American Fisheries Society Symposium 29:227–238, 2002

The Effect of Water Characteristics on Viability of the *Myxobolus cerebralis* Actinospore

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ABSTRACT The effect of pH, total hardness, salinity, and dissolved oxygen on viability of the triactinomyxon (TAM) stage of the Myxozoan parasite *Myxobolus cerebralis* was evaluated. The vital stains propidium iodide and fluorescein diacetate were used to determine if TAMs were alive or dead. Tests were conducted at 9°C and 14°C. Experiments were conducted at salinities of 5, 10, 15, 20, and 30 ‰, at pH from 4 to 10, at total hardness of 10 or 650 mg/L, and dissolved oxygen at 0–0.5 mg/L. Viability was not significantly affected by low dissolved oxygen or the total hardness levels tested. Few differences were observed related to temperature. Viability decreased over time in controls to roughly half after 72 h at either temperature. Salinity significantly affected mortality, even after 1 h. For disinfection, 20–30 ‰ salinity for at least 6 h would be required to effectively kill all TAMs. The further pH moved from circum-neutral, the more TAM viability decreased. Differences in water quality, especially pH, could partially explain differences observed in severity of whirling disease among geographic regions.

Myxobolus cerebralis is responsible for whirling disease in salmonid fishes. Native to Europe, the parasite was inadvertently introduced to the eastern United States in the mid-1950s (Markiw 1992a). The parasite has continued its western movement and to date can be found in states from coast to coast. However, the impacts of M. *cerebralis* on salmonid populations have varied widely with geographic location. In the northeastern United States and certain western waters, impacts of the disease on wild fish can be described as minimal (Hulbert 1996). However, many western trout waters have seen severe declines in wild trout populations. In Colorado, Nehring and Walker (1996) reported a nearly complete loss of four successive year classes (1991–1994) of rainbow trout fry in the upper Colorado River following the discovery of whirling disease. Similarly, on Montana's famed Madison River, discovery of whirling disease in 1991 is believed to be the chief cause of a 90% reduction in rainbow trout in two sections of the river by 1994 (Vincent 1996). To date there are no clear explanations for these regional variations in the impact of this parasite.

One possibility is that the characteristics of the water itself may provide the parasite better opportunities to infect its fish host. A more favorable water quality could extend the life of the infective stage, increasing the probability of finding a host. Many characteristics of surface water can be traced to the geology and climate of the region in which it lies. In the northeastern United States, trout streams are in general lower in pH and have softer water when compared with the majority of western streams (Schofield 1982). Exceptions to these generalizations can be found of course, but it is possible that geographic differences in water quality could partially help explain variations observed with whirling disease outbreaks.

The parasite M. *cerebralis* has a complex life cycle involving both an oligochaete worm and fish as hosts (Markiw and Wolf 1983). The infective stage for fish is the actinospore referred to as the triactinomyxon (TAM). After excretion from the oligochaete host (*Tubifex tubifex*), the TAMs are suspended in the water column and must encounter a fish in order to continue the life cycle of the organism. Once excreted, TAMs are short lived. Markiw (1992b) found that depending upon temperature, TAMs were viable for less than 8 d. El-Matbouli et al. (1999) found that more than 60% of TAMs were still viable after 15 d at water temperatures up to 15°C. In contrast, the resistant

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myxospore stage of the parasite may last for up to 12 years in wet mud (Bauer 1962). Because of a shorter window of viability, the TAM may be the weakest link in the life cycle of the organism.

In this study, the possibility that water quality may impact the life span of the actinospore, and thus the success of the parasite in a given water, is explored. Because of the TAM's fragile nature and the short period of opportunity it has to find a fish, interactions may be limited in environments that are not conducive to the actinospore. If water quality variables do negatively impact the actinospore, this may help explain the variability in infection rates seen across space and time. Additionally, such knowledge could be helpful in developing techniques aimed at disrupting the life cycle of the parasite.

The objective of this study was to examine the effects of salinity, pH, dissolved oxygen, and total hardness on TAM viability.

METHODS

Experimental Design

The effects of salinity, pH, dissolved oxygen (DO), and total hardness on TAM viability were tested at two temperatures, 9°C and 14°C. Duration of exposures were 1, 6, 24, 48, and 72 h. Live TAMs were obtained from laboratory oligochaete cultures 3 times per week (Monday, Wednesday, Friday) by siphoning water from the cultures through a 20 μm Nitex filter (Aquaculture Research/Environmental Associates, Homestead, Florida). TAMs were kept cold in a cooler with ice packs and used for experiments within 24 h.

For each experiment there were three treatments with three replicates per treatment. The three treatments were a baseline, control and the target group. Determiniation of the baseline variability was made at the beginning of each experiment (time = 0) from the TAM stock solution using staining methods described below. Target groups were exposed to the experimental water quality characteristic, and control groups were exposed to fresh well water.

Assessment of TAM Viability

After TAMs were exposed to their respective treatments, they were concentrated by filtering through a small 10 μm mesh filter, which was then thoroughly rinsed with 20 mL of fresh well water. As the last aliquot of water was filtered, 100 μL of retentate from the filter was transferred to each of three slides for staining.

Staining was based on the techniques of Jones and Senft (1985) and Markiw (1992b), where fluorescein diacetate (FDA) and propidium iodide (PI) are used simultaneously. An aqueous stock solution of PI (52 mg/mL) was made and kept frozen (–40°C) in 1 mL aliquots. Vials were then thawed on the test day. A concentrated stock solution of FDA (5 mg/mL acetone) was kept at –40°C. On the day of an experiment, 80–100 μL of the concentrated FDA solution was added to 8 mL of well water to make a working stock of FDA stain. This amount varied slightly to adjust for minor changes over time in the strength of the stain fluorescence and for the amount of organic matter in the sample. For each microscope slide, 50 μL of both FDA and PI were added to the 100 μL sample of recovered TAMs. Slides were cover slipped and incubated at 9–12°C in a dark, humid chamber for 45 min, then examined.

Using an epifluorescence microscope, live TAM sporozoites appear green with the FDA staining and dead sporozooites are red from the PI staining. On occasion sporozoites stained both red and green; these were recorded as possibly viable, while those stained green or red were recorded as viable and dead, respectively. All classifications of the possibly viable TAMs were included in the total TAM count. The total number of TAMs varied according to their availability and averaged 89, 99, 100, and 100 per slide in the salinity, pH, hardness, and DO tests, respectively.

Salinity Tests

Salinity experiments were conducted at concentrations of 5, 10, 15, 20, and 30‰. Stock solutions of salt water were made using noniodized rock salt dissolved in well water. For each salinity test, equal parts of TAM stock solution and salt water were combined in test tubes to achieve the required salinity. The test tube contents were thoroughly mixed, covered, and stored at experiment temperature. After the exposure time had elapsed, the TAMs were collected and enumerated as described.

pH Tests

The effects of pH on TAM viability were tested at pH 4.0, 5.0, 6.0, 8.5, 9.0, 9.5, and 10. The well water used for controls naturally varied from pH 7.5 to 8.2. The pH for treatments was maintained by addition of either 0.1 N NaOH or 5% H_2SO_4 to

hatchery well water. The pH was monitored throughout the exposure using a digital meter calibrated daily using standard pH buffers. Differences between initial and final pH levels of controls were minor, varying from 0.00 units in 1 h treatments to 0.1–0.25 units in 72 h treatments.

Small 10 μm filters were used for exposing TAMs to differing pH. These were constructed from plastic 50 mL centrifuge tubes. For each filter, the top 50 mm of the tube was cut off. Another 10 mm section of the tube was cut off and a small section removed to decrease the ring diameter to just fit within the 50 mm high section. The cut ring stretched and secured a small circle of 10-μm mesh Nitex snugly within the cylinder. The edges of the mesh were sealed with silicone to prevent loss of TAMs at the edges. The completed filters measured approximately 25 mm deep and 25 mm in diameter. Filters were weighted using small steel bolts taped to the outside to keep them neutrally buoyant. Lids from the centrifuge tubes were used to keep samples from leaking out.

Experiments were conducted by first filtering the TAM stock solution through the 10 μm filter and then refilling with water at the test pH. The filters were capped and placed into a 40 L tank containing water at the pH to be tested. Water from the tank was able to readily pass back and forth across the filter membrane. After the exposure time elapsed, filters were removed and the contents were rinsed with 20 mL fresh well water. Slides were made, read, and recorded as above.

Total Hardness Tests

The effects of total hardness on TAM viability was tested for water considered very hard and soft as measured using standard titration techniques (APHA 1989). Well water used for controls had a total hardness of approximately 260 mg/L. To test hard water, a stock solution of 1000 mg/L hard water was made using powdered $CaCl_2$, The stock solution was mixed in equal parts with TAM stock solution in test tubes, producing a 650 mg/L solution. The test tubes were covered and stored at 9°C and 14°C throughout exposure. After appropriate time had elapsed, TAMs were collected and enumerated.

The effects of soft water on TAM viability was tested using de-ionized well water mixed with regular well water to achieve 10 mg/L stock solution of soft water. TAMs were put onto small 10 μm filters and the water was allowed to drain off, as in pH experiments. The filters were then filled with soft water at the test temperature, covered and placed in 1-L containers filled with soft water. Following exposures, samples were stained, incubated, and enumerated as noted above.

Dissolved Oxygen Tests

The effect of low DO on TAM viability was tested at 0–0.5 mg/L oxygen. The well water used for control groups ranged from 6 to 8 mg/L oxygen. Low DO was accomplished by bubbling nitrogen (N_2) gas through an air stone in a small vial of TAM stock solution. After the oxygen was depleted, the vial contents were carefully transferred into small test tubes. Test tubes were filled to overflowing and sealed carefully to avoid trapping any air within them. Dissolved oxygen was measured with a digital meter (calibrated for elevation and temperature) at the beginning and end of each exposure. Dissolved oxygen was 0.0 mg/L at the end of the 72 h period for both temperature tests. After the exposure time had elapsed, test tube contents were poured onto filters and rinsed. Slides were prepared as noted above.

Statistical analysis

Data were analyzed using SPSS Advanced Statistics 7.0 (SPSS 1996). Data were arc-sine transformed and tested for normality using the Shapiro-Wilk test. The Mann–Whitney U-test was used to compare between freshwater controls at the two temperatures, separately for each time period. The same test was used to compare DO and total hardness treatments with their respective controls. The effects of salinity and pH were analyzed using a one-way analysis of variance (ANOVA) separately for each time period. Subsequent mean comparisons were conducted using the Least Significant Difference test. An α-level of 0.05 was used for all analyses.

RESULTS

Effect of Temperature on Controls

When the effect of temperature (9°C versus 14°C) was compared between controls for each exposure time (Figure 1), the only significant difference ($P \leq 0.05$) was at 24 h. Time did prove to negatively affect TAM viability at both temperatures with nearly 50% of TAMs from control groups dead after 72 h.

Salinity

All concentrations of salt water (5–30‰) had negative effects on TAM viability at both 9°C (Figure 2a) and 14°C (Figure 2b). Even at 1 h there were

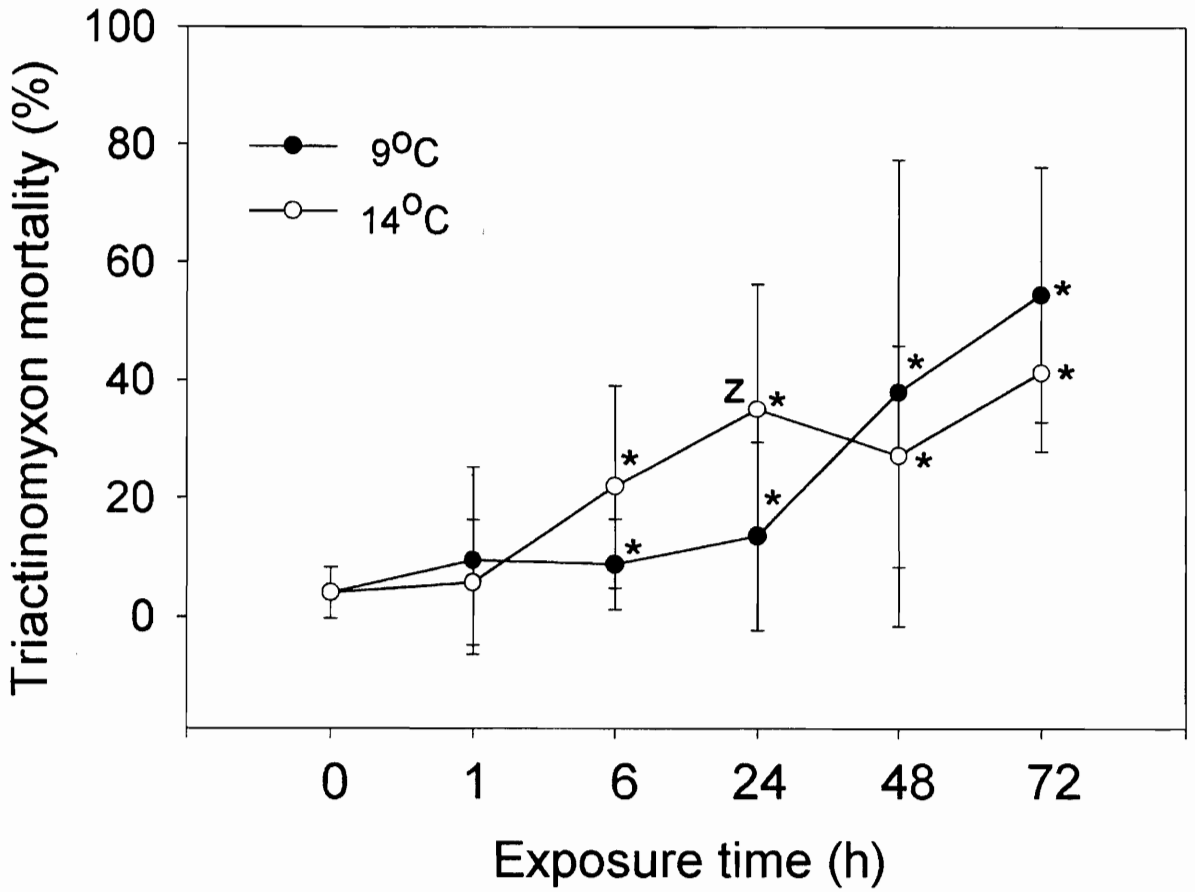

Figure 1. The effect of time and temperature on TAM viability for control groups from salinity tests. Reported as mean % of TAMs dead ± SD. Significant differences ($P \leq 0.05$) between temperature groups within a sampling time are noted by a letter. Asterisks denote significant differences from the baseline (Time = 0).

significant differences ($P \leq 0.05$) between all salt and freshwater treatments. Increasing the concentration of salt decreased the time required to kill TAMs. Salt concentrations of 20 and 30‰ had a rapid negative effect on TAM viability, killing more than 80% of TAMs within the first hour and nearly all TAMs after 6 h. Lower concentrations of salt (5 and 100/00) required 48 h to kill more than 80% of TAMs.

pH

TAM viability was impacted by pH. The further pH moved from circum-neutral, TAM viability decreased. On the basic end of the scale, pH of 8.5 and greater had significant ($P \leq 0.05$) negative effects on the viability of TAMs at 9°C (Figure 3) and 14°C (Figure 4). At 9°C, TAM viability was significantly lower than controls after 6 h of exposure to pH 9, 9.5, or 10. Viability of TAMs at pH 8.5 was slightly, but significantly, different from the controls (pH 7.95 and 8.1, respectively) at 24 and 72 h.

At 14°C, pH 9.5 and 10 treatments had significantly more dead TAMs than the control after 6 h. Viability at pH 9 was significantly reduced after 24 h; mortality increased to 79–88% compared with controls that had 16–30% mortality. Survival at pH 8.5 was only significantly reduced from the controls at 24 and 72 h (pH 7.5 and 8.0, respectively).

Acidity was also found to negatively affect TAM viability. At 9°C (Figure 5), pH 4 induced significantly more dead TAMs than the controls after only 1 h. Reduction in viability at pH 5 was significant after 6 h and at pH 6 was only significantly reduced from the controls (pH 8.2) at 72 h. At 14°C (Figure 6), there were significantly more dead TAMs in the pH 4 treatment than the control after only 1 h. Reduction in viability at pH 5 was significant after 6 h and at pH 6 viability was significantly lower than controls (pH 7.9–8.1) after 24 h.

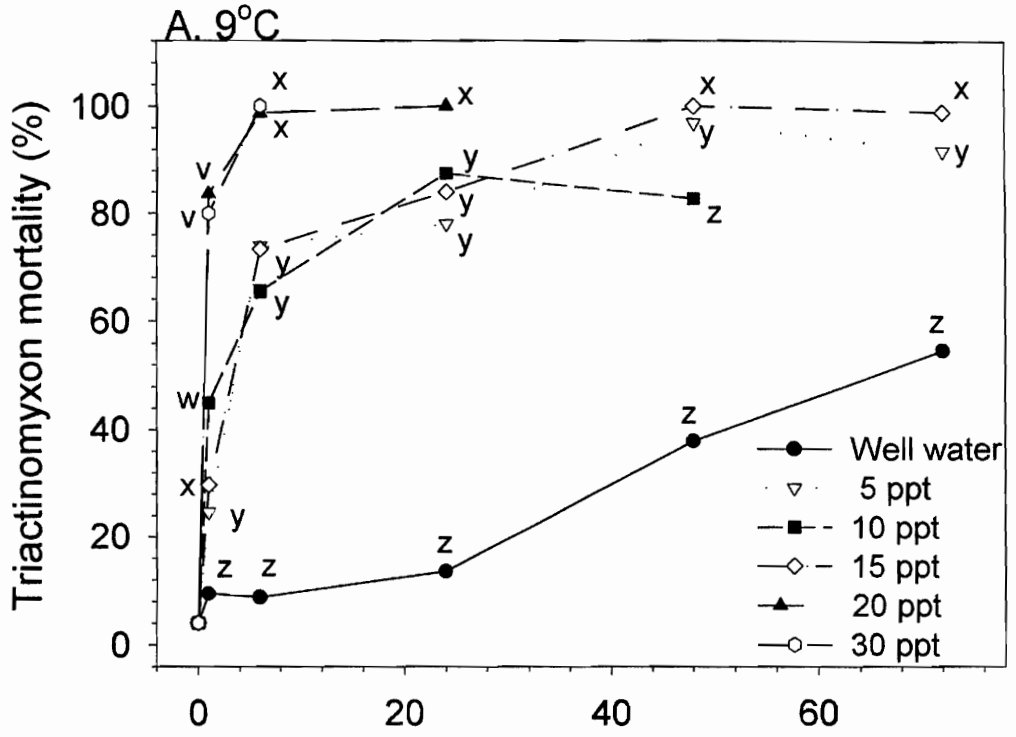

Figure 2a. Mean percentage of dead TAMs at reported saline concentrations over time, at 9°C. Significant differences ($P \leq 0.05$) between saline concentrations within a sampling time are noted by a different letter.

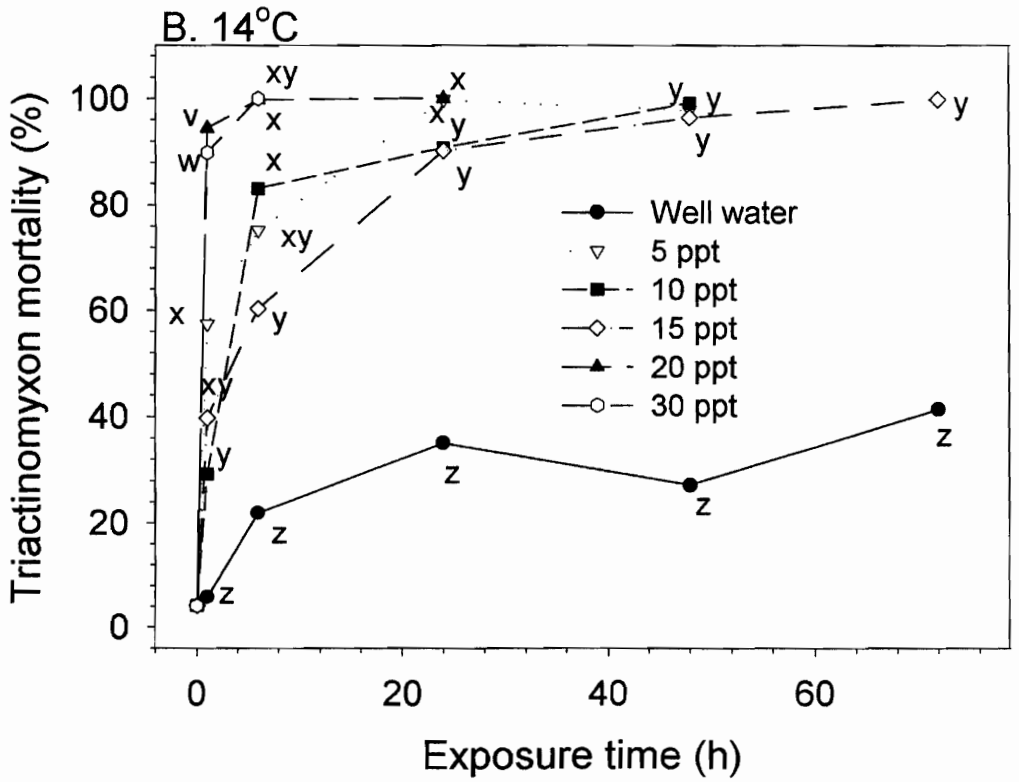

Figure 2b. Mean percentage of dead TAMs at reported saline concentrations over time, at 14°C. Significant differences ($P \leq 0.05$) between saline concentrations within a sampling time are noted by a different letter.

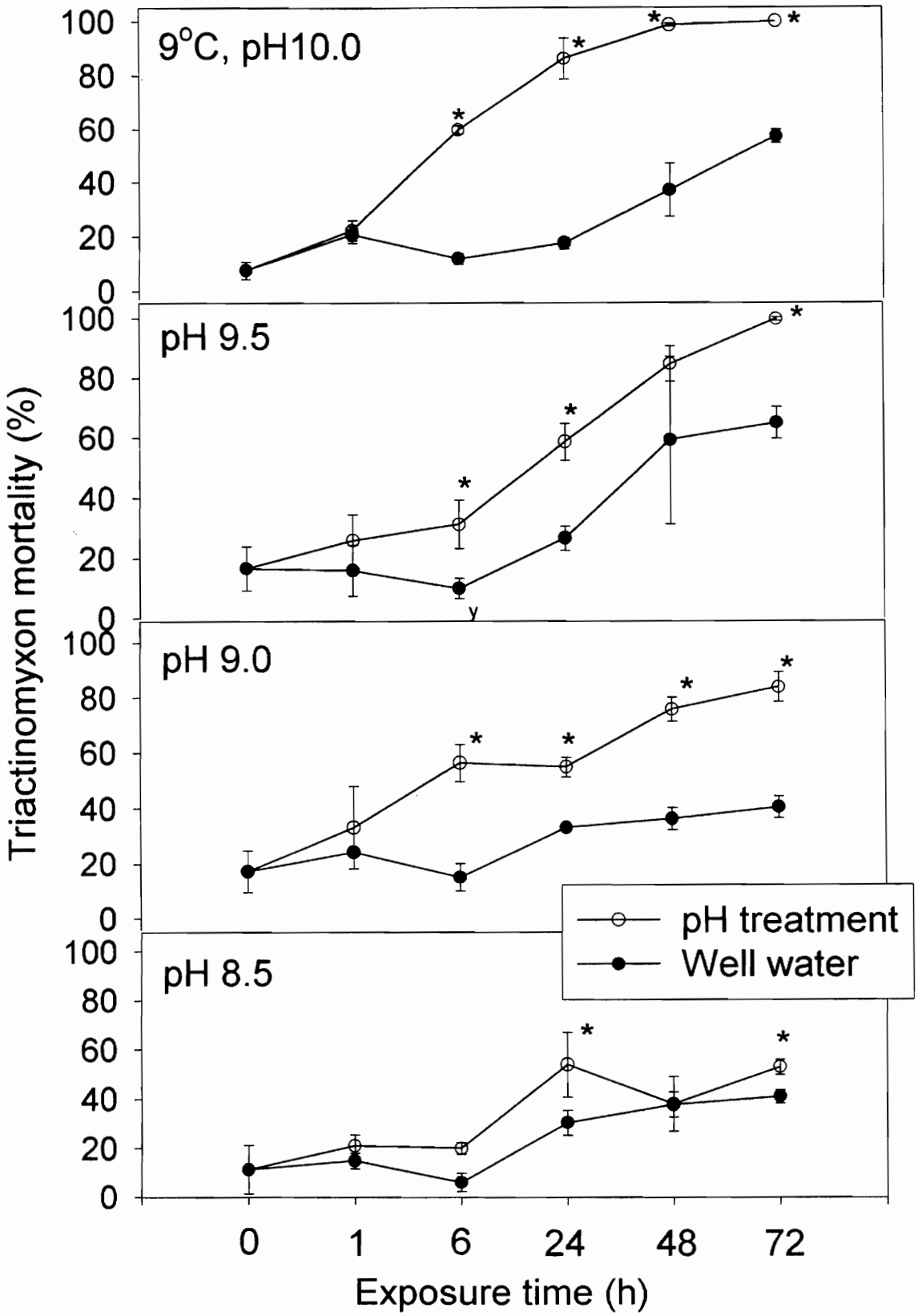

Figure 3. The effects of time and high pH on TAMs viability at 9°C, reported as mean percent of TA dead ± SD. Significant differences ($P \leq 0.05$) between well water and pH groups within a sampling time are noted by a asterisk.

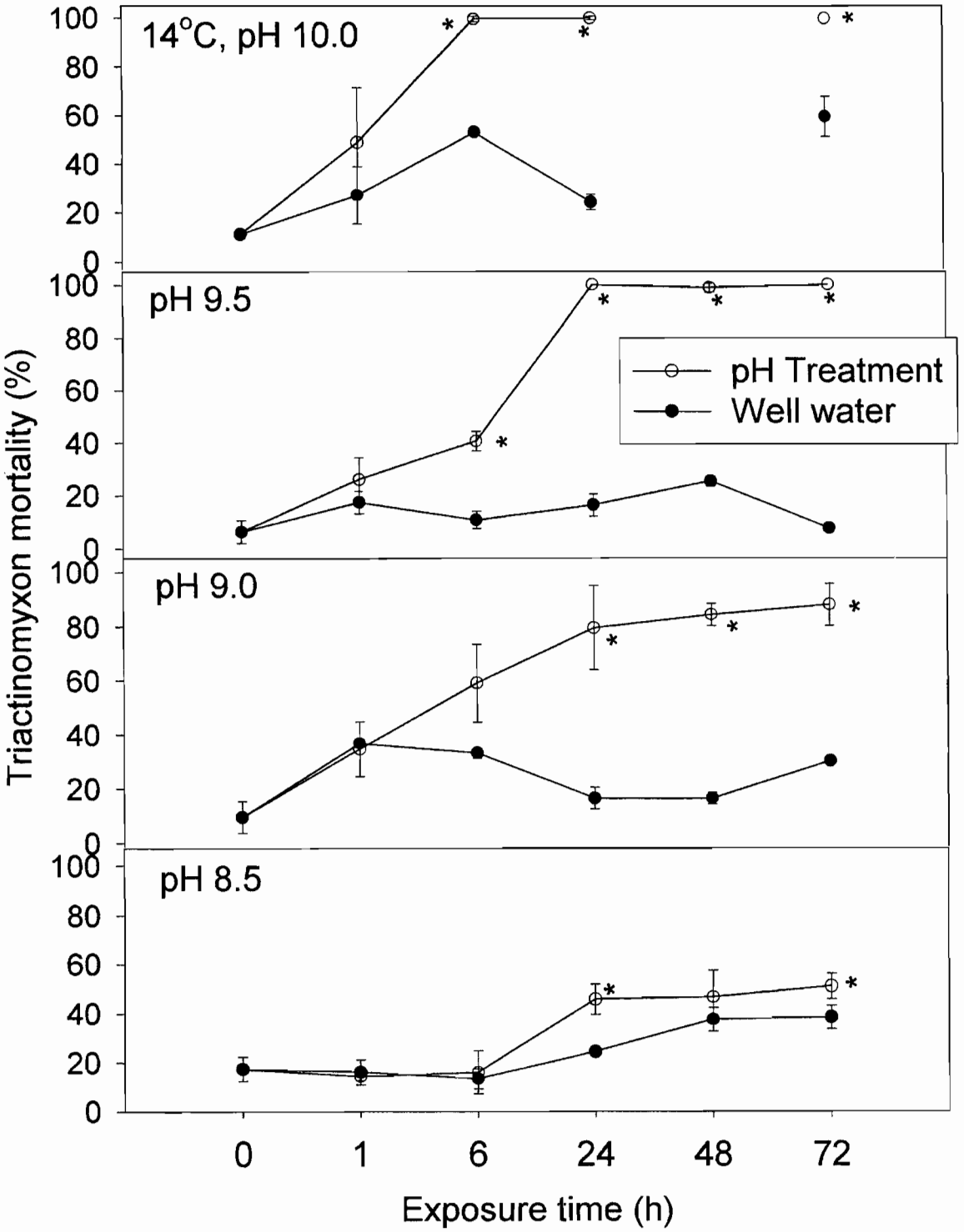

Figure 4. The effects of time and high pH on TAMs viability at 14°C, reported as mean percent of TA dead ± SD. Significant differences ($P \leq 0.05$) between well water and pH groups within a sampling time are noted by an asterisk.

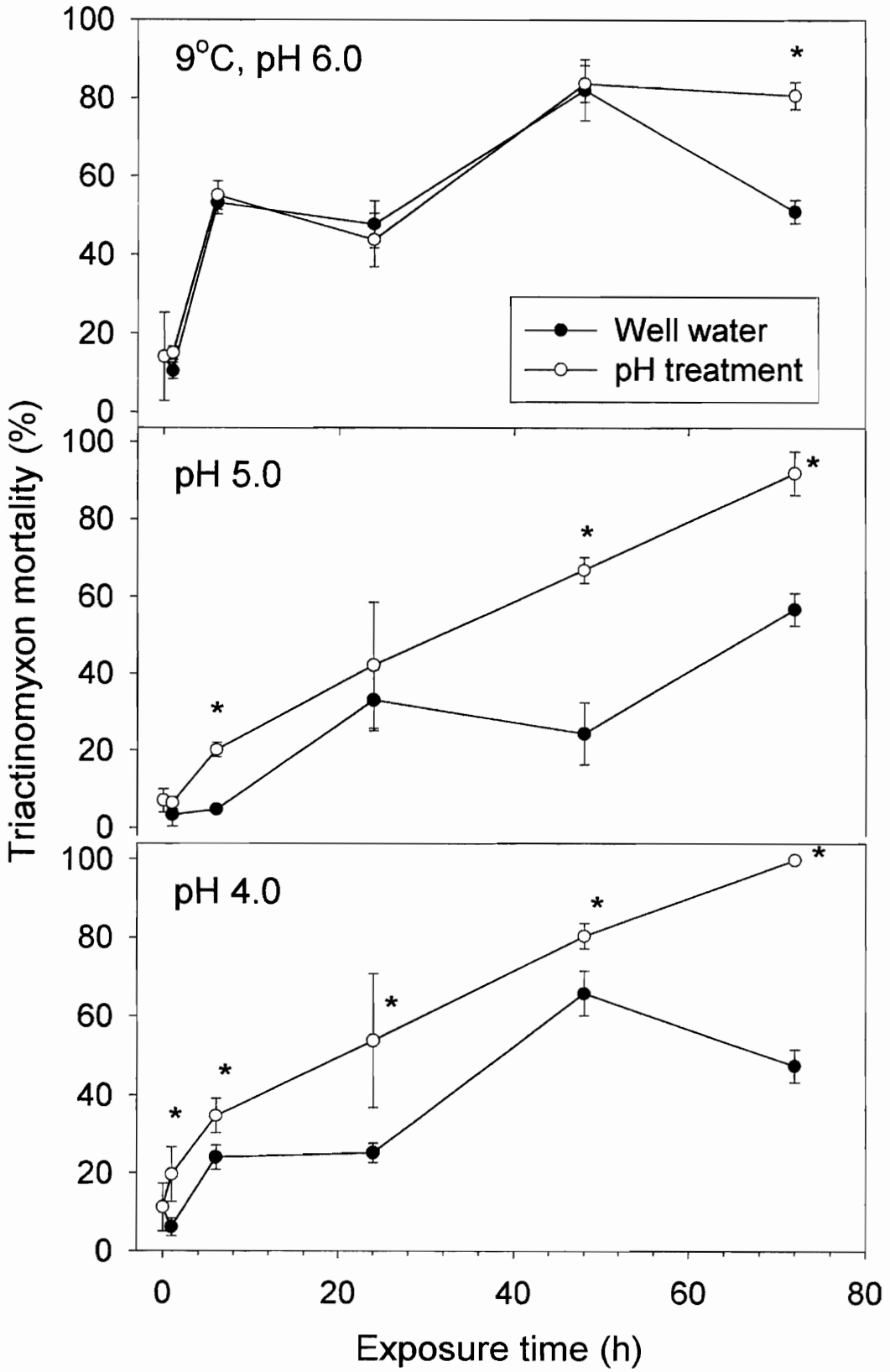

Figure 5. The effects of time and low pH on TAM viability at 9°C, reported as mean percent of TAMs dead ± SD. Significant differences ($P \leq 0.05$) between well water and pH groups within a sampling time are noted by an asterisk.

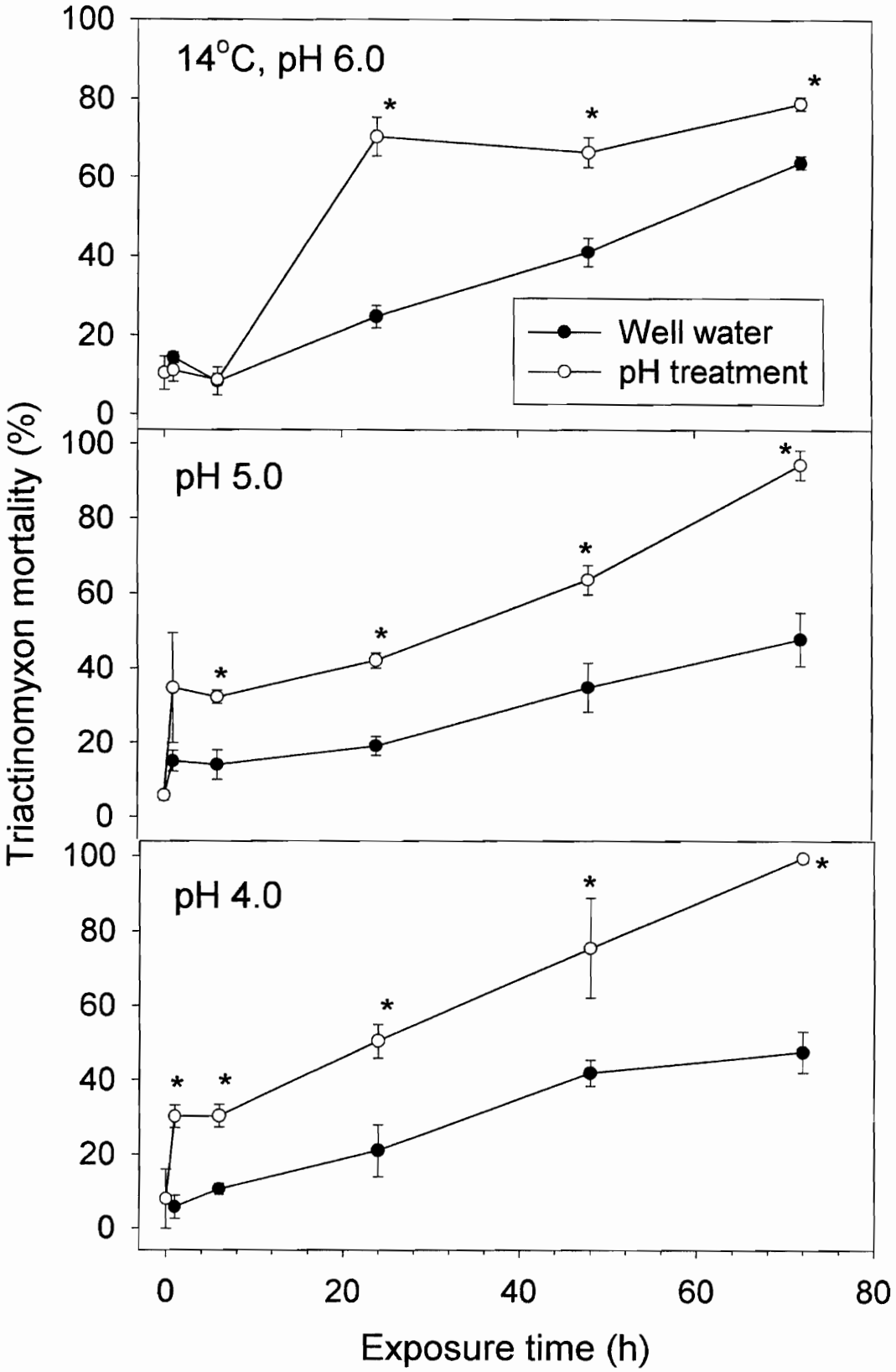

Figure 6. The effects of time and low pH on TAMs viability at 14°C, reported as mean percent of TA dead ± SD. Significant differences ($P \leq 0.05$) between well water and pH groups within a sampling time are noted by an asterisk.

Total Hardness

Total hardness was tested at extreme levels, evaluating both water considered very hard (650 mg/L) and soft (10 mg/L) at exposure times from 24 to 72 h. Even at these extreme levels of water hardness, there was no significant effect of either hard or soft water on the viability of TAMs ($P \leq 0.05$; Table 1). At 72 h, nearly 50% of observed TAMs died in the soft-water treatments, while approximately 30 and 45% mortality, respectively, occurred in the hard water and control treatments.

Dissolved Oxygen

Dissolved oxygen was tested at a very low level (0–0.5 mg/L) for a period of 72 h. At both 9°C and 14°C, DO decreased from 0.5 to 0.0 mg/L over the time period. In both exposures, low DO had no significant effect on the viability of TAMs (Table 1). In both well water and low DO treatments, 30–40% of TAMs were dead after the 72 h.

DISCUSSION

The epidemiology of whirling disease is still far from being fully understood. Factors that influence the viability and abundance of the alternate host, susceptibility of the fish host, and infection severity are still being explored. It is known that dose (i.e., the number of TAMs a fish is exposed to) has a significant impact on the development of clinical signs and mortality (Markiw 1992c). The research conducted in this study examined the effect of water quality characteristics on TAM viability, which affects the number of TAMs available to infect fish. Conditions that reduce viability would logically reduce the number of viable TAMs passing by a given point on a stream.

Vital staining using fluorochromes has been a useful method for viability assessment (Jones and Senft 1985). It has been used previously with *M. cerebralis* (Markiw 1992b) and viability has also been correlated with survival of *Giardia* cysts by Schupp and Erlandsen (1987). These authors noted that *Giardia muris* cysts that were stained red by PI were incapable of infecting mice, whereas green, FDA-stained cysts resulted in infection. Later work (Smith and Smith 1989; Labatiuk et al. 1991) has indicated that the dyes are conservative, overestimating actual viability of *Giardia* cysts. In this study, the TAMs staining both red and green were considered as potentially viable, but in reality may not be viable. Interpretation of the lethal effects of the variables tested are based on the vital staining result of 100% dead (all red).

In this study, pH was the variable most likely to influence TAM viability in freshwater. Both high and low values influenced viability in ranges that would not be deadly to fish. Some fishes have been found in certain lakes and streams at pH levels ranging from 4 to 10 (Cleary 1955). However, lethal levels may vary because sensitivity varies with species, presence of other ions such as Al+, and the stage of development (Doudoroff and Katz 1950). For example, Woodward et al. (1991) noted survival of greenback cutthroat trout *Oncorhynchus clarki stomias* alevins was reduced by 68% at pH 5.0. The data in this study suggest that low pH conditions typical in the eastern U.S. (Schofield 1982; Patrick 1996) would tend to reduce the lifespan of any TAMs released. This would be in agreement with the lack of severe population impacts noted for this region (Hulbert 1996). However, other variables such as relative

Table 1. Mean percentage (± SD) of dead TAMs for low dissolved oxygen, soft (10mg/L total hardness) and hard (650 mg/L total hardness) water exposed groups at given temperature and time. There were no significant ($P \leq 0.05$) differences between treatments or temperatures.

Water quality variable	Concentration (mg/L)	Temperature(°C)	Time (h)	% Mortality ± SD	
				Test	Control
Hardness[a]	650	9	24	23.3 ± 1.9	17.6 ± 2.0
Hardness	650	9	48	31.0 ± 2.2	28.3 ± 6.5
Hardness	650	9	72	27.1 ± 3.8	44.2 ± 4.3
Hardness	10	9	72	51.0 ± 1.4	38.4 ± 6.1
Hardness	10	14	72	61.7 ± 4.3	57.7 ± 0.5
DO	0	9	72	27.1 ± 3.8	44.2 ± 4.3
DO	0	14	72	27.7 ± 4.0	28.0 ± 5.1

[a]total hardness as $CaCO^3$

abundance of tubificid worms and temperature regimes may also act to limit the severity of the disease in the region. Also, not all eastern streams are acidic. Effects on TAMs were noted on the basic end of the pH scale as well, at levels that are not considered lethal to salmonids (≤ pH 9.5–9.8; Daye and Garside 1975; Murray and Ziebell 1984). Data from this study also indicated that high pH levels typically found in eutrophic waters in summer could reduce the lifespan of the infective stage. Studies of the effect of pH on *T. tubifex* indicate that lethal levels are more extreme than they are for salmonids; 96-h LC_{50} values were pH 3.6 and 10.5 without sediment or 2.5 and 10.7 with sediment (Chapman et al. 1982). Therefore, direct effects of pH on the worm host would not be expected, but the combined effect of parasitism and pH on TAM release has yet to be studied. The ultimate impact of the parasite under high or low pH conditions must also take into account worm population dynamics, temperature, natural predation, and a variety of other variables.

The effect of temperature on the disease has been examined in a few different studies. R. Vincent (Montana Fish, Wildlife, and Parks, personal communication) noted that the peak infection rates of sentinel fish occurred at about 12–13°C, from mid-May to Mid-July. Research with *T. tubifex* indicated that temperature played a key role in the development and production of TAMs. TAM production from worms transferred from 15°C to 25°C or 30°C stopped after 4 d, and after 15 d at 20°C (El-Matbouli et al. 1999). Worms transferred to 5°C, 10°C, or left at 15°C continued to produce TAMs. Markiw (1992b) noted that temperature influenced the lifespan of the TAM, ranging from only 2 to 3 d at 19–24°C to 7–8 d at 7°C. Similarly, we found that nearly 50% of TAMs from control groups at 9°C and 14°C were dead after 72 h. The lifespan data from this study may be conservative due to the collection of TAMs from cultures only three times a week (Monday, Wednesday, Friday). Therefore some TAMs may be 2–3 d old at temperatures from 13°C to 15°C, before initiating the test. This would mean the half-life of the TAMs could be from 3 to 6 d at 9°C or 14°C. El-Matbouli et al. (1999) found that TAMs may still be viable after 15 d at water temperatures up to 15°C.

The salinity data indicated that the TAMs are sensitive to salt, with higher concentrations causing significant mortality after only an hour. Lower concentrations at longer durations (e.g., 5–10 ‰ for 24 h or more) were similarly toxic. From a disinfection perspective, 20–30‰ salinity for at least 6 h would be required to effectively kill all TAMs. The sensitivity of TAMs to higher salinity is of interest relative to anadromous salmonids in important estuaries such as the mouth of the Columbia River. Salt may also be useful as a control measure in situations such as treatment of suspect water used in fish stocking trucks.

The lack of any effect of hardness at concentrations of 10 or 650 mg/L was not too surprising. Salmonids have a similar wide tolerance to hardness per se, with no effect noted until concentrations reach 1 mg/L (Brown and Lynam 1981). However, low hardness and alkalinity reduce the buffering capacity of the water, making any fish present very susceptible to drastic pH fluctuations. The lack of any effect of dissolved oxygen on viability was also not surprising. The worm host is typically found in sediments in which the DO can drop to zero. *Tubifex tubifex* is known to survive and even reproduce under anoxic conditions (Fox and Taylor 1955; Famme and Knudsen 1985). It would be a useful adaptation for the parasite to also survive such conditions, although this has yet to be evaluated *in vivo*.

The data presented here contribute to the understanding of the infection process in wild situations. Further research should elucidate other factors and relationships that affect disease severity. Once these are determined, these could be applied to fisheries management as it relates to whirling disease.

ACKNOWLEDGMENTS

We thank Grant White for the opportunity to collect infected worms from his farm for TAM production. We also thank Don Roberts at Utah State University for some of the TAMs used in the study. We thank Ronney Arndt and Quinn Cannon for their help in maintaining the worm cultures. The work was generously funded by the National Partnership for the Management of Wild and Native Cold Water Fisheries in cooperation with the Utah Division of Wildlife Resources. In coordination with matching funds from the Federal Aid in Sport Fish Restoration program, the Salt Lake County Fish and Game Association provided a generous donation used to purchase the epifluorescence microscope used in the study.