments tested are currently not practical. Sonication may be a useful technique for treating hatchery water supplies, but further research is needed before designing treatment systems. Hopefully this information can be used to prevent the spread of whirling disease to areas where it currently is not found.

Acknowledgements. This work was funded by the Utah Division of Wildlife Resources and the Federal Aid in Sport Fish Restoration Program. Thanks to the Salt Lake County Fish and Game Association for their generous contribution to whirling disease research and to Grant White for permitting collection of tubificid worms on his property. Thanks to Chris Wilson, Kent Thompson and Art Butts for the use of the epifluorescence microscope in their lab and to Mark Brough, Amy Stevenson, Amy Howa, Quinn Cannon, Jared Austin, Rhett D. Grover, and Tyler Gundersen for help with worm and TAM collection as well as maintenance of the worm cultures. John Wood and Janet Epp of Pisces-Molecular are gratefully acknowledged for the PCR analyses. Thanks to Kendall Hyde and the Leishman family for use of their ranch and spring water for conducting stain verification studies. Ronald Hedrick is gratefully acknowledged for his critical review of the manuscript.

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Editorial responsibility: Wolfgang Körting, Hannover, Germany

Submitted: January 21, 2002; Accepted: September 2, 2002
Proofs received from author(s): January 14, 2003