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[Article]

Effects of Freezing, Drying, Ultraviolet Irradiation, Chlorine, and Quaternary Ammonium Treatments on the Infectivity of Myxospores of *Myxobolus cerebralis* for *Tubifex tubifex*

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Abstract.—The effects of freezing, drying, ultraviolet irradiation (UV), chlorine, and a quaternary ammonium compound on the infectivity of the myxospore stage of *Myxobolus cerebralis* (the causative agent of whirling disease) for *Tubifex tubifex* were examined in a series of laboratory trials. Freezing at either −20°C or −80°C for a period of 7 d or 2 months eliminated infectivity as assessed by the absence of production of the actinospore stage (triactinomyxons [TAMs]) from *T. tubifex* cultures inoculated with treated myxospores over a 4–5-month period. Myxospores retained infectivity when held in well water at 5°C or 22°C for 7 d and when held at 4°C or 10°C d for 2 months. In contrast, no TAMs were produced from *T. tubifex* cultures inoculated with myxospores held at 20°C for 2 months. Drying of myxospores eliminated any evidence of infectivity for *T. tubifex*. Doses of UV from 40 to 480 mJ/cm^2 were all effective for inactivating myxospores of *M. cerebralis*, although a few TAMs were detected in one replicate *T. tubifex* culture at 240 mJ/cm^2 and in one replicate culture at 480 mJ/cm^2. Treatments of myxospores with chlorine bleach at active concentrations of at least 500 mg/L for 15 min largely inactivated myxospore infectivity for *T. tubifex*. Likewise, there was no evidence of TAMs produced by *T. tubifex* inoculated with myxospores treated with alkyl dimethyl benzyl ammonium chloride (ADBAC) at 1,500 mg/L for 10 min. Treatments of myxospores with 1,000-mg/L ADBAC for 10 min reduced TAM production in *T. tubifex* cultures sevenfold relative to that in cultures inoculated with an equal number of untreated myxospores. These results indicate that myxospores of *M. cerebralis* demonstrate a selective rather than broad resistance to selected physical and chemical treatments, and this selective resistance is consistent with conditions that myxospores are likely to experience in nature.

The life cycle of *Myxobolus cerebralis* (the causative agent of whirling disease) involves a series of developmental stages in salmonid and oligochaete hosts and in both cases culminates in the formation of spores that are infectious for the alternate hosts (Wolf and Markiw 1984). The actinospore or triactinomyxon (TAM) stage released from the oligochaete has been viewed as being more susceptible or vulnerable than the myxospore stage, which possesses hardened valves surrounding a binucleate sporoplasm (Hoffman 1990).

The use of fish-free water supplies and the application of ozone and ultraviolet irradiation (UV) treatments to water have been the principal methods for preventing contact between susceptible hatchery populations of salmonids and the waterborne TAMs of *M. cerebralis* (Hoffman 1990). Physical, chemical, and most recently biological means have been used to inactivate the *M. cerebralis* myxospores present in sediments or temporarily suspended in the water in areas where infected fish are present. Wagner (2002) provided a thorough review of the published works on the physical and chemical approaches to myxospore inactivation, and Beauchamp et al. (2006) and Steinbach-Elwell et al. (2006) described explorations into the potential use of *M. cerebralis*-resistant oligochaetes for biological removal of myxospores from sediment.

Several early studies examined the effects of freezing, drying, brining, smoking, and treatments with a range of different disinfectants and chemicals on

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myxospore viability. The two-host life cycle for *M. cerebralis* was not discovered until 1984 (Wolf and Markiw 1984), so earlier myxospore viability evaluations often depended upon vital staining approaches. More recent infectivity studies, however, have demonstrated that vital staining significantly underestimates the inactivation of waterborne cyst or spore stages that have traditionally been considered resistant among such human parasites as *Cryptosporidium* spp. (Campbell et al. 1995; Bukhari et al. 1999) and *Giardia* spp. (Craik et al. 2000; Linden et al. 2002) and the TAMs of *M. cerebralis* (Hedrick et al. 2007). Currently, the best metric for evaluating the viability of myxospores of *M. cerebralis* is to test their ability to infect the oligochaete host, *Tubifex tubifex*, under experimental conditions. This procedure was not available to investigators in many of the initial trials examining the effects of chemical disinfectants or other physical treatments on myxospores of *M. cerebralis*.

In the studies reported here, we examined the effects of selected chemical and physical treatments and a range of UV doses on the viability of *M. cerebralis* myxospores as assessed by their infectivity for known susceptible laboratory populations of *T. tubifex*. We report the efficacy of selected doses (concentration of disinfectant at selected duration of exposure) of commonly applied disinfecting agents, including UV, chlorine, and alkyl dimethyl benzyl ammonium chloride (ADBAC). In addition, we examined the effects of temperature and drying to provide additional insights into the survival of myxospores that might encounter such physical changes in the environment or under laboratory conditions. These studies reveal that the *M. cerebralis* myxospore stage's reputation for resistance to a broad set of chemical and physical treatments may not be completely deserved.

Methods

Preparation of M. cerebralis myxospores.—Myxospores were obtained from laboratory populations of rainbow trout *Oncorhynchus mykiss* infected with *M. cerebralis* by exposures to known concentrations of TAMs according to standard procedures (Beauchamp et al. 2002). Fish were held in aquaria receiving 15°C well water; at the time of myxospore extraction at 5–6 months postexposure, the fish were approximately 6–7 months old. Myxospore preparations generally consisted of homogenates made from two to three fish heads. Heads were homogenized with a blender, and the homogenate was filtered through gauze to remove larger fragments of hard and soft tissues. Myxospore concentrations were estimated by hemocytometer counts and adjusted so that the concentrations upon inoculation of *T. tubifex* test cultures would result in an infectivity ratio of 2,000 myxospores/worm except in the UV trials, where the ratio was 2,500 myxospores/worm. Myxospore preparations were held at 10°C and used on the same day of extraction from fish tissues. In one study, heads from rainbow trout infected with *M. cerebralis* were frozen intact and the myxospores were subsequently extracted by the same procedure described above. In each trial, replicate *T. tubifex* cultures prepared from the same bulk stock but not inoculated with myxospores were included as negative controls.

Cultures of T. tubifex.—Mitochondrial DNA lineage type III *T. tubifex* were originally derived from populations obtained from the settling ponds for the incoming water supply to the Mt. Whitney Fish Hatchery (Independence, California), as described by Beauchamp et al. (2001). The worms were maintained as bulk cultures in sediments within aquaria receiving 15°C, flow-through well water. For experimental treatments, 100 worms were separated into replicate 1-L containers with 300 mL of well water and 50 g of sterilized sand substrate. The worms were held at 15°C and were fed twice weekly, at which time 50% of the water was replaced with fresh well water. The worms were maintained in the smaller replicate culture containers for approximately 5 d prior to the initiation of each experiment. Although worm numbers were not counted after the initial establishment of the cultures, observations made concurrent with water changes indicated that many worms were present in all cultures at the time of the initial TAM releases (~80 d postexposure). Crude estimates of worm numbers from these visual observations suggested that up to 70% of the worms in all cultures were still present at the time of initial TAM releases.

Physical, chemical, and UV treatments of myxospores.—Freshly extracted myxospores were subjected to a range of different physical and chemical treatments to determine the effects on their viability. The physical treatments included holding at temperatures between −80°C and 22°C, drying, and exposure to a range of UV doses. The chemical treatments included exposure to selected concentrations of chlorine bleach and two preparations of ADBAC. The details of these treatments are provided below.

Temperature treatment study 1.—Eight replicate 1-mL suspensions of myxospores were placed into sealed 2-mL cryovials; two vials were then held at each of the following temperatures: −80, −20, 5, and 22°C. After 7 d, all vials were subjected to a temperature of 22°C for approximately 30 min. The entire myxospore suspension from each cryovial was then placed into one of eight *T. tubifex* test cultures (two worm cultures per treatment), and subsequent TAM production was evaluated as described below.

Temperature treatment study 2.—A second study was designed to evaluate the effects of temperature when myxospores were held either in suspensions (as in study 1) or in intact tissues (half-heads). For each temperature treatment ($n = 5$), the heads from two rainbow trout infected with *M. cerebralis* were cut into two equal halves. A half-head from each fish was homogenized to prepare a myxospore suspension, which was then placed into a sealed cryovial. The remaining half-head from each fish was placed into a sealed plastic bag with 50 mL of well water. The corresponding suspensions and half-heads from two replicate fish were held at −80, −20, 4, 10, or 20°C. The myxospore suspensions and half-heads were held at the treatment temperature for 2 months and then were brought to 22°C over a period of 4 h. Replicate half-heads held at each temperature were then homogenized to prepare individual suspensions of myxospores. The myxospore concentrations in all suspensions were then determined and adjusted so that *T. tubifex* cultures would receive the appropriate concentrations.

Effects of drying.—The effect of drying on myxospore infectivity was evaluated under two conditions: drying in sunlight under ambient air temperatures and drying under artificial light on the laboratory benchtop at 22°C. A myxospore suspension was prepared from the heads of two rainbow trout infected with *M. cerebralis*. After counting, myxospore concentrations were adjusted to 2×10^5 myxospores/mL. The open bottom halves of four petri dishes (60 mm) each received 1 mL of the myxospore suspension. Two petri dishes were placed in the sunlight, and two were placed on the laboratory benchtop. Temperature under both conditions was recorded at the beginning and end of the drying period by use of a thermometer placed on the surface of the petri dishes. Time to desiccation was recorded for suspensions under each condition. At the end of the drying period, the dried myxospores were resuspended in 1 mL of well water and placed into a cryovial. Untreated myxospores placed into 1 mL of water and held at 4°C (with no light) for 18.5 h served as positive controls. After myxospores were dried and resuspended in water, the contents of each cryovial, including the positive control vials, were added to individual *T. tubifex* cultures.

Effects of UV irradiation.—Myxospore suspensions prepared as previously described from four rainbow trout heads were diluted 1:5 with well water, and larger particulates were then removed by passage through 20- and 10-μm Nitex screens. Myxospores were collected by centrifugation at 500 × gravity (*g*) for 10 min and then resuspended in well water. Replicate 50-mL suspensions, each containing 2.5×10^5 myxospores in well water, were placed onto petri dishes for UV treatments at five different doses delivered by a low-pressure mercury vapor lamp in a collimated beam apparatus. The methods used for UV treatments of myxospores were the same as those previously described by Hedrick et al. (2007) for TAMs of *M. cerebralis*. The UV dose was determined by using a calibrated IL1700 radiometer and accounting for nonuniform intensity in the horizontal plane (petri factor), divergence of light rays through the sample depth (divergence factor), reflection of light from the sample surface (reflection factor), and attenuation of light through the water (water factor) due to absorbance (Bolton and Linden 2003). The UV doses examined were 0, 40, 120, 240, and 480 mJ/cm^2 (mJ/cm^2 = mW/cm^2). Petri dishes containing the myxospore suspensions were held at 4°C after UV treatments and then were used directly to inoculate replicate *T. tubifex* cultures. At the end of the 5-month observation period for TAM production, three to five individual worms were removed from the 0-, 40-, and 480-mJ/cm^2 treatment replicates and were tested for the presence of *M. cerebralis* DNA by the polymerase chain reaction (PCR) method described by Andree et al. (1998).

Effects of chlorine bleach.—Dilutions of commercial chlorine (6.15%) bleach (The Clorox Company, Oakland, California) were prepared in 18°C well water (pH = 8.1). Concentrations of active ingredient in the dilutions were evaluated with the CN-66 test kit (Hach Company, Loveland, Colorado) for free chlorine. Differences between expected and measured concentrations of chlorine for each dilution were less than 10%. Myxospore suspensions were partially clarified by centrifugation at 600 × *g* for 10 min and then resuspended in well water. Replicate 2.5-mL suspensions containing 5×10^4 myxospores each were then prepared. Dilutions of bleach were mixed 1:1 (volume/volume [v/v]) to provide the final concentrations of active ingredient (0, 200, 500, 1,250, 2,500, and 5,000 mg/L). Two replicate tubes were used for each concentration, and each tube was mixed gently three times over a 15-min contact period. Residual chlorine was neutralized with the addition of an equal volume of a 1.25% solution of sodium thiosulfate for 30 min prior to addition of the myxospore suspensions to the worm cultures. Controls consisted of replicate *T. tubifex* cultures inoculated with (1) myxospores mixed only with well water and (2) myxospores mixed with 0.625% sodium thiosulfate for 30 min. Replicate *T. tubifex* cultures that did not receive myxospores served as negative controls. Beginning at 82 d postinoculation, TAMs released into the water of *T. tubifex* cultures were recorded weekly and then summed over a period of 7 months.

Effects of quaternary ammonium compound.—Two preparations of quaternary ammonium compound, ADBAC (50% C_{14}, 40% C_{12}, 10% C_{16}), were examined. One was a commercially available cleaner, 409 (Clorox), and the second was provided by a fishing rod manufacturer (Scott Fly Rod Company, Montrose, Colorado). The two preparations had the same formulation except that the Clorox cleaner had 0.3% active ingredient and the Scott preparation had 0.2% active ingredient. Both preparations were tested at a 1:1 v/v dilution based on the recommended dose indicated on the container. Replicate myxospore suspensions in conical tubes were mixed with an equal volume of full-strength quaternary ammonium compound such that the final contact concentrations with the myxospores were 1,500 mg/L for the Clorox formulation and 1,000 mg/L for the Scott formulation. Duration of contact between the quaternary ammonium compounds and the myxospores was 10 min at 22°C. To remove residual ADBAC, the samples were diluted 1:500 with 18°C well water. The myxospores were then collected by centrifugation at $600 \times g$ for 10 min. The myxospores were counted and resuspended in well water to obtain concentrations for inoculation of the worm cultures. At the end of the 4-month observation period for TAM production, five individual worms were removed from each replicate of the myxospores treated with 1,500-mg/L ADBAC and tested for the presence of *M. cerebralis* DNA by PCR (Andree et al. 1998).

Assessments of infectivity of myxospore stage.—The infectivity of myxospores after the various experimental or control treatments was assessed by inoculation of replicate *T. tubifex* cultures. Concentrations of myxospores in the suspensions before treatment or after selected treatments were adjusted so that upon inoculation of *T. tubifex* cultures, the infectivity ratio would be 2,000 myxospores/worm. At the time of exposure, the water level in the worm cultures was reduced by approximately 75% and the myxospore suspension was added. After 4 h, fresh well water was added to obtain the original water level. The worms were maintained in 15°C incubators, and each container received supplemental air supplied by air stones. The photoperiod was maintained at 12 h light : 12 h dark. After inoculation of worm cultures with myxospores, a 25% water change and two feedings were conducted each week. Monitoring for the presence of TAMs in the water commenced at 80–84 d postinoculation and continued weekly for 4–5 months (7 months in the chlorine bleach study). Total TAM counts represent an estimate based on subsamples of water removed at the time of each water change. Briefly, the TAM enumeration methods included filtration of the water collected (~200 mL) from each worm culture through a 20-μm Nitex screen and recovery of the trapped materials (including TAMs) in 10 mL of well water. The TAMs were suspended by gentle mixing; a 100-μL quantity was removed and placed on a petri dish, and the total number of TAMs in the entire volume was counted. This was repeated three times per sample for each suspended filtrate, and a mean TAM value was calculated. Only TAMs that were structurally intact (sporoplasm and polar capsules not discharged and in the appropriate locations) were included in the counts. At the end of the study, the sum of the TAMs over the recording period was calculated for each replicate worm culture and a mean for each treatment was then determined. The effect of a given treatment on total TAM production was expressed as a log reduction, calculated by determining the $\log_{10}$ of the quotient of total TAM numbers after treatment divided by total TAM numbers in positive control cultures.

Statistics.—Statistical tests were applied only to the data from the chlorine bleach and ADBAC trials, because in all other trials TAM production in treatment groups was zero or negligible. Paired *t*-tests (Microsoft Excel) were used to compare the mean number of TAMs between the 0- and 200-mg/L chlorine treatments and between the 0- and 1,000-mg/L ADBAC treatments. Significant differences were those with a *P*-value of 0.05 or less.

Results

Temperature Treatments

There was no evidence for residual infectivity of *M. cerebralis* myxospores that were frozen at −20°C or −80°C either in suspensions or in fish tissues (i.e., half-heads; Table 1). There was no TAM production by any of the *T. tubifex* cultures inoculated in temperature studies 1 and 2 with myxospore suspensions that were frozen for 7 d or 2 months. Thus, the log reduction in viable myxospores resulting from freezing and thawing in the two studies was greater than 4 (Table 1). In contrast, myxospore suspensions held at 5°C or 22°C for 7 d or at 4°C or 10°C for 2 months were infectious for *T. tubifex*, as indicated by TAM production that began at 84–91 d postinoculation (Table 1). The greatest mean number of TAMs (71,735) was produced by worms inoculated with myxospores held at 5°C for 7 d in study 1. Fewer TAMs (25,840) were detected with myxospores held at 22°C for 7 d (study 1). Myxospores, either in suspension or in tissues (half-heads), showed minimal residual infectivity (log reduction > 4, relative to myxospores held at 4°C) when the holding duration at 20°C was extended to 2 months (study 2). Three TAMs were detected (one TAM in 3 of 30 total sampling times) for the replicate

TABLE 1.—Effects of temperature on *Myxobolus cerebralis* myxospore infectivity for *Tubifex tubifex*, as indicated by the estimated number of triactinomyxons (TAMs) produced in *T. tubifex* replicate cultures (R1 and R2). Replicate myxospore suspensions or myxospores present in rainbow trout tissues (half-heads) were held for either 7 d (study 1) or 2 months (study 2) at a range of temperatures and then were used to inoculate individual cultures of 100 *T. tubifex* at 15°C. Beginning at 84 d postinoculation, TAMs released into the water of *T. tubifex* cultures were recorded weekly and summed for the 5-month observation period.

Temperature (°C)	Myxospore suspensions				Myxospores in tissues[b]			
	R1	R2	Mean	Log reduction[a]	R1	R2	Mean	Log reduction
				Study 1				
−80	0	0	0	>4.85	nd[c]	nd		
−20	0	0	0	>4.85	nd	nd		
5	77,970	65,500	71,735		nd	nd		
22	34,100	17,580	25,840	0.47	nd	nd		
				Study 2				
−80	0	0	0	>4.31	0	0	0	>4.30
−20	0	0	0	>4.31	0	0	0	>4.30
4	23,750	17,350	20,550		47,200	8,300	27,750	
10	18,800	16,100	17,450	0.07	0	3	1.5	0.07
20	3	0	1.5	>4.13	0	0	0	>4.30

[a] Log reduction (defined in Methods) is in relation to TAMs produced at 4°C or 5°C.
[b] Myxospores in half-heads were placed into sealed plastic bags with 50 mL of well water.
[c] In study 1, only myxospore suspensions were examined (nd = not done).

worm cultures inoculated with myxospore suspensions held at 20°C. No TAMs were detected among worm cultures treated with myxospores from half-heads held at 20°C for 2 months. Myxospore suspensions held at 10°C produced TAMs, although fewer than were produced by myxospores held at 4°C, and the TAMs were present earlier rather than more uniformly throughout the observation period (data not shown). In a manner similar to the myxospore suspensions held at 20°C, only four TAMs were detected in cultures treated with myxospores from half-heads held at 10°C for 2 months. Neither temperature study had evidence of TAM production from replicate worm cultures that were not inoculated with myxospores (negative controls).

Effects of Drying

Replicate myxospore suspensions (1 mL) placed into uncovered petri dishes dried over a period of 105 min when held in direct sunlight. The temperature at the level of the petri dishes was 18°C at the beginning of the desiccation period and 42°C at the end. Drying on the laboratory benchtop occurred over a period of 18.5 h at a constant temperature of 22°C. Over the 5-month observation period, no TAMs were produced in negative control *T. tubifex* culture replicates or in cultures that received myxospore suspensions that were dried in sunlight or on the laboratory benchtop. Therefore, the log reduction in TAM production was at least 4 relative to TAM production in the two replicate worm cultures inoculated with myxospore suspensions held at 4°C for 18.5 h (51,300 and 24,000 TAMs).

Effects of UV Irradiation

No TAMs were observed from *T. tubifex* inoculated with myxospore suspensions treated with UV at 40 or 120 mJ/cm^2 (Table 2). Over the 5-month observation period, only one and three TAMs were detected from the replicate *T. tubifex* cultures inoculated with myxospores treated at 240 mJ/cm^2. The UV doses tested achieved a log reduction in TAM production of at least 4 relative to TAM production from myxospores not treated with UV (Table 2). In *T. tubifex* cultures receiving myxospores treated with UV at 480 mJ/cm^2, four TAMs were detected in one replicate and none were found in the second replicate (Table 2). No *M. cerebralis* DNA was detected by PCR among five *T. tubifex* examined from each replicate approximately 5 months after inoculation with myxospore suspensions treated with UV at 40 or 480 mJ/cm^2 (Table 2). Also, *M. cerebralis* DNA was not present among 10 negative control *T. tubifex* (5 worms/replicate) examined by PCR. In contrast, all eight *T. tubifex* from cultures inoculated with untreated myxospores were PCR positive for the presence of *M. cerebralis* DNA.

Effects of Chlorine Bleach

Chlorine at concentrations of 200 mg/L (active ingredient) for 15 min (dose = 200 mg/L × 15 min =← 3,000 mg/L-min) provided partial inactivation of myxospores, as measured by a reduction in TAM

TABLE 2.—Effects of ultraviolet irradiation (UV) on *Myxobolus cerebralis* myxospore infectivity for *Tubifex tubifex*, as indicated by the estimated number of triactinomyxons (TAMs) produced in *T. tubifex* replicate cultures (R1 and R2). Replicate myxospore suspensions treated with a range of UV doses were used to inoculate individual cultures of 100 *T. tubifex* at 15°C. Beginning at 84 d postinoculation, TAMs released into the water of *T. tubifex* cultures were recorded weekly and summed over the 5-month observation period. At 4 months postinoculation, worms from selected treatments were tested for the presence of *M. cerebralis* DNA by use of polymerase chain reaction (PCR; given as number positive/number sampled).

UV dose (mJ/cm^2)	R1	R2	Mean	Log reduction[a]	PCR
0	216,050	10,450	113,250		8/8
40	0	0	0	>5.05	0/10
120	0	0	0	>5.05	nd[b]
240	1	3	2	4.75	nd
480	0	4	2	4.75	0/10
Negative control[c]	0	0	0		

[a] Defined in Methods.
[b] nd = not done.
[c] Negative controls were *T. tubifex* cultures that did not receive myxospores.

production (log reduction = 1.3) relative to positive controls (Table 3). The difference in the mean number of TAMs between the 200-mg/L treatment and the positive control was statistically significant ($P < 0.05$). At the higher concentrations of chlorine tested, either there was no indication of TAM production (2,500 and 5,000 mg/L) or a maximum of two TAMs was detected (500 and 1,250 mg/L); therefore, the log reduction was 5 or greater, and mean number of TAMs was significantly different than that of the positive controls. Two TAMs were detected in one replicate *T. tubifex* culture 4 months after receiving myxospores treated with 500-mg/L chlorine; likewise, two TAMs were seen in one replicate culture 5.5 months after inoculation with myxospores treated with 1,250-mg/L chlorine. Treatments of myxospores with only sodium thiosulfate resulted in substantial TAM production (mean = 34,591 TAMs) over the 7-month period, which was considerably less ($P < 0.05$) than that from untreated myxospores (mean = 118,788 TAMs; Table 3). No TAMs were detected from negative control *T. tubifex* cultures.

TABLE 3.—Effects of chlorine bleach on *Myxobolus cerebralis* myxospore infectivity for *Tubifex tubifex*, as indicated by the estimated number of triactinomyxons (TAMs) produced in *T. tubifex* replicate cultures (R1 and R2). Replicate myxospore suspensions were treated for 15 min at 15°C, and the chlorine was then neutralized with sodium thiosulfate ($Na_2S_2O_3$). Treated myxospore suspensions were used to inoculate individual cultures of 100 *T. tubifex* at 15°C. Beginning at 82 d postinoculation, TAMs released into the water of *T. tubifex* cultures were recorded weekly and summed over the 7-month observation period.

Chlorine dose (mg/L)	R1	R2	Mean	Log reduction[a]
0	127,375	110,200	118,788	
200	30,052	1,200	15,625	1.30
500	2	0	1	5.07
1,250	0	2	1	5.07
2,500	0	0	0	>5.07
5,000	0	0	0	>5.07
0 ($Na_2S_2O_3$ only)	37,806	31,375	34,591	0.54

[a] Defined in Methods.

Effects of Quaternary Ammonium

Treatments of myxospores with quaternary ammonium preparations (initial 0.2% and 0.3% active ingredient prior to dilution) for 10 min considerably reduced the numbers of TAMs produced by inoculated *T. tubifex* cultures in comparison with worm cultures receiving untreated myxospores (Table 4). The ADBAC preparation with the initial 0.2% active ingredient (final dose = 10,000 mg/L-min) resulted in TAM production in both replicate *T. tubifex* cultures. The mean production of 2,009 TAMs over the 4-month observation period represented a log reduction of 1.06 relative to TAM production from cultures receiving untreated myxospores, but this difference was not statistically significant (Table 4). In contrast, the stronger ADBAC concentration with an initial 0.3% active ingredient (final dose = 15,000 mg/L-min) eliminated any evidence of infectivity. No TAMs were observed at any sampling time; the resulting mean number of TAMs represented a log reduction of over 4.14 relative to mean TAMs from positive control cultures, and the difference in TAM production was significant ($P < 0.05$). In contrast, *T. tubifex* cultures inoculated with untreated myxospores produced a mean of 14,050 TAMs over the period of observation. No TAMs were detected from replicate *T. tubifex* cultures that were not inoculated with myxospores. A PCR test at 4 months postinoculation indicated that 10 worms from *T. tubifex* cultures inoculated with 0.3% ADBAC-treated myxospores were all negative for the presence of *M. cerebralis* DNA.

Discussion

The successful spread and establishment of *M. cerebralis* in both artificially propagated and naturally reproducing salmonid populations are partially attributable to the formation and persistence of the resistant myxospore stage (Hoffman 1970; Bartholomew and Reno 2002; Bartholomew et al. 2005). Myxospores of *M. cerebralis* can persist in skeletal tissues of fish over their entire lifetime; once outside the host, the myxospores can survive a range of environmental extremes (Hoffman 1990). The inactivation of myxospores by either chemical or physical treatments, or potentially by novel biological means (Beauchamp et al. 2006), is therefore important in minimizing the spread and potential impacts of whirling disease on artificially propagated and naturally reproducing salmonid populations.

In our temperature studies 1 and 2, freezing the myxospores at −20 or −80°C for either 7 d or 2 months inactivated any evidence of infectivity, as assessed by the lack of TAM production in *T. tubifex* cultures after inoculation (Table 1). Our results differ from those reported for freezing of *M. cerebralis* myxospores at −20°C by Hoffman and Putz (1969, 1971) and then later by El-Matbouli and Hoffmann (1991). The latter study was the more conclusive, as the authors were able to exploit knowledge of the two-host life cycle that was unknown at the time of the prior studies (Hoffman and Putz 1969, 1971). El-Matbouli and Hoffmann (1991) used 10 rainbow trout heads frozen intact for 2–4 months. They mentioned that their stock of mixed tubificids (90% *T. tubifex*) was periodically tested and found to be free of actinospore production; thus, we assume there were no TAMs produced among negative control aquaria in their trials. Potential explanations for the conflicting results between their study and ours may involve either differences in freezing conditions or the sheer numbers of myxospores and oligochaetes used. In our trials, we adjusted our final myxospore numbers to 2,000 myxospores/worm ($n = 100$ worms) for infectivity assessments. El-Matbouli and Hoffmann (1991) did not report myxospore concentrations or worm numbers, but if 10 highly infected rainbow trout heads and 100 g of worms were used, the myxospore and worm concentrations would be much greater than those used in our trials. Such high concentrations of myxospores, particularly if freezing inactivates most (e.g., about 99.99–99.999%; total log reduction = 4–5) but not all, might have allowed a sufficient number to survive and initiate infections in the oligochaetes. Although freezing of myxospores in the sediments of natural aquatic systems can occur, most myxospores probably do not freeze and thus natural selection does not favor a strong resistance to freezing. Although freezing may eliminate most (log reduction = 4), some myxospores may survive, particularly when present in large numbers in intact fish tissues. Therefore, caution should be applied to prevent the spread of whirling disease in cases where myxospores from fish tissues are apt to come in contact with suitable numbers of susceptible tubificids (Hoffman 1970; El-Matbouli and Hoffmann 1991).

TABLE 4.—Effects of a quaternary ammonium compound, alkyl dimethyl benzyl ammonium chloride (ADBAC) on *Myxobolus cerebralis* myxospore infectivity for *Tubifex tubifex*, as indicated by the estimated number of triactinomyxons (TAMs) produced in *T. tubifex* replicate cultures (R1 and R2). Replicate myxospore suspensions were treated with ADBAC at either 1,000 or 1,500 mg/L (active ingredient) for 10 min at 22°C. Excess ADBAC was removed by dilution and collection of myxospores by centrifugation. Treated myxospores resuspended in well water were used to inoculate individual cultures of 100 *T. tubifex* at 15°C. Beginning at 83 d postinoculation, TAMs released into the water of *T. tubifex* cultures were recorded weekly and summed over the 4-month observation period.

ADBAC (mg/L)	R1	R2	Mean	Log reduction[b]
0	15,700	12,400	14,050	
1,000	412	3,606	2,009	1.06
1,500	0[a]	0[a]	0	>4.14

[a] At 4 months postinoculation, samples of 5 worms/replicate (1,500-mg/L treatment only) were negative for the presence of *M. cerebralis* DNA based on polymerase chain reaction tests.
[b] Defined in Methods.

The effects of temperatures other than freezing on myxospore inactivation have included evaluations by viable staining (Hoffman and Markiw 1977) as well as infectivity for *T. tubifex* (Wolf and Markiw 1982; Wolf et al. 1986). Those studies demonstrate that myxospores can withstand temperatures approaching 60°C for short periods of time and that viability is longer at lower temperatures. As controls for our freezing trials, myxospores incubated at 5°C and 22°C survived quite well over a 7-d period (Table 1). Extending the incubation period to 2 months did not appear to affect stability of myxospores at 4°C or 10°C. In contrast, 2 months at 20°C substantially reduced the viability of myxospores (log reduction > 4.3) held either in suspension or in fish tissues (Table 1). This result suggests that even subtle temperature increases, particularly if prolonged (as seen in some salmonid habitats), can significantly reduce myxospore infectivity.

Desiccation effectively inactivates many pathogens, including those from aquatic environments (Mara and Cairncross 1989). Hoffman and O'Grodnick (1977) concluded that *M. cerebralis* myxospores were inacti-

vated via thorough drying of contaminated mud in aquaria over 13–19 months. Susceptible rainbow trout added to aquaria with rehydrated mud substrates failed to develop whirling disease over a 4.5-month observation period, whereas the disease did develop among fish in aquaria in which the mud was not dried. These studies were conducted prior to knowledge that *T. tubifex* was an essential host in the life cycle, and drying probably removed all viable worms from the mud. Our study resolves that experimental uncertainty and demonstrates that drying, either in or out of sunlight at the temperatures tested, effectively inactivates the myxospores of *M. cerebralis*.

Ultraviolet irradiation has been demonstrated in earlier and more recent studies as providing protection of rainbow trout from infection with the TAMs of *M. cerebralis* (Hoffman 1974, 1975; Hedrick et al. 2000, 2007). Only limited experimentation has been reported on trials to inactivate the myxospore stage as judged by vital staining (J. Bartholomew, Oregon State University, Corvallis, personal communication). In that work, a UV dose of 1,700 mJ/cm^2 was reported for complete inactivation of myxospores in suspension; this UV level is similar to that initially thought necessary for inactivation of TAMs in the early trials of Hedrick et al. (2000). However, more recent studies (Hedrick et al. 2007) have indicated that substantially lower UV levels (40 mJ/cm^2 and perhaps even lower) are adequate to inactivate TAMs when present at very high levels. In our current trial with myxospores, all doses of UV tested were effective (Table 2); thus, the 40-mJ/cm^2 commercial standard for water treatments is sufficient for complete inactivation of myxospores at the densities (2,500 myxospores/worm) tested. Our initial assumptions that the thickened valves would protect the sporoplasm from UV were unfounded, and the results are instead consistent with the relative susceptibility to lower UV doses (e.g., 11–12 mJ/cm^2 for a log reduction of 3) seen in other cyst-forming parasites, such as *Giardia* and *Cryptosporidium* (Campbell et al. 1995; Bukhari et al. 1999; Craik et al. 2000; Qian et al. 2004). The presence of a single TAM or a few TAMs produced by one replicate at 240 mJ/cm^2 and one replicate at 480 mJ/cm^2 (Table 2) was observed but is difficult to explain. It remains possible that a few myxospores, even at the higher doses of UV, were spared from the full exposure despite our attempts to keep the suspension in motion during treatment with a magnetic stirrer. This may have resulted in a very mild infection in a single oligochaete in each case. We presume that our experimental conditions, particularly the high infectivity ratio (2,500 myxospores/worm), were sensitive enough to detect even the fewest viable myxospores in our treatments. Reducing this infection ratio (i.e., to a level that approximates natural contacts between myxospores and oligochaetes) would probably have eliminated these isolated TAM production episodes.

Chlorine at 500 mg/L for 15 min was required for the near elimination of myxospore infectivity in our trials (Table 3). Three TAMs were observed in *T. tubifex* cultures receiving myxospores treated with chlorine at 500 and 1,250 mg/L, but this occurred only at one time point. In well water like that used to suspend myxospores in our study (pH = 8.1; temperature = 18°C), the principal aqueous form of chlorine would be hypochlorite (OCl^-) at approximately 80% and a lesser amount (20%) of hypochlorous acid (HOCl) would be present. We assume that even greater myxospore inactivation would occur in waters with lower pH values, because concentrations of HOCl, the stronger disinfectant of the two chlorine forms, would increase substantially. At a pH of 7.0, the principal form (80%) would be HOCl (Tucker 1993). An incidental finding from the current study was the inactivating effect of sodium thiosulfate on the myxospores (Table 3). An approximately fourfold reduction in TAM production from sodium thiosulfate-treated myxospores relative to positive control cultures was observed (Table 3). This implies that the reduced TAM production from 200-mg/L chlorine treatments was more due to the sodium thiosulfate than to the chlorine, which further emphasizes the necessity of 500-mg/L or greater chlorine applications.

In early studies, treatments of mud with up to 1,200-mg/L chlorine were largely ineffective in protecting trout from infections (Hoffman and O'Grodnick 1977). Treatments of myxospore suspensions with 400-mg/L chlorine resulted in only a 90% reduction of viable staining myxospores (Hoffman and Hoffman 1972). Distortion of myxospores was observed microscopically after treatments with 1,600 mg/L (Hoffman and Putz 1969); at 5,000 mg/L, all myxospores took up the dye methylene blue (B. MacConnell, U.S. Fish and Wildlife Service, personal communication). Taken together, these studies suggest that myxospores, both in mud and in suspensions, are resistant to chlorine. Binding of chlorine to organics present during treatments probably reduces the active concentrations that reach the myxospores, resulting in a compromised effectiveness of the disinfection process. In mud, infected *T. tubifex* may also escape chlorine exposure by burrowing into deeper sediments, which would partially explain why relatively high concentrations of chlorine are ineffective in pond disinfection procedures (Hoffman and O'Grodnick 1977). Our trials, however, suggest that once in suspension and somewhat free of

major organic substrates, myxospores are vulnerable to inactivation by moderate levels of chlorine.

Quaternary ammonia compounds, particularly the third generation or "dual quats," are widely used as industrial disinfectants. They serve as cationic surface-active agents that absorb to and penetrate cell membranes and then disrupt cell membrane functions (Merianos 1991). In our studies, ADBAC, a commonly used quaternary ammonium compound, was tested at 1,000 and 1,500 mg/L for 10 min (Table 4). The concentration of 1,500 mg/L used in our trial was greater than the 200 and 800 mg/L that were reported by Hoffman and Putz (1969) to cause "distortion and probable death" of myxospores examined microscopically. Subsequent studies with ADBAC in trials by Hoffman and Hoffman (1972) at 50, 100, and 200 mg/L found only minimal myxospore killing, as evaluated by viable staining methods. Our trials suggest that the myxospores treated with ADBAC concentrations less than 1,500 mg/L are not fully inactivated, even if some distortion or vital dye uptake is observed (Table 4).

The discovery of the life cycle of *M. cerebralis* has made possible numerous studies that have improved our knowledge of the biology of this important pathogen of salmonids. By exploiting the life cycle for our current studies, we have avoided key experimental difficulties encountered in earlier studies examining physical and chemical inactivation of the myxospores of *M. cerebralis*. Our results support, in part, earlier hypotheses that the myxospore is a strongly resistant stage and is able to withstand environmental extremes that would significantly hamper control or eradication of the parasite. However, in several trials, myxospores were considerably more labile than we presumed, as evidenced by their susceptibility to drying, freezing, and UV exposures. This apparent dichotomy between resistance and susceptibility may result from the evolution of selective resistance to conditions that are most likely to be encountered and that are critical to completion of the life cycle. This might include the ability to tolerate temperatures and chemical conditions encountered when passing through key vectors like predatory birds and fish (El-Matbouli and Hoffmann 1991), but not conditions that are less likely to be routinely encountered in salmonid habitat, such as water temperatures above 20°C, freezing, and drying. The labile nature of myxospores to some of these factors (e.g., temperature) may contribute to differences in disease severity as observed among natural salmonid habitats. Also, the vulnerability of myxospores to certain factors (e.g., UV-C) provides effective approaches for preventing whirling disease by arresting or eliminating myxospore spread within or to habitats where salmonids occur naturally or are artificially propagated.

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