DYNAMICS OF THE WATERBORNE STAGE OF *MYXOBOLUS CEREBRALIS* ESTIMATED DIRECTLY BY PACKED-BED FILTRATION

by

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ABSTRACT

Past assessments of whirling disease infection risk on wild trout populations have relied on disease severity experienced by cultured fish exposed in sentinel cages or flat-screen filtration of *Myxobolus cerebralis* triactinomyxons. The former technique is indirect and inexpedient whereas the latter is imprecise and inaccurate. I examined spatial and temporal dynamics of *Myxobolus cerebralis* triactinomyxon concentrations directly using packed-bed filtration at five mainstem sites on the upper Madison River and at Willow Creek, a tributary to the Jefferson River. Packed-bed filtration provides accurate and precise estimates of ambient triactinomyxon concentrations. I also compared findings inferred from indirect sentinel-cage trials and my concurrent direct measurements. Triactinomyxon concentrations were consistently higher at Willow Creek than at the five sites on the Madison River, at which concentrations did not differ spatially. Concentrations at all sites peaked in spring and autumn. Variation in concentrations among five consecutive days was absent, but strong diel variation was evident; at Willow Creek, concentrations were elevated during periods of low light intensity. Indirect sentinel-cage sampling and simultaneous, direct packed-bed filtration largely corresponded, except in one low-temperature sample when a low percentage of fish became infected despite high concentrations of triactinomyxons. Packed-bed filtration proved to be a rapid, efficient, and effective method for assessing whirling disease infection risk among wild trout.

1
INTRODUCTION

Salmonid whirling disease is caused by the myxozoan parasite *Myxobolus cerebralis* (Hofer 1903). The parasite is thought to have spread to the United States in the 1950s through the transport of fish from Europe (Hoffman 1990). Recent declines among wild rainbow trout populations in the Intermountain West have been linked to the disease (Nehring and Walker 1996; Vincent 1996) and have led to heightened interest in the ecology of the parasite. Managers require information on distribution and abundance of the parasite to develop effective management strategies to mitigate effects of the disease on wild trout populations.

Myxobolus cerebralis has a two-host life cycle with two different spore stages, the myxospore and actinospore. The actinosporean stage, also called the triactinomyxon spore, infects a salmonid fish whereas the myxospore stage infects the oligochaete *Tubifex tubifex* (Wolf and Markiw 1984; El-Matbouli and Hoffmann 1989). Triactinomyxons develop within the oligochaete host and are released directly into the water column (El-Matbouli and Hoffmann 1998) where they float freely until coming in contact with a fish. Upon contact, the triactinomyxon fires its "nematocysts," disrupting the epidermis of the fish and injects its 64 sporoplasms (Markiw 1989a; El-Matbouli et al. 1995). Individual germ cells from the sporoplasms disperse, penetrate host cells of the epidermis, and divide by mitosis and endogeny (cells arising within cells) (Hedrick and El-Matbouli 2002). The parasites migrate via the peripheral nerves and the central nervous system and eventually invade and destroy cartilage before developing into

mature myxospores (El-Matbouli and Hoffman 1995). Cartilage destruction and inflammation resulting from the developing myxospores is associated with mortality in young fish (Markiw 1991). Severity of the disease in salmonids is dependent on age (Ryce et al. in press), size (Ryce 2003), fish species (MacConnell and Vincent 2002), and especially on the density of triactinomyxons to which the fish are exposed (Markiw 1992a; Markiw 1992b; Ryce et al. in press).

Estimation of infection risk to wild trout requires effective methods for quantifying densities of *M. cerebralis* triactinomyxons in natural systems. Relative densities of triactinomyxons in open waters have heretofore been estimated indirectly by exposing cultured rainbow trout of standardized sizes and ages to ambient conditions in sentinel fish cages. Disease severity of caged fish, typically measured by the qualitative MacConnell-Baldwin histological scale (Hedrick et al. 1999) or myxospore burdens (O'Grodnick 1975), is assumed to reflect triactinomyxon densities at exposure sites and times, and also therefore to correspond to infection risk experienced by co-occurring wild fish. Sentinel fish exposures indicate that *M. cerebralis* infection risk may vary spatially within and among watersheds and over time (Hiner and Moffitt 2001; Sandell et al. 2001). However, different water temperatures, water quality conditions, and water velocities at cage sites may affect infection intensities in caged fish apart from absolute triactinomyxon densities present and these effects may differ among sentinel trout strains, sizes, ages, and physiological conditions (El-Matbouli et al. 1999; Vincent 2002b; Ryce 2003; Ryce et al. in press). Fish sizes and ages are difficult to match precisely for such exposures, and slight differences (e.g., a week or several mm) can affect disease severity

significantly (Ryce 2003; Ryce et al. in press). Therefore, comparisons based on sentinel fish exposures are potentially confounded by these difficult-to-control variables and may provide an inaccurate and imprecise estimate of actual infection risks experienced by wild fish. Furthermore, sentinel-cage fish studies are time consuming, expensive, and provide results only after a minimum of at least three months because exposed fish must be held long enough for the parasite to develop to the myxospore stage in the cartilage of the fish before histological examination or myxospore isolation can be performed. In most cases, the lag time is considerably longer because sample preparation and examination are not conducted immediately. Polymerase Chain Reaction (PCR) is a more rapid parasite detection technique (Andree et al. 1998). PCR can detect *M. cerebralis* in trout or worms at any time after exposure, but provides no information on the degree of exposure, indicating only presence or absence of the parasite. A newly developed quantitative PCR approach may be able to assess some degree of infection severity in moderately to heavily infected rainbow trout but it is still being modified to evaluate infections among rainbow trout that receive low dose exposures to triactinomyxons (Kelley et al. 2003).

Use of water-filtering techniques to directly estimate triactinomyxon concentrations would eliminate confounding variables involved with sentinel cage exposures and more exactly estimate ambient infection risk to wild fish. Simple 20-µm mesh flat screens are commonly used for collection and concentration of laboratory-reared triactinomyxons (El-Matbouli et al. 1995; Hedrick et al. 1999). Triactinomyxons have been filtered from open waters with such screens; however, concentrations

recovered tended to decrease as the volume of water filtered increased (Thompson and Nehring 2000). Three-dimensional material has been a better filter to capture triactinomyxons. For example, sand-charcoal filters (Hoffman 1974), passive sand filtration (Nehring et al. 2003), and sand filters with grain size greater than 180 μm (Arndt and Wagner 2003) were successful in hatcheries for preventing triactinomyxons from reaching vulnerable fish. Packed-bed filtration is commonly employed in water and wastewater treatment to remove particles and reduce the concentration of microbial pathogens (Redman et al. 2001). It is also used to remove sub-micron and nanometer particles from solution (Sisson et al. 1995). It involves the use of uniform-sized, spherical glass beads packed under vacuum in a column. Interstitial spaces among the spheres create a tortuous, three-dimensional path whose length is dependent on the depth of the bed. Volumes to be filtered are drawn through the packed bed under vacuum and target particles become trapped in the bed. After the volume has been filtered, the bed is back-washed, thereby destabilizing it and suspending trapped particles in the supernatant above the bed. This fluid is then removed and the particles quantified. Packed-bed filtration to concentrate and quantify *M. cerebralis* triactinomyxons from opens waters could be a quick, accurate, and precise method to directly estimate infection risk to wild fish. Potential uses of a packed-bed filtration system to study *M. cerebralis* are manifold. It could be used to quickly estimate temporal and spatial variation in infection risk, for example, in different rivers, in different places within a system river, and at different times and seasons. It may be an especially useful tool for evaluating how habitat and water quality conditions (degraded habitats, high *T. tubifex* densities, myxospore

deposition sites, altered discharge and thermal regimes, etc.) affect infection intensity. In infected systems, estimates would provide a better measure of effects of habitat improvement and water discharge management programs on disease, survival, and recruitment of wild salmonid populations.

I conducted preliminary laboratory studies using known quantities of triactinomyxons to develop a packed-bed filtration system that could be used in the field. Results of these studies are described in the Development and Testing chapter of this thesis. I subsequently field-tested the system in the upper Madison River system and at Willow Creek, Montana, at sites that had previously been used for sentinel cage exposures and were known to be positive for the parasite. These field tests constitute my thesis research. My specific objectives were:

Objective 1

To determine spatial distributions of triactinomyxon abundances in the wild; Drainage-wide studies using sentinel cages have shown spatial variation in disease severity (Sandell et al. 2001; Hiner and Moffitt 2001; Hubert et al. 2002; Anderson 2004). Direct estimation of actual triactinomyxon densities at specific locations would allow for a better understanding of spatial variation in infection risk for wild fish.

Objective 2

To determine the seasonal, daily, and diel temporal distributions of triactinomyxons in the Madison River and Willow Creek; Large temporal variation in disease severity of sentinel fish has been documented, with higher levels of infection in the spring and autumn than in summer months in Montana (Vincent 1999; Downing 2000). My direct measure of triactinomyxons would determine if temporal variation in triactinomyxon densities follows a similar pattern. Furthermore, disease severity in wild fish is assumed to reflect continuous and cumulative exposure to ambient triactinomyxon concentrations. Accordingly, an understanding of temporal dynamics of triactinomyxon concentrations is necessary to determine appropriate time scales over which concentrations should be sampled.

Objective 3

To determine if water temperature and stream discharge are correlated with variation in triactinomyxon densities in the wild; Water temperature has been shown to correlate with infection intensity of rainbow trout in sentinel-cage studies (Baldwin et al. 2000; Vincent 2002a). Stream discharge has also been shown to be a factor in disease severity of exposed sentinel fish in the laboratory and in a Madison River field study (Vincent 2002b). However, water temperature and stream discharge may affect infection intensities in caged fish apart from triactinomyxon concentrations present. This objective

will enhance our understanding of the effects of water temperature and stream discharge on triactinomyxon concentrations.

Objective 4

To assess congruence of measured triactinomyxon concentrations with results of concurrent sentinel-cage fish exposures; This objective will allow for an evaluation of the packed-bed filtration method in relation to the sentinel cage exposures for estimating infection risk in wild trout populations.

DEVELOPMENT AND TESTING

Previously, Frederic T. Barrows of the Bozeman Fish Technology Center, Alexander V. Zale of the Montana Cooperative Fishery Research Unit, and Thomas E. McMahon of Montana State University conducted two laboratory studies to develop methods to directly estimate triactinomyxon abundances accurately and precisely. The Montana researchers first sought to improve on the technique of filtering triactinomyxons in open waters with simple 20-µm mesh flat screens (Thompson and Nehring 2000) by using dual rotating-drum screen filtration, a technique commonly employed by the aquaculture industry to filter and concentrate large quantities of zooplankton for feeding larval fish. This method was unsuccessful, achieving recovery rates of known numbers of triactinomyxons ranging from only 4.9 to 47.5% in the laboratory when triactinomyxons were diluted in volumes greater than a few liters (Barrows et al. 1999). This failure led them to test the technique of filtering triactinomyxons with flat screens for accuracy. Mesh sizes of 15, 20, 30, 35, 41, 53, 80, and 100 µm were tested for recovery of known amounts of triactinomyxons. These different mesh sizes were tested using 1.2 and 12-liter volumes of water. Highest triactinomyxon recovery was achieved using 20-µm mesh screens when filtering both volumes (Figure 1). However, recovery decreased when filtering a 12-liter volume of water and was highly variable (mean = 65.5%, range 44-94%) compared to 1.2 liters (mean = 101%, range 95-109%) (Figure 1). Mean recovery of triactinomyxons diluted in 120 liters of water filtered through 20-µm mesh screens was only 21% (Figure 1). Therefore, as the volume of

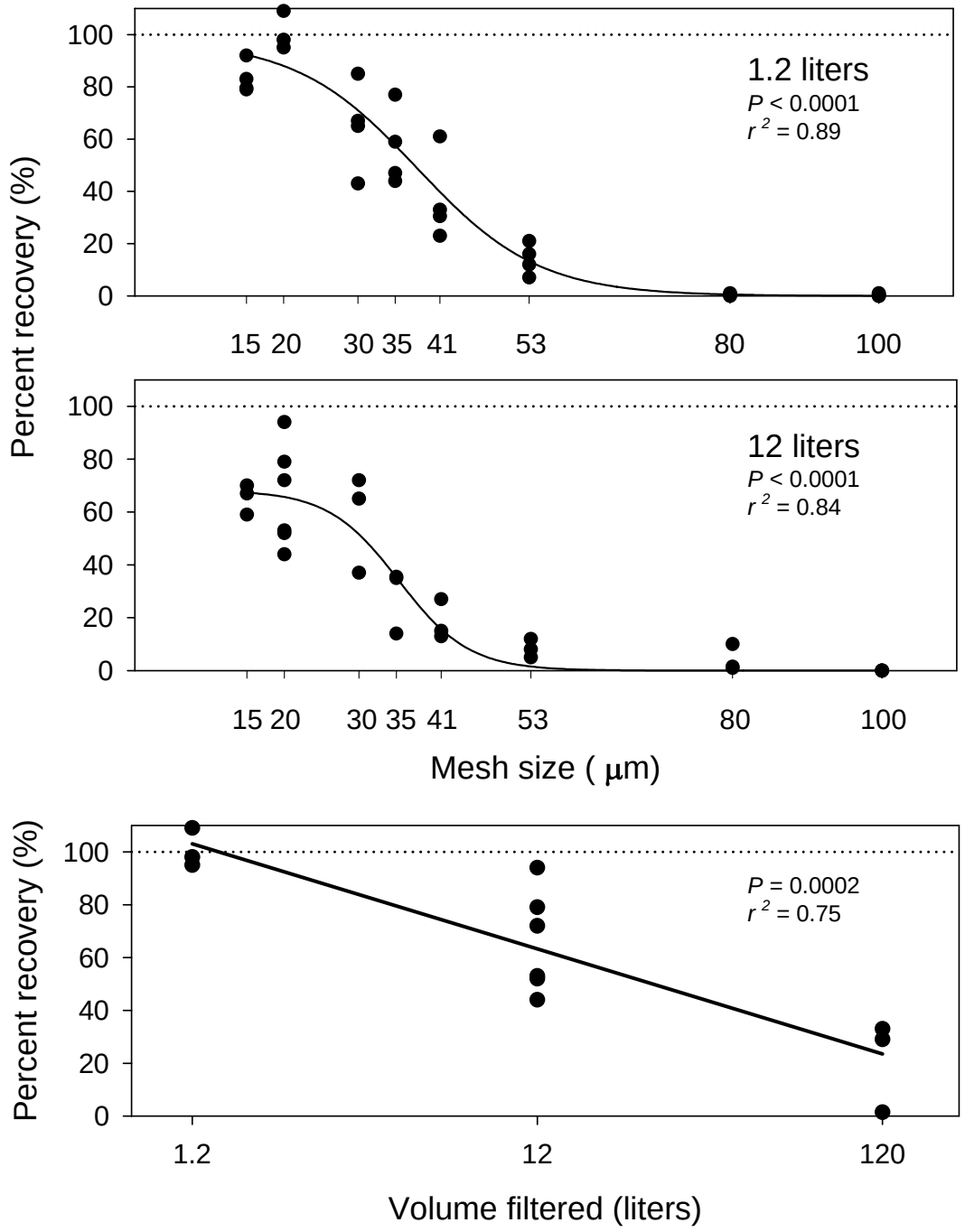

Figure 1. Percent of triactinomyxon concentrations recovered when filtering 1.2 (upper) and 12-liter (middle) volumes of water through different size mesh screen filters, and when filtering 1.2, 12, and 120-liter volumes of water through a 20-μm mesh screen filter (lower).

water being filtered increased, triactinomyxon recovery decreased and varied unpredictably. Thompson and Nehring (2000) and Wagner (2002) noted similar decreases in recovery rates with increasing water volumes filtered. Screen filtration was judged by the Montana researchers to be useful only for pre-filtering particles larger than triactinomyxons from water samples, but not for the concentration step of the collection process.

A second study examined use of packed-bed filtration for filtering and concentrating triactinomyxons (Barrows et al. 2001). I joined this team of researchers and conducted a series of laboratory trials using a 153-mm diameter packed-bed filter column to evaluate and optimize the technique for triactinomyxon filtration (Barrows et al. 2002). We used known amounts of laboratory-produced triactinomyxons to optimize accuracy, precision, and sensitivity of the system. We initially filtered 50,000 to 250,000 laboratory-produced triactinomyxons per trial, and subsequently used 2,000 triactinomyxons per run to test the sensitivity of the system. Sample volumes tested were 12 and 120 liters. A vacuum pump drew water through the packed bed and into dual alternating vacuum chambers (Figure 2). Backwash was provided by an electric water pump controlled by a timed relay switch and flow valve. We tested different bed depths and the duration, volume, and discharge rate of backwash in the laboratory to find a combination of these parameters that consistently resulted in complete recovery of introduced triactinomyxons. Our configuration optimization trials revealed that a bed depth of 8 mm of 200-µm glass beads, a backwash time of 10 seconds, and a backwash volume of 1000 ml consistently provided almost

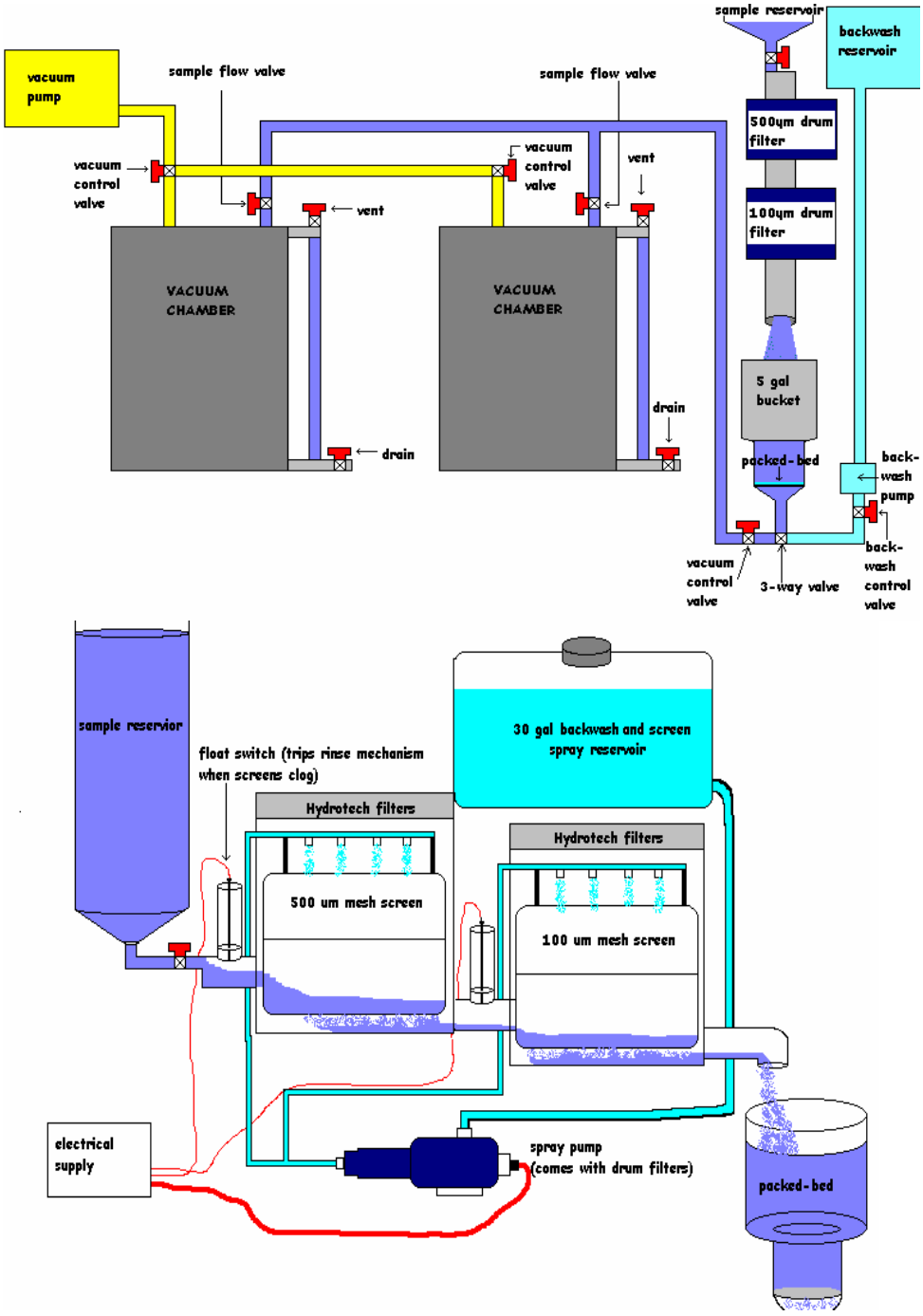

Figure 2. The packed-bed filtration system showing the dual-alternating vacuum chambers and backwash system (upper) and the dual-rotating drum filters (lower).

total recovery of introduced triactinomyxons in the laboratory. Because triactinomyxon densities in the 1000-ml backwash volumes were too low for precise enumeration by microscopy, we filtered this volume through a small 25-mm diameter packed-bed column, again using a bed depth of 8 mm of 200-µm glass beads and backwashed with a volume of only 35 ml. This 35-ml supernatant was then centrifuged to further concentrate the triactinomyxons. Successful centrifugation of triactinomyxons was achieved through the addition of sodium hexametaphosphate to samples at a concentration of 6.2 g per liter. The sodium hexametaphosphate caused collapse of the triactinomyxons and therefore increased their density sufficiently to allow migration during centrifugation. Mean recovery rates of triactinomyxons in paired trials were 102% (range 96% to 106%, n = 3) with and 56% (39% to 67%, n = 3) without sodium hexametaphosphate. Although collapsed, the triactinomyxons were still readily recognizable following treatment. Optimal centrifugation was achieved at 6000 x gravity (g) for 30 minutes. Following centrifugation, all triactinomyxons in the 35-ml supernatant were concentrated in 3.5 ml. Three 80-ml aliquots of this concentrate were removed with a micropipette and examined under a phase-contrast microscope to count the number of triactinomyxons present.

Modifications for use in the field were subsequently added and tested. Rotating-drum screen filtration was incorporated to act as a pre-filter, removing debris and organisms larger than triactinomyxons from stream water before it flowed to the packed-bed filter. Sample volumes passed through 500-µm and 100-µm mesh rotating-drum filters (Figure 2) before flowing into the packed-bed. Also, a laboratory test evaluated

the effect of pumping on triactinomyxons because field samples would need to be moved from the stream to the filtration unit. Significant losses and damage to triactinomyxons were inflicted by peristaltic and diaphragm pumps. Triactinomyxons were not damaged or lost during pumping with an electric, submersible, centrifugal pump.

The complete filtration system was tested in the laboratory by diluting 50,000 triactinomyxons in 120-liters of water. This sample was pumped using a centrifugal pump through an irrigation hose to another 120-liter sample reservoir. The water sample flowed by gravity through 500 and 100-µm mesh dual rotating-drum screen filters to the packed-bed filter. The water was drawn through the bed under vacuum pressure, trapping the triactinomyxons in the filter. The bed was backwashed, re-suspending the trapped triactinomyxons in one liter of clean water. The one-liter field sample was filtered through a small-diameter packed-bed system and backwashed with a volume of 35 ml. This 35-ml volume was centrifuged and further concentrated to 3.5 ml. Three 80-µl aliquots were taken from the 3.5 ml sample and examined using phase-contrast microscopy to enumerate triactinomyxon concentrations. These tests of the complete packed-bed filtration system resulted in a mean recovery rate of 91.28% (SD ± 9.99%, range 77-107%, n = 14) with a minimum detectable concentration of 0.04 triactinomyxons per liter.

The filtration system was mounted on a flatbed trailer for transport to the field and was successfully field tested in October 2001 at Willow Creek, Montana. Intense infestation of this stream by *M. cerebralis* has been documented (Baldwin et al. 2000). It was operated in the field using parameters optimal for triactinomyxon recovery as

determined by our laboratory trials. The field samples were collected in sample bottles and stored on ice in a cooler for return to the laboratory. The duration of collecting one field sample averaged 12 minutes. Triactinomyxons were recovered at densities of 1.4/L on 11 October 2001 and 0.7/L on 23 October 2001.

Enumerated triactinomyxon concentrations were corrected for sampling error associated with the filtration process using the mean and variance of our laboratory trials (Mood et al. 1974). The expected value of predicted triactinomyxon concentrations was

$$\mathrm{E}\left(\hat{\mu}_{TAMs}\right) = \frac{\bar{x}_{FieldTAMs}}{\bar{x}_{Lab}}\left(1 + \frac{s^2_{Lab}}{\bar{x}^2_{Lab}}\right) \tag{1}$$

where observed mean triactinomyxon densities from the field were divided by the mean percent recovered from the filter in laboratory trails to estimate the value of triactinomyxons that would be expected with 100% recovery. This value was then corrected for error caused by laboratory variance. The variance of predicted triactinomyxon concentrations was

$$VAR\left(\hat{\mu}_{TAMs}\right) = \left(\frac{\bar{x}_{FieldTAMs}}{\bar{x}_{Lab}}\right)^2 \times \left(\frac{s^2_{FieldTAMs}}{\bar{x}^2_{FieldTAMs}}\right) + \left(\frac{s^2_{Lab}}{\bar{x}^2_{Lab}}\right) \tag{2}$$

where the corrected mean triactinomyxon concentration, the variance of the replicate field samples, and the variance of my laboratory trials are used to estimate total variance.

STUDY AREA

I collected samples at five sites in the upper Madison River system in Madison County, Montana. The five sites (Lyons Bridge, Kirby Bridge, Pine Butte, Raynolds Pass, and North Slide) are distributed along a 17-km reach of the river (Figure 3). They were chosen because they correspond to sites used by E. Richard Vincent of Montana Fish, Wildlife and Parks for sentinel-cage fish exposures in previous years and during my study period.

The Madison River originates at the confluence of the Firehole and Gibbon Rivers in Yellowstone National Park and flows north into southwestern Montana. It flows 193 km until it meets the Gallatin and Jefferson Rivers to form the Missouri River. The total drainage area of the Madison is 6475 km^2 (Vincent 1987). The five sites are distributed along a 17-km reach of the river below Hebgen and Quake Lake. The elevation in this reach is between 1665-1995 m with an average gradient of 6 m/km (Vincent 1987). Lyons Bridge (UTM 12 453288E 4971833N), Pine Butte (UTM 12 456730E 4967395N), and Raynolds Pass (UTM 12 464269E 4963792N) are main-channel sites dominated by cobble substrate and fast moving water. Kirby Bridge (UTM 12 45422E 4970688N) and North Slide (UTM 12 464269E 4963715N) are side channels dominated by gravel and cobble with fine-sediment deposition.

Hebgen Dam controls the flow of the upper Madison River below Hebgen Lake, with an annual average discharge of 48 m^3/sec and maximum of 99 m^3/sec typically occurring in late May or early June. The dam is regulated by a water-flow agreement that

dictates an annual minimum discharge of at least 17 m^3/sec (Vincent 1996). Water temperatures range from 0 ^oC in December and January to 20 ^oC in July and August.

The watershed of the upper Madison consists of alkaline soils on broad terraces that were formed by fault movements along the Madison Range front (Alt and Hyndman 1997) and is bounded by mountain ranges. The watershed consists of primarily field pasture and forested areas with residential and commercial areas consisting of less than 15% of the total landscape.

I also collected samples at Willow Creek, Montana, a tributary to the Jefferson River (Figure 3). This site was also used for sentinel-cage fish exposures in previous years and during my study period. The site has a high prevalence of *M. cerebralis*-infected wild trout (Baldwin et al. 2000) and *T. tubifex* (Rognlie and Knapp 1998). The stream here drains an area of 217.04 square kilometers. The Willow Creek site (UTM 12 442824E 5063266N) is 2.8 kilometers upstream from Willow Creek Dam and 4 kilometers northeast of Harrison, Montana, at an elevation of about 1450 m. This site is a riffle/pool habitat dominated by gravel and fine sediments. The average discharge at this site is 1.025 m^3/sec and water flows range from 9.1 m^3/sec in June to 0.25 m^3/sec in August (USGS Station Number 06035000). Water temperatures range from 0 ^oC in December and January to 26 ^oC in July and August.

The Willow Creek watershed consists of sediments associated with basin and range faulting and glacial deposits lying on a bedrock substrate of Precambrian gneisses and granitic igneous rocks (http://www.indiana.edu/~iugfs/wcdwgeol.htm, January 2004). The watershed consists of primarily field pasture bounded by rolling hills with

residential and commercial areas consisting of less than 10% of the total landscape. Livestock grazing and agricultural activity are prevalent throughout this watershed.

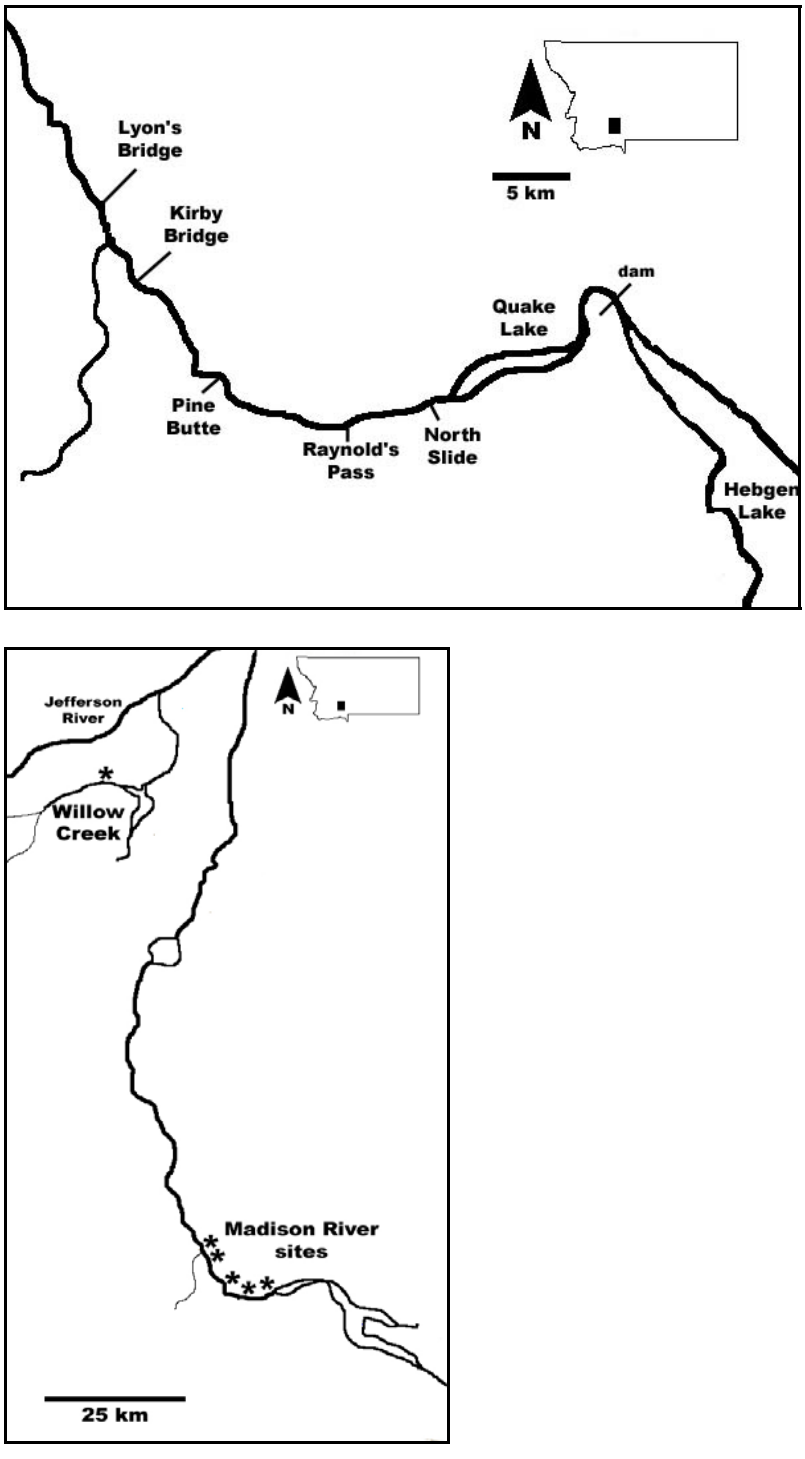

Figure 3. Map of the study area along the upper Madison River, Montana (upper), and Willow Creek in relation to Madison River sites (lower).

METHODS

Objectives 1 and 2

To determine spatial distributions of triactinomyxon abundances and the seasonal, daily, and diel temporal distributions of triactinomyxons in the Madison River and Willow Creek; I collected triplicate 120-L samples at five sites in the upper Madison River system and at one site on Willow Creek, Montana, every two weeks from May through November 2002 and March through July 2003. I quantified triactinomyxon concentrations using the packed-bed filtration methods described in the previous section. Triactinomyxon concentrations were examined in two ways. First, the concentrations of triactinomyxons per liter of sampled stream water were estimated. Subsequently, the concentrations per liter were multiplied by mean daily instantaneous stream discharge to estimate triactinomyxon abundances passing the study site each second. I tested for significant differences in triactinomyxon concentrations per liter and abundances per second among sites and dates using multiple-factor analysis of variance (SAS version 8.2). Significant differences among sites and dates were distinguished using Bonferroni's multiple comparisons test. For all tests, significance was set at $\alpha = 0.05$.

The dates of peak triactinomyxon concentrations differed among sites. Therefore, relationships between the timing of peak triactinomyxon concentration and distance below Quake Lake were examined using simple linear regression (SAS version 8.2). Timing of peak triactinomyxon concentrations was determined as the date of highest

triactinomyxon concentrations during Spring and Autumn 2002 and Spring 2003 at each site. Raynold's Pass was only included in this analysis during 2002 because no distinct spring peak at Raynold's Pass was apparent in 2003.

I estimated triactinomyxon concentrations on five consecutive days at the same time of day at all six sites to estimate day-to-day variability in infection risk. I tested for significant differences in triactinomyxon concentrations per liter among days using single-factor analysis of variance (SAS version 8.2). Similarly, I sampled every 4 hours for 24 hours at each of the sites beginning and ending at noon to evaluate diel cycling of triactinomyxon concentrations. Diel patterns in triactinomyxon concentrations were examined by calculating the mean and SE of the 24-hour triactinomyxon concentrations and comparing these to each 4-hour sample concentration. Concentrations greater than 3 SE above the daily mean were deemed substantially higher than the mean (Jeff Banfield, Department of Mathematical Science, Montana State University, personal communication).

Objective 3

To determine if water temperature and stream discharge are correlated with variation in triactinomyxon densities in the wild; The time-series samples collected throughout the season were used to evaluate the effects of temperature and streamflow discharge on triactinomyxon densities. During sampling periods, water temperature was measured with a mercury thermometer and recorded. In addition, United States

Geological Survey gauging stations are located adjacent to the Kirby Bridge site on the Madison and just downstream of the Willow Creek site. These stations recorded water temperature and streamflow every 15 minutes and these data were accessed on the USGS water data website (*http://waterdata.usgs.gov/mt/nwis/sw*). Water temperature during sampling at each of the five Madison River sites was compared with Kirby Bridge gauging station data using simple linear regression (SAS version 8.2). The resulting regression equations were used to predict mean water temperatures during the 24 hours prior to sampling periods for each site. The Willow Creek gauging station discontinued recording temperature in September 2002 because of funding cuts. Therefore, a linear regression of water temperature during sampling periods and mean temperature 24 hours prior to sampling from May to September 2002 was used to predict means for October 2002 and March through July 2003. Relationships between triactinomyxon concentrations and mean water temperatures 24 hours prior to sampling were examined using peak non-linear regression (TableCurve 2D Version 5.01). Peak non-linear regression was used based on the hypothesis that triactinomyxon concentrations would be highest at a range of water temperatures and drop off above and below that range. I also tested for significant correlations between triactinomyxon densities during sampling periods and the mean daily stream discharge using simple linear (SAS version 8.2) and non-linear regression (TableCurve 2D Version 5.01). Additionally, I examined the combined effect of temperature and stream discharge on triactinomyxon abundance using multiple regression (SAS version 8.2).

Objective 4

To assess congruence of measured triactinomyxon concentrations with results of concurrent sentinel-cage fish exposures; E. Richard Vincent of the Montana Department of Fish, Wildlife, and Parks conducted time-series sentinel-cage exposures at the five Madison River sites concurrent with some of my sampling periods in 2002. I collected triplicate triactinomyxon samples once during each exposure period. In addition, I estimated triactinomyxon abundances at Willow Creek parallel with Vincent's cage exposures there beginning in early May 2002 and ending in late June 2002. He conducted four 10-day exposures during that 2-month period. I collected triplicate triactinomyxon samples twice during each 10-day exposure period. I examined relationships between mean triactinomyxon concentrations during exposure periods and the median microscopic pathology grades of the exposed fish using single-factor analysis of variance (SAS version 8.2). I also examined relationships between triactinomyxon densities during exposures and the percentage of fish with microscopic pathology grades of moderate, high, and severe in the exposed sentinel fish (Anderson 2004) using simple linear regression (SAS version 8.2).

RESULTS

Spatial Variability

Mean and peak triactinomyxon concentrations per liter at Willow Creek consistently exceeded those at the Madison River sites. The mean concentration at Willow Creek over all sampling periods (mean = 3.08, SE ± 0.95, range 0.00 to 69.70) was significantly higher than the mean at all five Madison River sites combined (mean = 0.06 SE ± 0.01, range 0.00 to 1.95) ($P < 0.0001$; Figure 4). However, stream discharges at Willow Creek were lower than at the Madison River. Therefore, mean triactinomyxons passing a sampling site per second were not significantly different among the six sites (Figure 4). No significant difference in mean triactinomyxon concentrations per liter and per second existed among the five Madison River sites ($P = 0.6934$; Figure 4). However, the furthest upstream site (North Slide) tended to have a higher mean concentration and more variation than the other Madison sites.

Temporal Variability

Whereas mean triactinomyxon concentrations at the five Madison River sites were similar, seasonal temporal variation in mean triactinomyxon concentrations per liter (Figure 5) and abundances per second (Figure 6) was significant at each Madison River

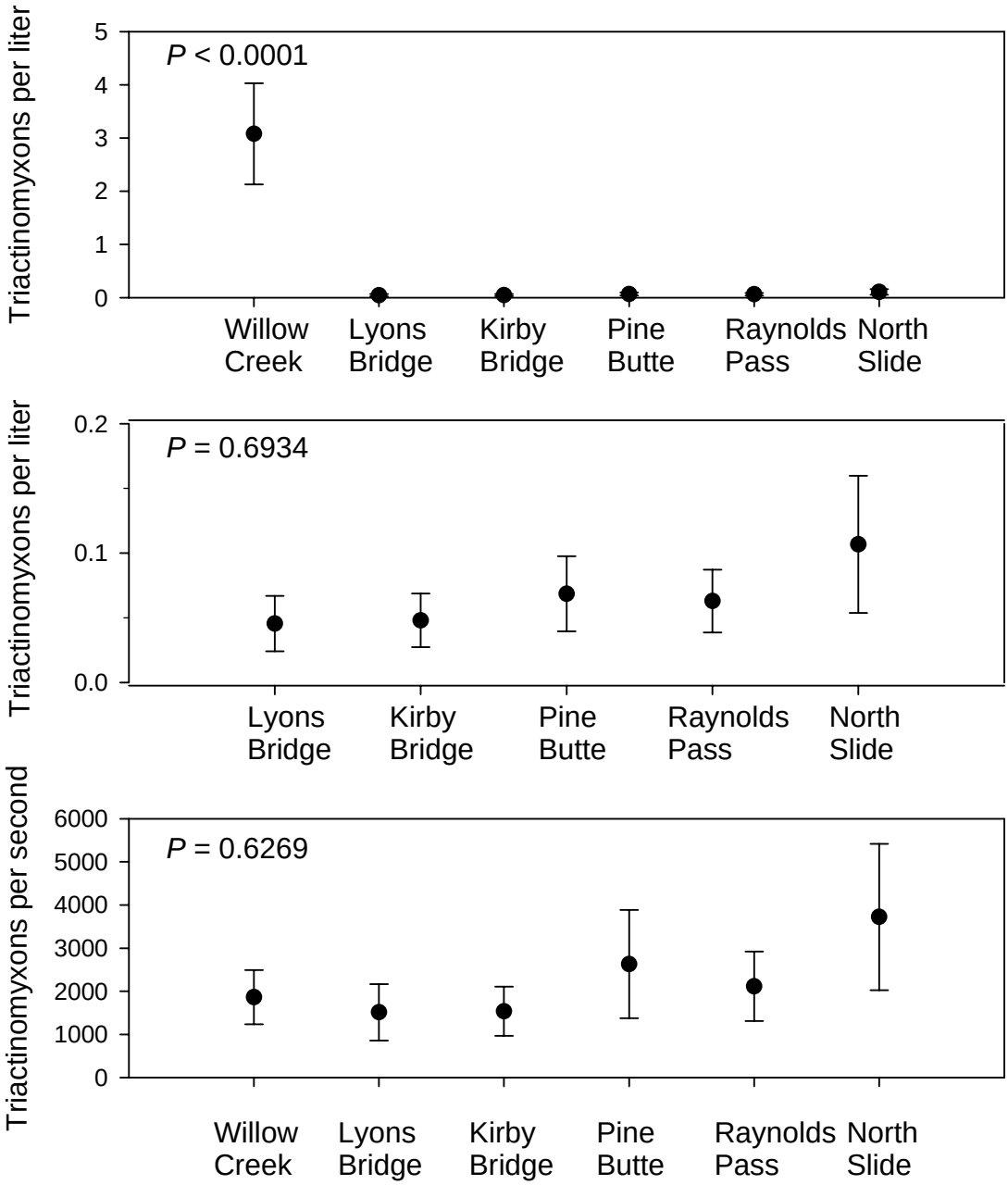

Figure 4. Mean triactinomyxons per liter (± SE) over the seasonal sampling periods at Willow Creek and the Madison River sites (upper), the Madison River sites (center), and the mean triactinomyxons per second (± SE) at Willow Creek and the Madison River sites (lower).

site (all $P < 0.0001$). Also, a significant interaction existed between date and site ($P < 0.0001$). In other words, triactinomyxon concentrations at all five sites fluctuated seasonally, and the temporal fluctuations were asynchronous among sites (Figure 5). Significantly higher concentrations tended to occur during spring (May to July) and autumn (September and October); concentrations at all sites were low (typically absent) during summer (late July through early September) and in early spring (March and April). At most sites, concentrations were higher in Spring 2002 than in Autumn 2002 or Spring 2003 (Figure 5). A similar seasonal pattern was evident at Willow Creek (Figure 7). Triactinomyxon concentrations were highest in May and June 2002, October 2002, and May and June 2003. However, Spring and Autumn 2002 peak concentrations per liter were similar at Willow Creek, and Spring 2003 concentrations were significantly lower (Figure 7). Spring 2002 peak abundances per second were significantly higher than both Autumn 2002 and Spring 2003 (Figure 7) because of high triactinomyxon concentrations in combination with high stream discharge that spring.

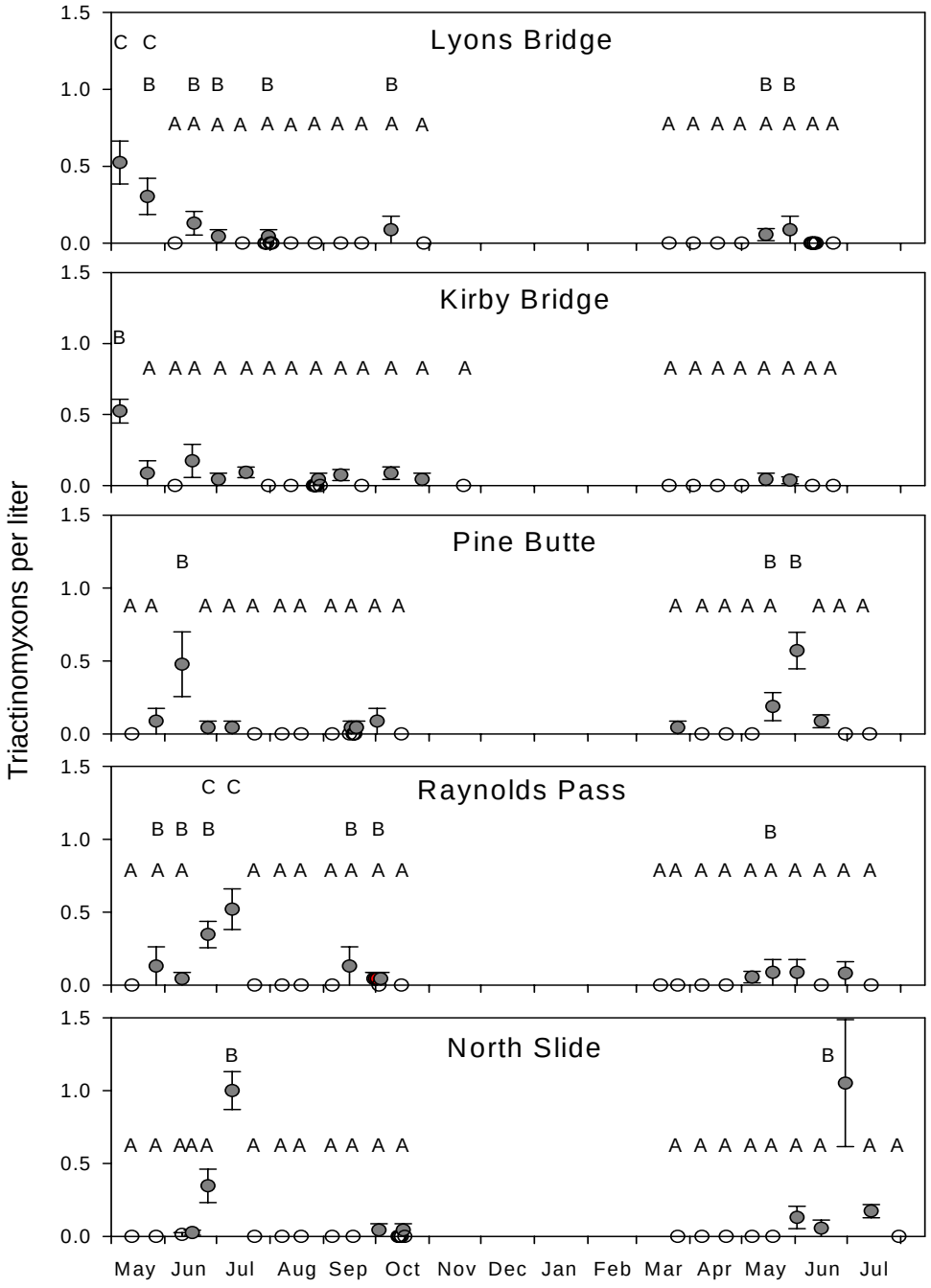

Figure 5. Mean triactinomyxon concentrations (± SE) at five Madison River sites, 2002 and 2003. Means within a site with the same letter are not significantly different. Open symbols denote absence of triactinomyxons. Overlapping symbols represent five consecutive days of sampling associated with the day-to-day variation investigation.

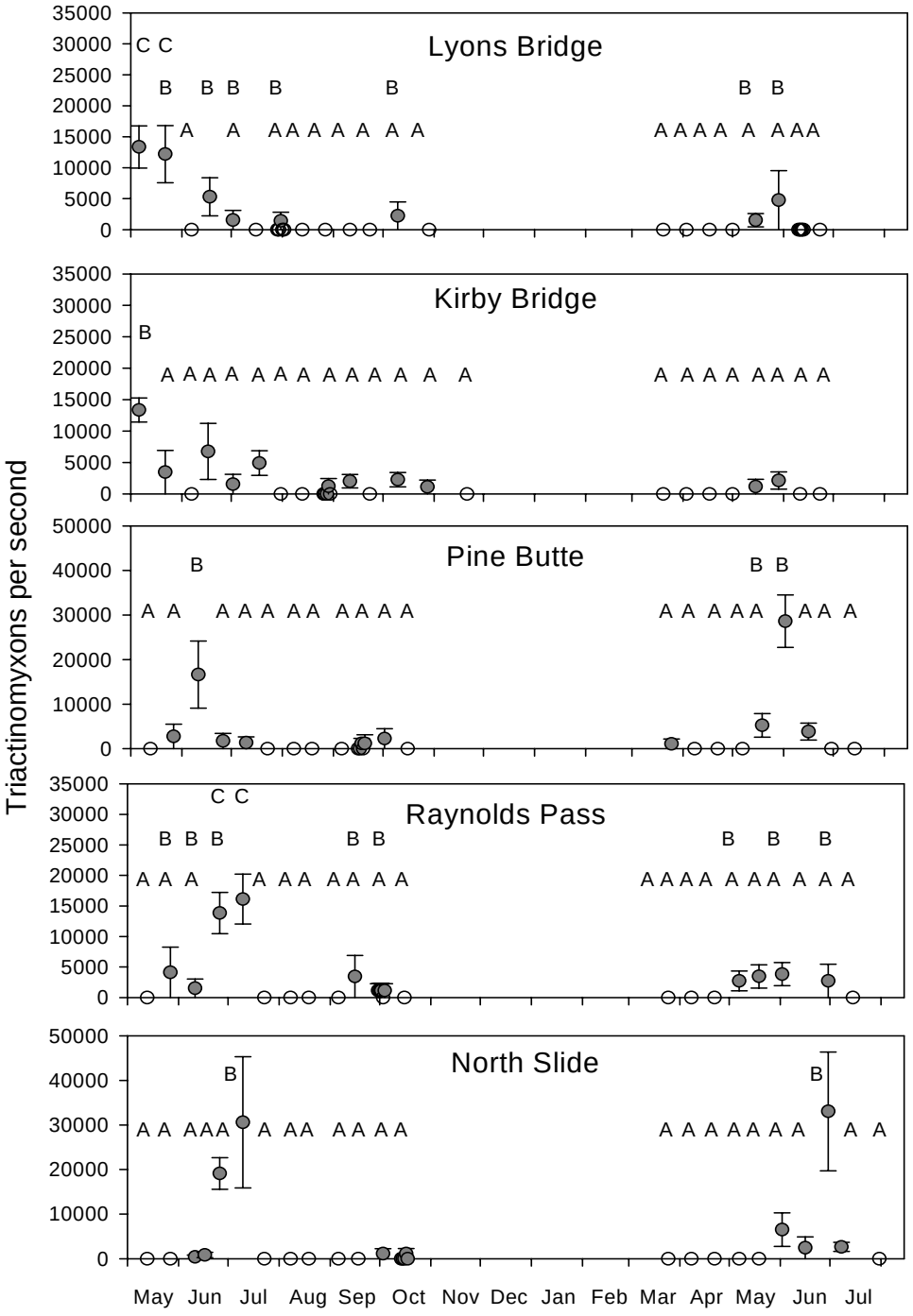

Figure 6. Mean triactinomyxons per second (± SE) at five Madison River sites, 2002 and 2003. Means within a site with the same letter are not significantly different. Open symbols denote absence of triactinomyxons. Overlapping symbols represent five consecutive days of sampling associated with the day-to-day variation investigation.

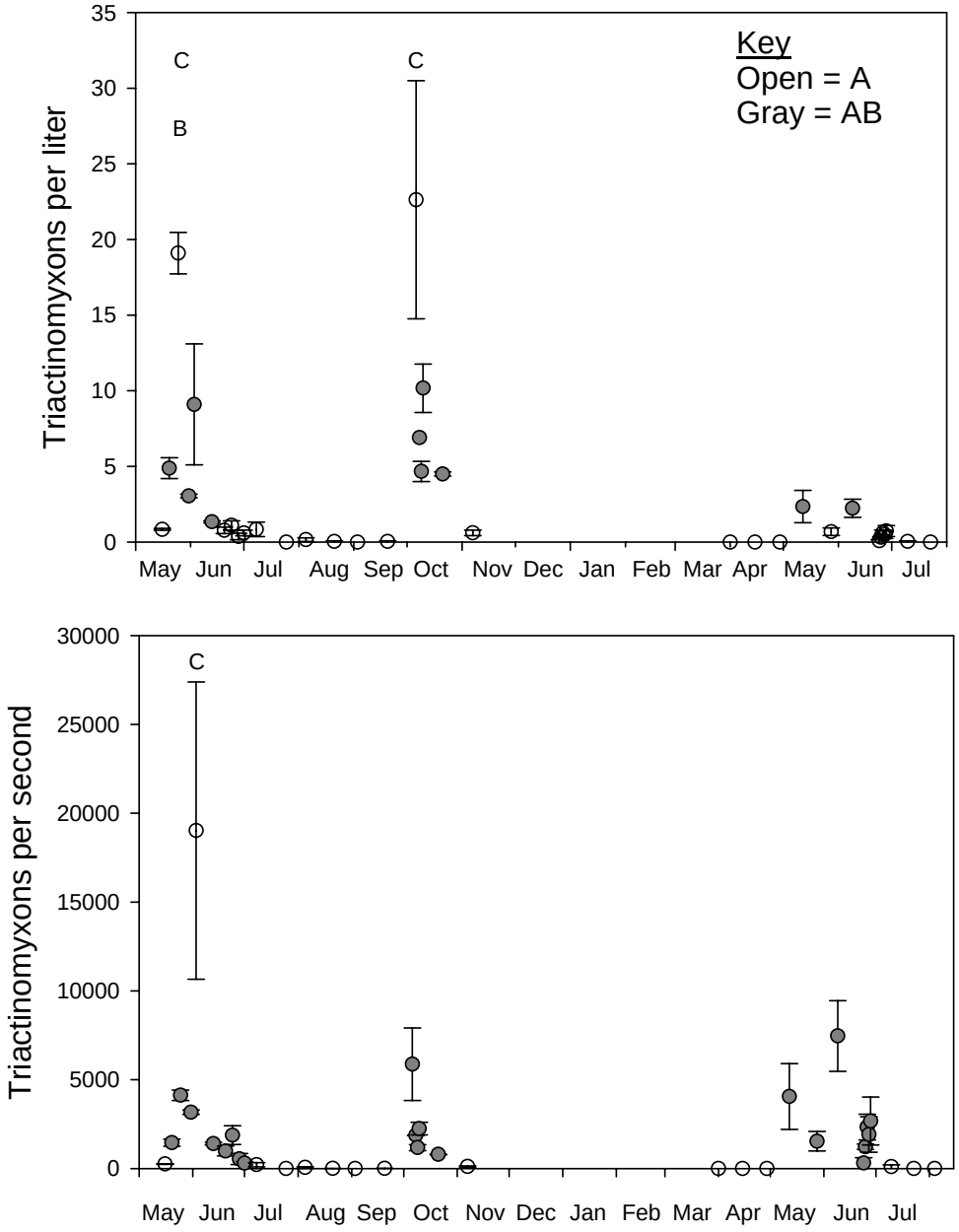

Figure 7. Mean triactinomyxon concentrations per liter (± SE) (upper) and mean triactinomyxon abundances per second (± SE) (lower) at Willow Creek, 2002 and 2003. Means with the same letter within a graph are not significantly different. Unlabeled open symbols are designated A; shaded symbols are designated AB. Overlapping symbols represent five consecutive days of sampling associated with the day-to-day variation investigation.

Triactinomyxons were detected over a broad range of temperatures in my study and no consistent relationship existed between water temperature and triactinomyxon concentrations. At Willow Creek, elevated triactinomyxon concentrations occurred at temperatures between about 5 and 14 ^{o}C, with a calculated peak at 10.7 ^{o}C ($P < 0.0001$; Log normal 4-parameter regression; Figure 8). The mean water temperature during the 24 hours prior to sampling explained 78% of the variation in the data. The same relationship was not evident at the Madison River at all sites in aggregate. Triactinomyxons were detected at temperatures between 3.5 and 17.0 ^{o}C, with a calculated peak at 13.0 ^{o}C ($P <0.0001$; Extra value 4-parameter tailed regression; Figure 8). Although significant, temperature explained only 28% of the seasonal variation in the aggregate data.

Thermal relationships also differed among the five Madison River sites (Figure 9). No significant relationship existed between triactinomyxon concentrations and water temperature at Lyons Bridge and Pine Butte. Non-linear regression did show a significant relationship at Kirby Bridge ($P = 0.013$; Exponentially modified Gaussian regression), Raynold's Pass ($P = 0.0005$; Extra value 4-parameter tailed regression), and North Slide ($P = 0.002$; Exponentially modified maximum power-peak regression), but the calculated peaks occurred at 4.6, 14.5, and 12.9 ^{o}C, respectively.

No significant relationship existed between mean daily stream discharges and triactinomyxon concentrations at any of the six sites ($P > 0.05$). Mean daily stream discharge in combination with the mean water temperature 24 hours prior to sampling

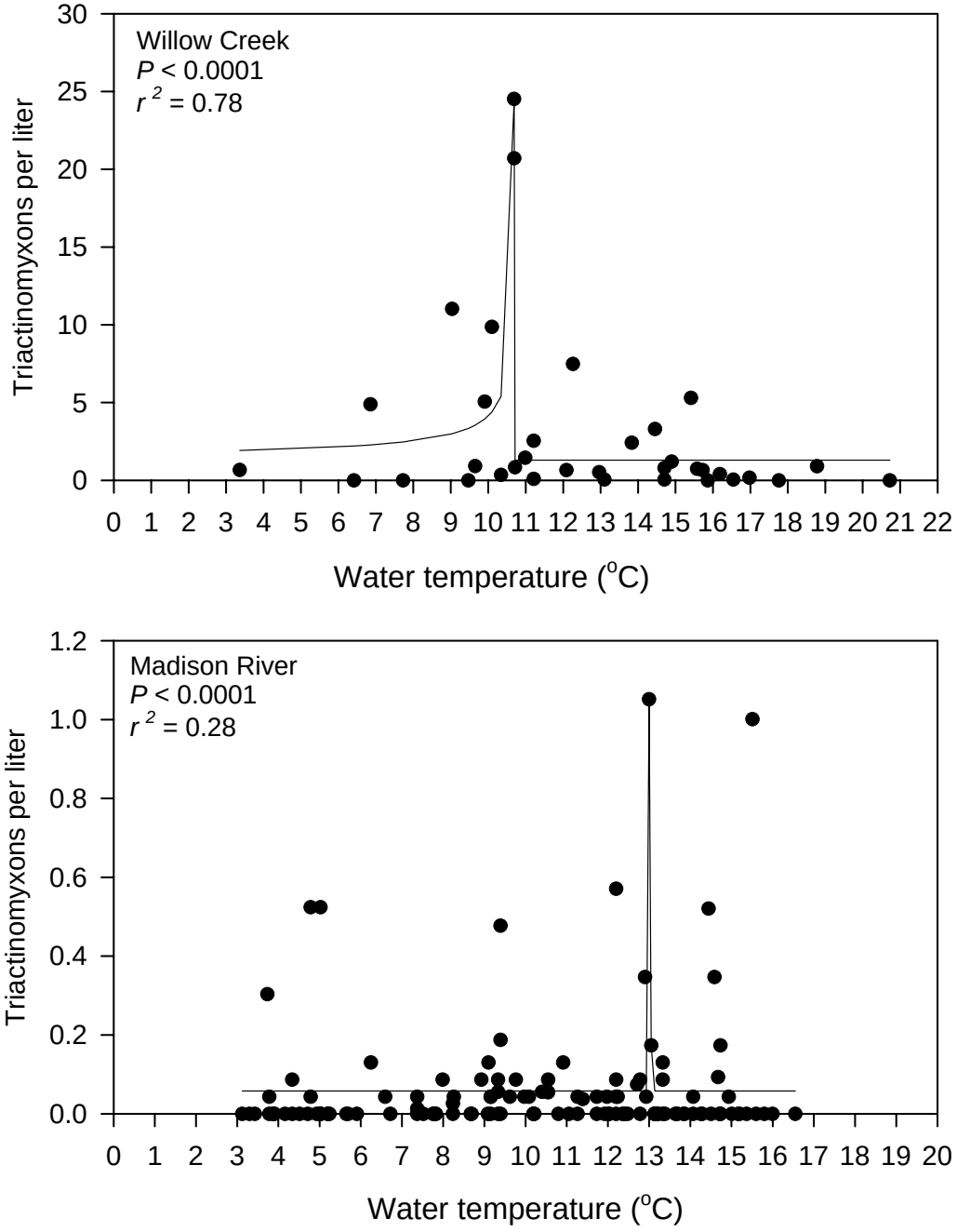

Figure 8. Relationships between triactinomyxon concentrations and mean water temperature 24 hours prior to sampling at Willow Creek (upper) and five sites in aggregate on the Madison River (lower). Curves were fitted using non-linear regression. The calculated peaks occurred at Willow Creek and the Madison River and 10.7 and 13.0 ^{o}C, respectively.

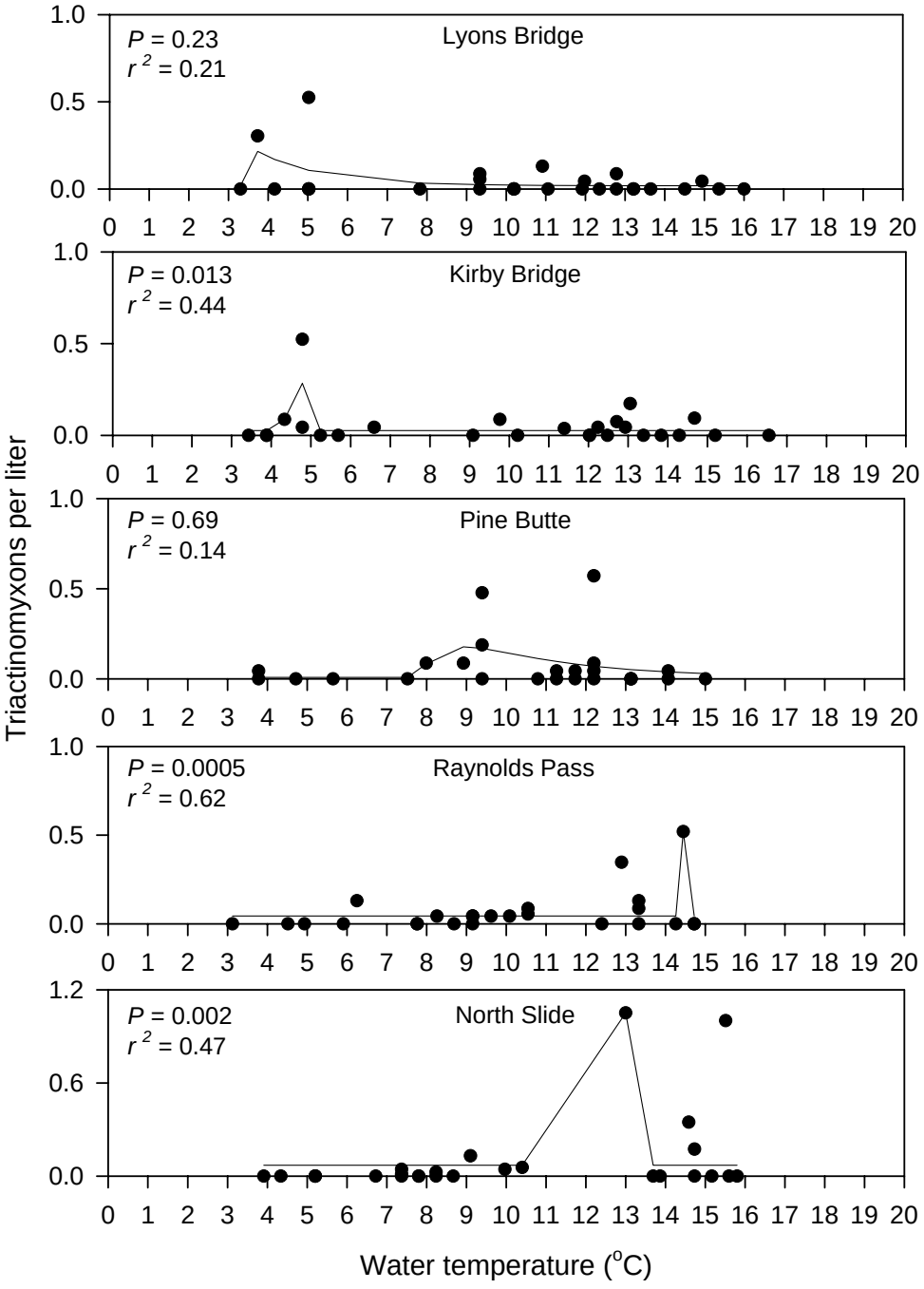

Figure 9. Relationships between triactinomyxon concentration and mean water temperatures 24 hours prior to sampling at five sites on the Madison River.

also did not significantly explain variation in triactinomyxon concentrations when using linear or non-linear multiple regression at all six sites ($P > 0.05$).

During both springs, peak concentrations at all sites tended to occur earlier at the downstream sites than at the upstream sites. This relationship was not evident in autumn. A significant relationship existed between the dates of peak concentrations in the spring and distances below the Quake Lake outlet (Figure 10).

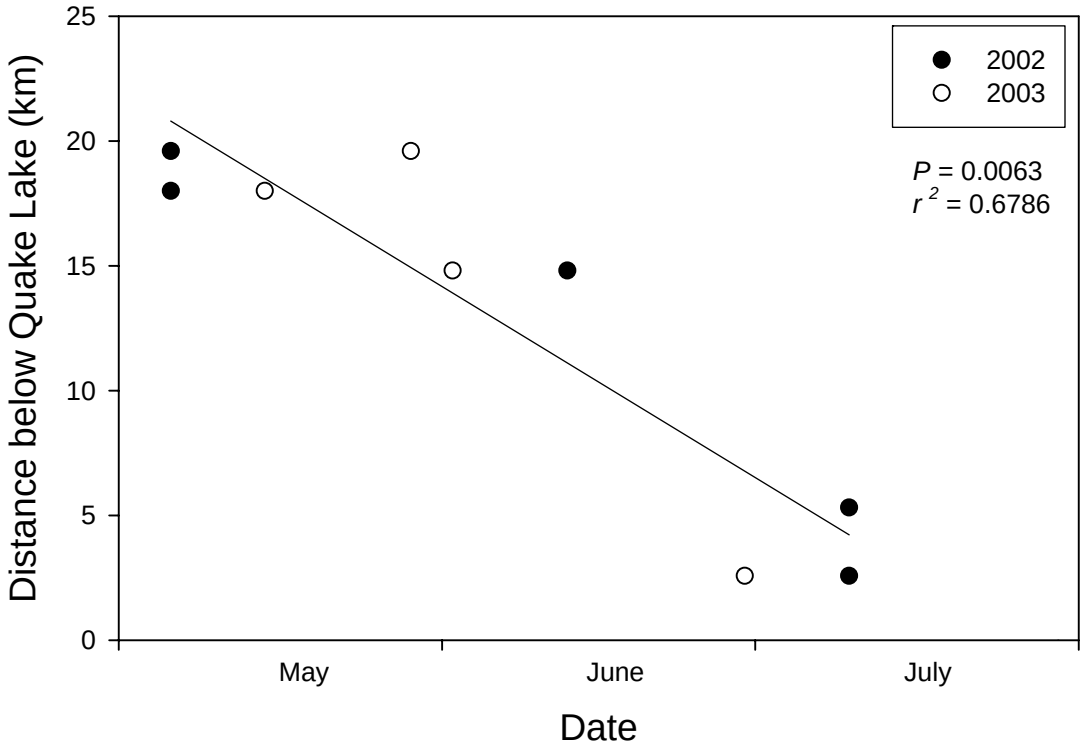

Figure 10. Relationship between the date of peak triactinomyxon concentrations and the distance downstream from the Quake Lake outlet.

Triactinomyxon concentrations varied little among consecutive days. Triactinomyxon concentrations tended to increase slightly over the five-day period at

Willow Creek. However, mean daily triactinomyxon concentrations were not significantly different ($P = 0.33$; Figure 11). Daily triactinomyxons concentrations varied between absence and low levels at the five Madison sites. Mean daily concentrations were not significantly different at any site (all $P > 0.05$; Figure 11).

Diel variation was evident. Triactinomyxon concentrations increased over 3 SE from the daily mean during one time of day at all sites. Triactinomyxon concentrations tended to be higher than the daily mean during the evening, night and morning hours at Willow Creek (Figure 12). Concentrations were consistently low during the afternoon and early evening. This relationship was not apparent at the Madison River sites (Figure 13), where concentrations were often high in the afternoon and early evening and tended not to show distinct diel patterns.

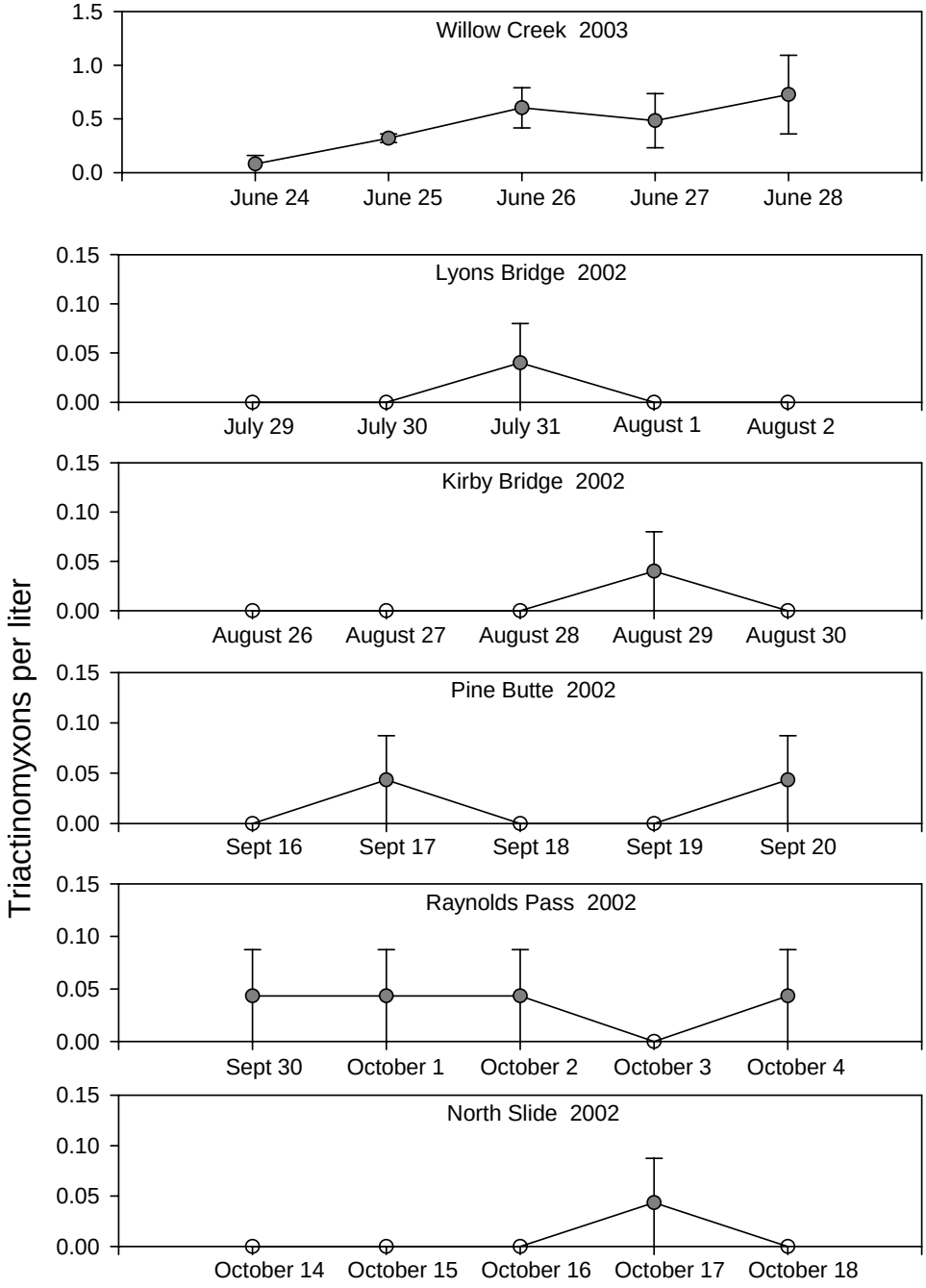

Figure 11. Mean triactinomyxon concentrations (± SE) on consecutive days at Willow Creek and five sites on the Madison River, 2002 and 2003. Open symbols denote absence of triactinomyxons.

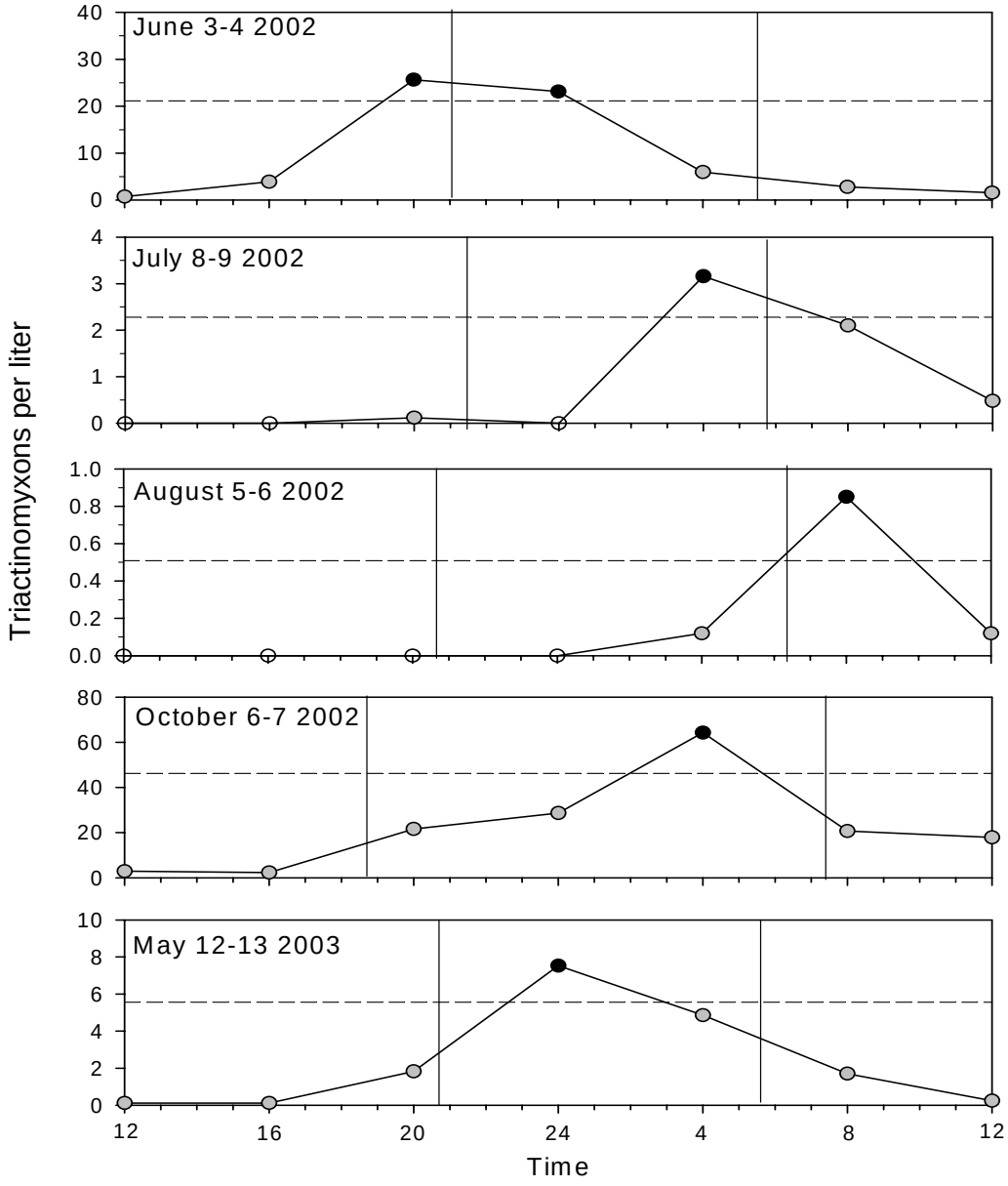

Figure 12. Diel triactinomyxon concentrations at Willow Creek on five dates in 2002 and 2003. Vertical lines are sunset and sunrise. Horizontal dashed line indicates +3 SE from the daily mean. Black symbols are times when concentrations exceeded 3 SE. Open symbols denote absence of triactinomyxons.

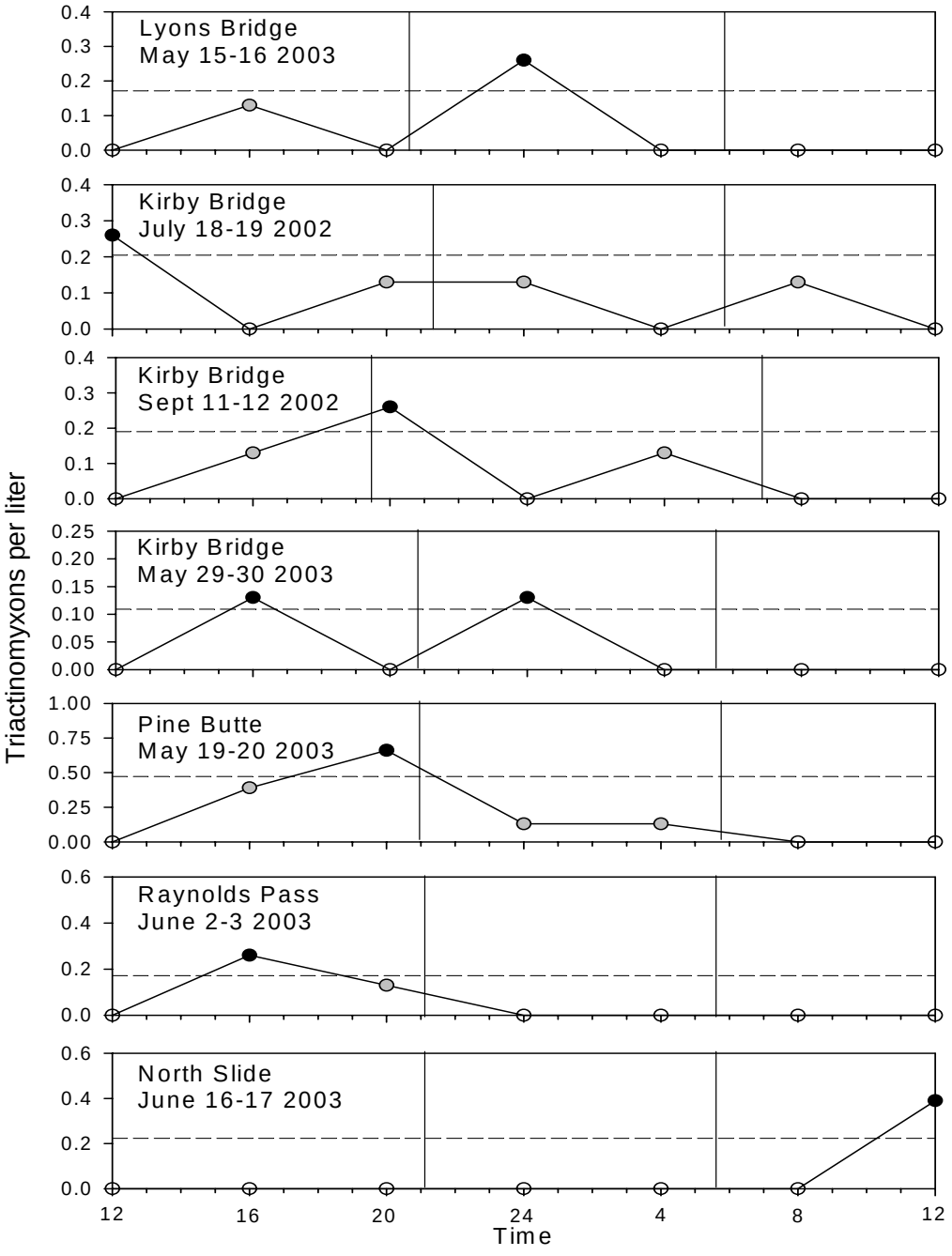

Figure 13. Diel triactinomyxon concentrations at five sites on the Madison River in 2002 and 2003. Vertical lines are sunset and sunrise. Horizontal dashed line indicates +3 SE from the daily mean. Black symbols are times when concentrations exceeded 3 SE. Open symbols denote absence of triactinomyxons.

Triactinomyxon Abundance Compared to Disease Severity

In general, triactinomyxon concentrations in the Madison River were high during sentinel-cage exposure periods that resulted in median microscopic pathology grades of high and severe or when more than 85% of fish in each cage had microscopic histology grades of moderate, high, or severe (Figure 14). However, it was also high during a single period that resulted in a low percentage of fish infected, with a median grade of none, which suggests that factors other than triactinomyxon concentrations may affect infection severity in caged fish.

Triactinomyxon concentrations were high during all cage exposures at Willow Creek. All exposures resulted in median microscopic pathology grades of severe, and greater than 90% of fish in each cage had pathology grades of moderate, high, or severe. Combined Willow Creek and Madison River data again showed that triactinomyxon concentrations were highest during exposures that resulted in severe median microscopic pathology grades and when greater than 85% of caged fish had pathology grades of moderate, high, or severe (Figure 15). Although not statistically significant ($P = 0.0562$), these combined data reinforced the relationship between triactinomyxon concentrations and microscopic pathology grades.

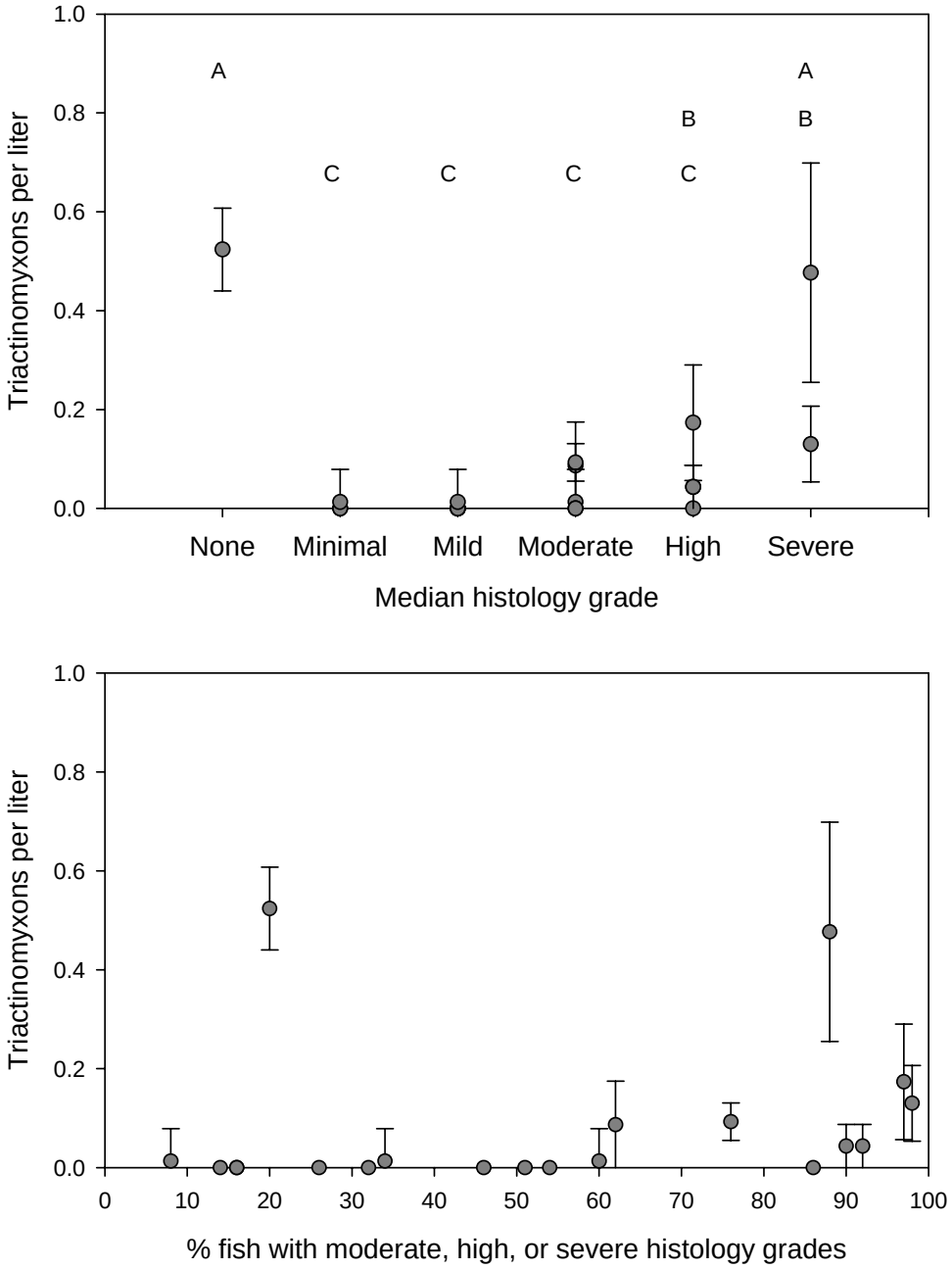

Figure 14. Relation between triactinomyxon concentrations (± SE) during exposures and median histology grades of sentinel fish (upper). Means with the same letter are not significantly different. Relation between triactinomyxon concentrations (± SE) during exposures and the percentage of fish with histology grades of moderate, high, or severe in each cage (lower), Madison River.

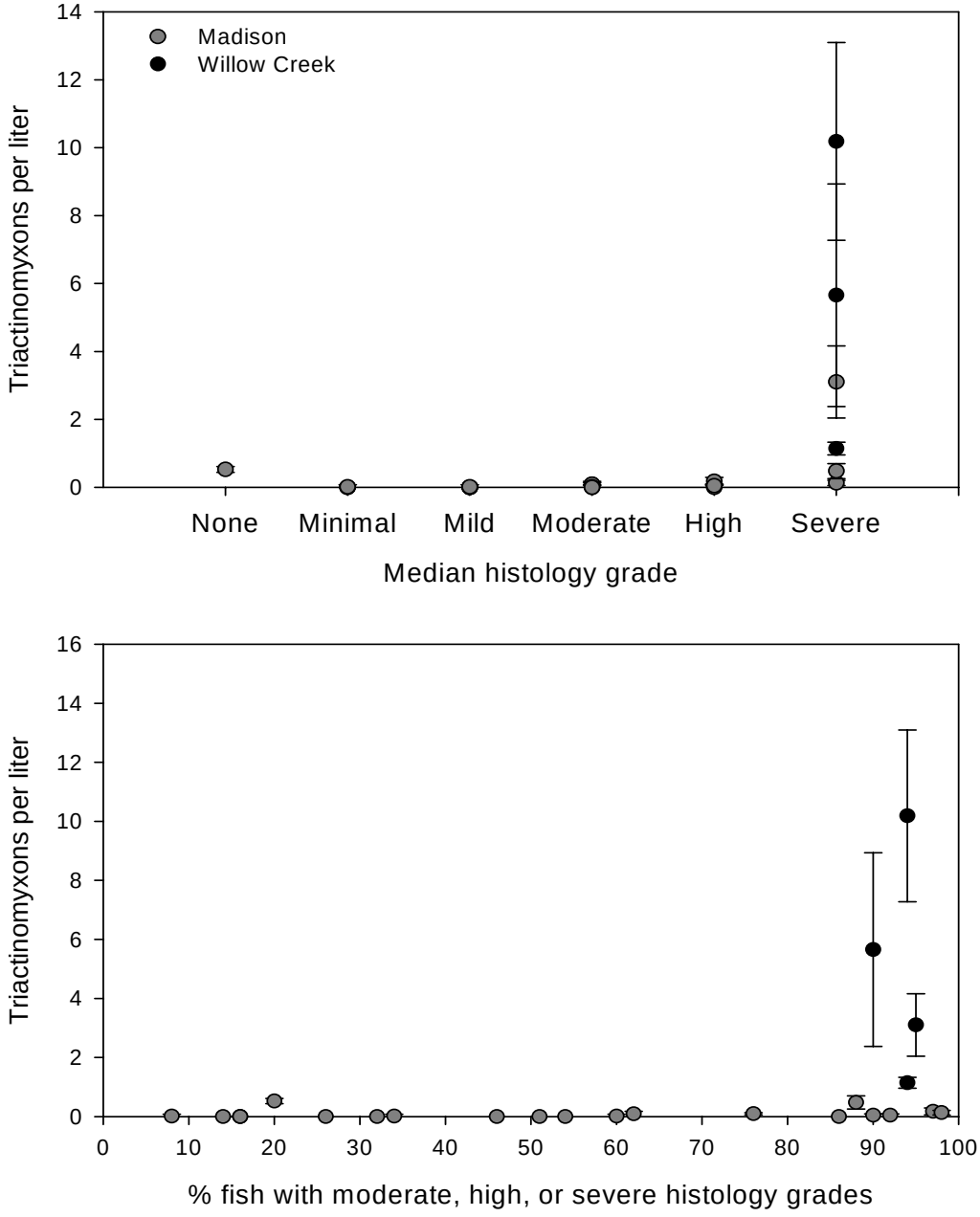

Figure 15. Relations between triactinomyxon concentrations (± SE) and median histology grades of sentinel fish (upper), and triactinomyxon concentrations (± SE) and the percentage of fish with histology grades of moderate, high, or severe in each cage (lower), Madison River and Willow Creek combined.

DISCUSSION

Spatial Variability

Mean and peak triactinomyxon concentrations at Willow Creek consistently exceeded those at the Madison River sites. These findings conform with expectations based on previous studies in these systems. Variation in the density of *Tubifex tubifex* and amount of *M. cerebralis* myxospore loading are likely mechanisms leading to higher triactinomyxon concentrations in one area but not another (Zendt and Bergersen 2000; Kerans and Zale 2002). Willow Creek is a degraded and organically enriched stream with livestock grazing and agricultural activity in the watershed (personal observation). These conditions have been shown to encourage an abundance of *T. tubifex* and to decrease aquatic oligochaete species diversity (Zendt and Bergersen 2000; Sandell et al. 2001). Willow Creek also has a high prevalence of *M. cerebralis*-infected wild trout (Baldwin et al. 2000) and *T. tubifex* (Rognlie and Knapp 1998). Myxospores are released into sediments upon death of infected salmonids (Hedrick et al. 1998) and are dispersed and deposited like other fine sediments in streams. Low flows and numerous beaver dams above the Willow Creek site facilitate sediment and myxospore deposition. High infections can coincide with these conditions. For example, sentinel fish exposed in the South Fork of the Boise River developed severe infections at a site containing high amounts of fine sediment immediately downstream of a beaver dam (Hiner and Moffit 2001). Also, the percentage of *T. tubifex* releasing triactinomyxons was positively

correlated to the relative abundance of fine sediments and their content of organic material and negatively correlated to water velocity (Krueger 2002).

Willow Creek is a lower order stream than the Madison River. The Madison River drains an area of 6475 km^2 and Willow Creek drains an area of only 217.04 km^2. High concentrations of triactinomyxons in combination with lower discharge caused the mean triactinomyxons per second at Willow Creek to be similar to mean triactinomyxons per second at the five Madison River sites. However, wild trout in Willow Creek would probably have a higher chance of contact with triactinomyxons because of their higher concentrations there.

We expected triactinomyxon concentrations to differ among the individual Madison River sites. Drainage-wide studies using sentinel cages have shown variation in disease severity on similar spatial scales. For example, the percent of *M. cerebralis*-positive fish varied widely over a 28-km reach of the Lostine River, Oregon (Hiner and Moffitt 2001), and over a 25-km section of the South Fork of the Boise River, Idaho (Sandell et al. 2001). Triactinomyxon concentrations varied significantly between two spring creeks in Wyoming and concentrations declined with upstream progression within each creek (Hubert et al. 2002). However, no difference in mean or peak triactinomyxon concentrations existed among the five Madison River sites. This finding corresponds with analyses of sentinel-cage trials that found no significant differences in mean microscopic pathology grades among study reaches on the Madison River when data from multiple cages at sites were combined within months, even though grades varied widely among microhabitats and among dates at each site (Downing et al. 2002). Lack of

spatial variation in infection severity was also found among eight sites distributed along a 30-km reach of Little Prickly Pear Creek, Montana (R. Vincent, Montana Fish, Wildlife and Parks, Bozeman, unpublished data). Spatial variation in parasite prevalence, or lack thereof, could be explained by numerous factors such as widespread or patchy myxospore availability, and variation in abundances of susceptible worm and fish hosts.

Temporal Variability

Seasonal variation in triactinomyxon concentrations paralleled inferences from sentinel-cage studies on the Madison River (Vincent 1999; Downing et al. 2002). Triactinomyxon concentrations were higher during spring and autumn than during summer months. Moreover, I observed higher mean concentrations of triactinomyxons at the Madison River sites (but not at Willow Creek) during spring than in autumn, again consistent with findings of Downing et al. (2002). I also observed annual variation in triactinomyxon concentrations, similar to variation in disease severity (Downing et al. 2002). At most sites, peak concentrations were higher in Spring 2002 than in Spring 2003. I did not observe annual shifts in timing of peak infectivity as reported by Downing et al. (2002), but such shifts likely correspond to specific annual differences in factors that elicit triactinomyxon release.

Seasonal timing of infection risk in these systems was different from that previously reported in other systems. Disease severity in sentinel fish exposures in Montana spring creeks was highest in the winter and early spring (Anderson 2004).

Disease severity of sentinel fish was highest in June, July, and August on the Lostine River, Oregon (Hiner and Moffitt 2001), and triactinomyxons were most abundant throughout the summer and early fall in the upper Colorado River drainage (Thompson and Nehring 2000). Timing of infection risk may be related to system-specific factors such as *T. tubifex* maturity cycles or the availability of viable myxospores (Markiw 1986).

Water temperature has been shown to correlate with infection intensity of rainbow trout in several sentinel-cage studies. However, triactinomyxons were detected over a broad range of temperatures in my study. At Willow Creek, elevated triactinomyxon concentrations occurred at temperatures between about 5 and 14 ^{o}C, with a calculated peak at 10.7 ^{o}C. Concurrent water temperatures did not explain the seasonal variation in triactinomyxon concentrations at the Madison River sites. My findings are similar to results of Anderson (2004) where infection in fish exposed in spring creeks was detected at temperatures ranging from 4 ^{o}C to 14 ^{o}C and in rivers from 0.1 ^{o}C to 17.5 ^{o}C with peak infection occurring between 6-12 ^{o}C. Other studies have also shown inconsistent relationships between disease severity and water temperatures. Vincent (2002a) found highest infection intensities occurred at water temperatures between 12 and 16 ^{o}C and Baldwin et al. (2000) found disease severity was positively correlated with water temperatures of 6-11 ^{o}C at Willow Creek, Montana. The severity of infection in rainbow trout was not related to mean water temperatures in Idaho (Hiner and Moffitt 2001). Other factors, such as accumulated degree days, myxospore availability, and day length,

perhaps in combination, may be responsible for the variation in triactinomyxon concentrations I saw.

Spring peak concentrations at the Madison River sites tended to occur earlier at the downstream sites, suggesting that accumulated degree days may be an important determinant of the timing of triactinomyxon release. Myxospores may be available seasonally for *T. tubifex* to ingest after spring and autumn spawning because of increased mortality of infected trout (Kerans and Zale 2002). Water temperature influences the development time of the parasite to the triactinomyxon stage in *T. tubifex*. Triactinomyxons were released 170 days post-exposure from *T. tubifex* reared at 8 ^{o}C (R. Stevens and B. L. Kerans, unpublished data), whereas those reared at 15 ^{o}C released triactinomyxons at 90 days post exposure (El-Matbouli and Hoffmann 1998). In both studies, triactinomyxons were released between 1,320-1,360 degree days. Therefore, slight differences in temperatures among sites may influence the date of triactinomyxon release. Water temperatures among the Madison River sites were similar throughout the spring, summer, and autumn, but winter temperatures at the upstream sites were consistently colder and less variable than the downstream sites because of the influence of surface discharges from ice-covered Quake Lake.

Previously, stream discharge was inferred to affect disease severity of fish both in the laboratory and in a Madison River field study. As discharge increased, disease intensity decreased, apparently as the result of dilution (Vincent 2002b). Thompson et al. (1999) suggested that higher water velocities associated with higher flows might provide a more efficient suspension of triactinomyxons in the water column. However, we saw

no relationship between stream discharges and triactinomyxon concentrations. The timing of peak triactinomyxon concentrations among sites occurred before, during, and after peak spring runoff.

Packed-bed filtration provided an instantaneous assessment of triactinomyxon concentrations and therefore also of infection risk experienced by wild fish. However, disease severity in wild fish is assumed to reflect continuous and cumulative exposure to ambient triactinomyxon concentrations. Accordingly, an understanding of temporal dynamics of triactinomyxon concentrations is necessary to determine appropriate time scales over which concentrations should be sampled. We observed no day-to-day variation in triactinomyxon concentrations over five day sampling periods, suggesting that daily sampling is not necessary and sampling once a week would be sufficient to estimate infection risk to wild fish. However, diel variation was evident at all sites. At Willow Creek, triactinomyxon concentrations were elevated during periods of low light intensity, similar to common patterns of aquatic invertebrate drift, which typically peak at dusk (Elliot 1967; Collier and Wakelin 1992). Such crepuscular activity is typically considered to be an anti-predator response (Holt and Waters 1967). No clear diel pattern was evident at the Madison River sites. However, the study reach of the Madison River has numerous side channels, which may be source areas for triactinomyxons (Krueger 2002). Once released from *T. tubifex*, triactinomyxons are neutrally buoyant and float freely in the water column. The distance downstream triactinomyxons travel is unknown but may be limited only by predation and physical agitation. Triactinomyxons collected

at a Madison River site during one time of day may have been released far upstream hours earlier.

Compensation for diel variation could be achieved by continuously collecting water to be filtered from a site over a 24-hour period. This composite sample could be filtered through the packed-bed filter at the end of the 24-hour period to get a complete assessment of triactinomyxon concentrations experienced by wild fish during that period. I developed a siphon for such sampling, but did not have the opportunity to test it during this study.

Triactinomyxon Abundance Compared to Disease Severity

In general, triactinomyxon concentrations were high during periods that corresponded to median microscopic pathology grades of high or severe and that resulted in greater than 85% percent of fish with microscopic histology grades of moderate, high, or severe. Packed-bed filtration is therefore likely a suitable substitute for sentinel cages. However, this relation did not hold true for one paired packed-bed and sentinel-cage sample period on the Madison River. Low infection in caged fish resulted from that exposure, despite a relatively high concurrent triactinomyxon concentration. Packed-bed filtration provides an instantaneous assessment of triactinomyxon concentrations, whereas disease severity of sentinel fish reflects cumulative exposure to ambient triactinomyxon concentrations during the exposure period. Variation in triactinomyxon concentrations allows the possibility that triactinomyxons were high when I sampled and low or absent during the remainder of that exposure period. Also, other myxozoan

species with actinospores similar to *M. cerebralis* triactinomyxons may be present in the Madison River (Billie Kerans, personal communication). Similar actinospores may have been quantified along with *M. cerebralis* triactinomyxons during this exposure. Water temperature during that sample period (5 ºC) was the lowest for which we had paired samples, suggesting that triactinomyxon concentrations may not reflect infection risk for sentinel-caged fish at low water temperatures. Furthermore, sentinel cage exposure results may differ among sentinel trout strains, sizes, ages, and physiological conditions. Slight differences in fish sizes and ages can affect disease severity significantly (Ryce 2003; Ryce et al. in press). Therefore, comparisons of triactinomyxon concentrations to sentinel fish exposures are potentially confounded by these variables.

Packed-bed filtration proved to be a rapid, efficient, and effective method for direct quantification of triactinomyxon abundances in the field. It will be a useful tool to examine relations between actual triactinomyxon abundances and factors that affect disease severity in wild fish. It is an easy diagnostic tool for determining concentrations of the parasite in different systems and at different times. It will be useful for evaluating how habitat and water quality conditions (degraded habitats, high *Tubifex* densities, myxospore deposition sites, altered discharge and thermal regimes, etc.) affect infection intensity. Accordingly, the filtration system can be used to test the efficacy of various management strategies and techniques designed to develop practical management solutions that maintain viable, self-sustaining wild trout fisheries.

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