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A Simple Technique Used to Filter and Quantify the Actinospore of *Myxobolus cerebralis* and Determine Its Seasonal Abundance in the Colorado River

KEVIN G. THOMPSON

Colorado Cooperative Fish and Wildlife Research Unit, Colorado State University, 201 Wagar Building, Fort Collins, Colorado 80523, USA

R. BARRY NEHRING*

Colorado Division of Wildlife, 2300 South Townsend, Montrose, Colorado 81401, USA

Abstract.—We used a simple technique for filtering the actinospores of *Myxobolus cerebralis* from natural waters to observe seasonal periodicity at eight sites in the upper Colorado River drainage. We used a tub lined with 20-μm-mesh Pecap screen to concentrate actinospores from 1,900-L samples and estimate density by microscope count. Identity of the observed actinospores as those of *M. cerebralis* was confirmed in 86 samples by the use of a polymerase chain reaction test. The 42-ha Windy Gap Reservoir appeared to be a point source of actinospores; the highest densities observed were consistently from samples taken at sites just below the reservoir. Both densities and the frequency of detection were much lower 26 km below the reservoir. The actinospores first appeared in abundance after the runoff in both years of the study. Actinospore densities tended to be greatest during summer and early fall and lowest during spring. In August 1997, a series of significant flow fluctuations and attendant water temperature swings appeared to alternately inhibit and stimulate the release of actinospores. Populations of rainbow trout *Oncorhynchus mykiss* continue to suffer recruitment failures throughout the study reach, apparently because of the effects of whirling disease in age-0 fish. This suggests that the detection of low numbers of actinospores by this technique at some sampling locations may indicate a level of infectivity that is destructive for the susceptible rainbow trout.

Wolf and Markiw (1984) first demonstrated that *Myxobolus cerebralis,* the salmonid parasite that can cause whirling disease, has a two-host life cycle that alternates between salmonid fishes and oligochaete worms and produces spores in each host that can only infect the other host. (Hereafter, we refer to the spore of *M. cerebralis* as myxospore and the triactinomyxon spore as actinospore [Lom et al. 1997]). This life cycle was independently confirmed by El-Matbouli and Hoffmann (1989). Recently, Andree et al. (1997) used DNA-based techniques to further demonstrate that the alternating myxospore and actinospore stages are the same organism. El-Matbouli and Hoffmann (1998) detailed the development of the organism in the worm host from the myxospore stage to the actinospore stage by the use of light and electron microscopes. These studies firmly established that the actinospore is *M. cerebralis*; consequently, detecting this actinosporean in a natural water indicates that *M. cerebralis* is enzootic in that water.

Although *M. cerebralis* was once considered a threat only in cultured fish environments, recent studies and observations in Colorado, Utah, and Montana have shown that whirling disease can affect wild salmonid populations. The presence of whirling disease and the concomitant collapse of recruitment of rainbow trout *Oncorhynchus mykiss* have been documented in several streams in the intermountain west (Walker and Nehring 1995; Nehring and Walker 1996; Vincent 1996; Nehring et al. 1998). The parasite was first detected in the upper Colorado River drainage in 1988 among hatchery rainbow trout stocked in the fall of 1987 on private property at two separate locations (Walker and Nehring 1995). Wild adult rainbow trout in the Colorado River first tested positive for the parasite at the annual disease inspection during egg-taking operations in April 1992 (Walker and Nehring 1995).

Unfortunately, little is understood about the ecology of the parasite in the wild. Do localized areas of infectivity influence wider areas of some streams? What densities of the actinospores of *M. cerebralis* are sufficient to cause the impacts on salmonid populations observed in the Colorado River? Are there seasonal patterns to the release

* Corresponding author: barry.nehring@state.co.us

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of actinospores from the tubificid host? Because of the work of those who demonstrated the alternating stages of the life cycle of *M. cerebralis* (Wolf and Markiw 1984; El-Matbouli and Hoffmann 1989), we hypothesized that it should be possible to identify enzootic areas and examine the seasonal dynamics of the parasite in natural settings by observing the patterns of release of the actinospore from the tubificid host. Use of water filtration techniques to detect actinospores could also provide a nonlethal alternative to fish sampling to establish the distribution of *M. cerebralis*.

Our objectives in the present study were twofold: first, to develop a simple method for filtering and quantifying the actinospores of *M. cerebralis* from riverine water samples, and second, to use the filtration method to observe the production of actinospores of *M. cerebralis* at eight locations in the upper Colorado River drainage, Grand County, Colorado.

Methods

Study area.—The study area encompassed 28 km of the main-stem Colorado River and the Fraser and Williams Fork rivers, just upstream of their confluences with the Colorado River (Figure 1). Windy Gap Reservoir, a shallow, 42-ha impoundment on the Colorado River near the upper end of the area, is about 1 km downstream of the Fraser River confluence.

Of the eight sampling sites selected (Figure 1), three were above Windy Gap Reservoir. These were Horn Ranch on the Colorado River above the Fraser River confluence, Fraser River above the Colorado confluence, and Above Windy Gap on the Colorado River below the Fraser River confluence. Two were immediately below Windy Gap Reservoir in the Spill Basin and in the North Outlet, a separate channel from the Spill Basin. One site was 1.8 km downstream of Windy Gap Reservoir at the Hitching Post Bridge. The remaining two sites were about 26 km downstream of Windy Gap Reservoir at Breeze Bridge on the Colorado River and on the Williams Fork River 2 km above its confluence with the Colorado River.

Discharge data for the Colorado River at the Hitching Post Bridge and the Williams Fork River below Williams Fork Reservoir were obtained from U.S. Geological Survey Water Resources Data publications (Crowfoot et al. 1998, 1999). Discharge data for the Fraser River and for the Windy Gap Reservoir Spill Basin and North Outlet were obtained from the Northern Colorado Water Conservancy District. Discharge for the Colorado River at Horn Ranch was derived by subtracting Fraser River discharge from Hitching Post discharge. Breeze Bridge discharge was approximated by Williams Fork discharge added to Hitching Post discharge. The contributions of tributaries between Hitching Post and Breeze Bridge are inconsequential during fall and winter, and they are negated by agricultural irrigation over the same reach during late spring and summer.

The Above Windy Gap site was sampled in August and September 1997 and July and August 1998. The Spill Basin site was sampled at least monthly except when prevented by ice cover. The other six sites were sampled at least monthly from April 1997 through December 1998.

Water filtration.—The filter used for this work consisted of 20-μm-mesh Pecap screen material (AREA, Inc., Homestead, Florida) fitted inside a heavy plastic utility tub measuring 66 cm long, 51 cm wide, and 15 cm deep. The sides and bottom of the utility tub were perforated with 13-mm holes to maximize water passage efficiency. Filter screens matched the dimensions of the tub and were fitted with an elastic collar for secure attachment to the tub.

Methods used to deliver water to the filter were simple bucketing, siphons, and battery-operated bilge pumps. For bucket samples, 19-L buckets of river water were poured through the filter until the desired volume had been filtered. For siphon and bilge pump samples, the rate of flow was determined by timing a known volume several times, and the average fill rate was used to compute the required time of operation for the siphon or bilge pump to deliver the desired volume. On each filtering occasion, data recorded included site, water temperature, amount filtered, amount of filtrate, date, time of day, and method of filtration.

After the entire volume of water was passed through the filter, wash bottles were used to thoroughly rinse the screen and move all filtrate into one corner of the filter tub. The sample was then rinsed through a funnel into a sample jar, resulting in a concentrated filtrate ranging from 40 to 550 mL. Filtrates were kept cool during transport and examined by dissecting microscope (40–80×) for actinospores within 24 h of collection.

Before examination, filtrates were thoroughly mixed to resuspend organic and nonorganic matter, allowed to settle for 20–30 s to eliminate the heaviest debris, and then subsampled 20 times by pipetting 1-mL volumes into plastic scintillation vials. The process was then repeated, adding another 1-mL volume to each vial in reverse order, in an

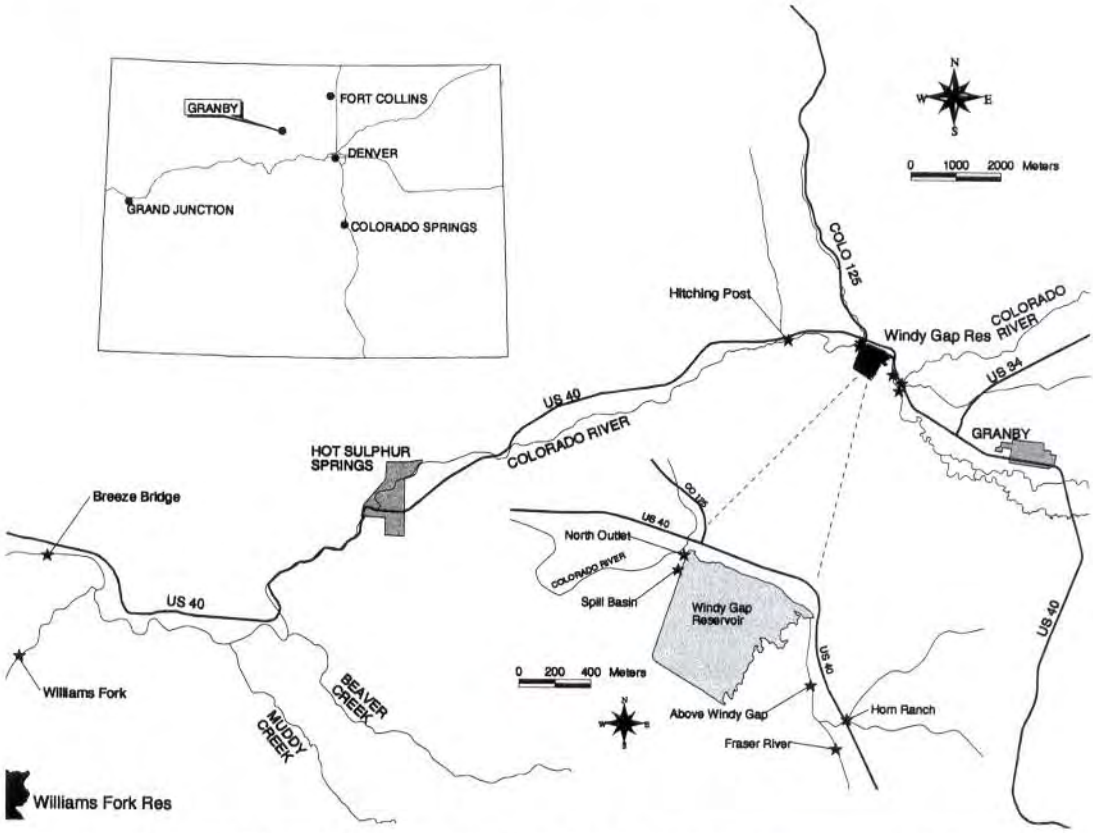

FIGURE 1.—The upper Colorado River study area. Stars show the locations of sampling sites. Abbreviations are: CO 125 and COLO 125, Colorado state highway 125; US 40, U.S. Route 40.

effort to reduce variability of actinospore densities that could arise from possible settling of actinospores. Each resulting 2-mL aliquot was stained with 60 or 120 μL of an aqueous solution saturated with crystal violet. Each aliquot was mixed again before one 82.4- or 84.8-μL sample, respectively (80 μL = actual river water volume), was withdrawn and placed on a petri dish with a grid under a 24 × 40 mm cover slip. All grids were examined for actinospores, and the average number counted in the 20 subsamples was used to generate an estimate of actinospore density in the water that was filtered.

On two occasions we sequentially filtered four samples of 190, 570, 1,135, and 1,900 L at a single site. These samples were compared for each occasion by analysis of variance to see whether there was any effect of filtering differing quantities of water on the resulting actinospore density estimate.

Data analysis.—We used actinospores per liter and actinospores per day as response variables to examine the influence of several factors on the production of actinospores. Independent variables considered in the modeling were site, year, season of the year, flow (only for actinospores per liter), water temperature, and water temperature squared.

From October 1997 to December 1998, 127 filtered samples were tested for the presence of the nucleic acid of *M. cerebralis* by polymerase chain reaction (PCR) to confirm that actinospores observed were those of *M. cerebralis*. Because 1.6 mL of water from each sample was examined by microscopy for each sample, an equal volume was obtained from the remaining sample filtrate, preserved in 3.6 mL of ethanol, and submitted for PCR testing. Negative control samples made of tap water and a small amount of soil or organic debris were submitted on several occasions along with the true field samples. All samples were assigned an alphanumeric code to mask the identity of the sample from laboratory personnel performing the PCR test.

The test used was based on the PCR procedure

developed by Andree et al. (1998); only a single round rather than a nested procedure was used. Samples were initially centrifuged at 1,000 × gravity (g) for 5 min. Most of the supernatant was then aspirated off, and then 1–1.5 mL and any pellet material were transferred to a microfuge tube. These were centrifuged at 14,000 × g for 3 min, and the pellet was then resuspended in 200 μL of lysis buffer. Total DNA was extracted from each sample by the use of a spin-column purification procedure (Qiagen, Inc., Valencia, California).

Results

The bucket method proved to be the simplest and fastest filtration technique. Most 1,900-L samples could be obtained in less than 30 min, compared with 2 h or more for pumped or siphoned samples. Consequently, after just a few pumped or siphoned samples, all further samples were obtained with the bucket method. Pumped and siphoned samples were not considered in the analysis.

The 20 subsample counts from each filtrate were adequate to provide low-variance estimates of actinospores per liter in individual samples, except when densities were very low (see SE markers, Figure 2). At low densities, confidence limits of plus or minus 100% of the point estimate were common.

Actinospore density estimates tended to decrease as the quantity filtered increased on the two occasions when differing quantities were filtered (Table 1). On the first occasion, when the overall F-test was significant ($P = 0.042$), multiple comparison tests indicated that the density estimate from the 190-L filtration was significantly greater than that from the 1,900-L filtration ($P < 0.05$), but all other comparisons were nonsignificant. Because of this evidence that the volume filtered might affect efficiency, further analyses were based on 1,900-L samples only.

Statistical analysis of the data set from all collection sites was complicated by large differences in actual values of density estimates and numbers of nonzero estimates. These differences precluded the pooling of data from all sites. There was no consistent relationship between temperature, or any other continuous predictor variable, and actinospore density at any site. Consequently, presentation of statistical results is limited to the gross scale of the categorical predictor variables season or year.

The first detection of abundant actinospores occurred immediately below Windy Gap Reservoir at the North Outlet and Spill Basin sites (Figure 2) in July 1997 and appeared to coincide closely with the cessation of runoff. Generally, actinospore densities were highest during the fall and summer and were lowest during spring at six of the seven sites where spring samples were obtained. These seasonal differences were significant at the Fraser River, North Outlet, Spill Basin, and Hitching Post sites (all $P < 0.002$). Actinospore densities were higher in 1998 than in 1997 at these same four sites, as well as at Williams Fork (all $P < 0.042$). Qualitatively, the sites below Windy Gap Reservoir (North Outlet, Spill Basin, and Hitching Post) clearly show higher densities of actinospores than do the other sites (Figure 2).

Attenuation of spore densities downstream of Windy Gap Reservoir was very rapid; densities at the Hitching Post site 1.8 km downstream were consistently lower than those observed at the North Outlet or Spill Basin sites. At the Breeze Bridge, 26 km downstream, only two times during the entire study did actinospore density estimates exceed 1 actinospore/L.

Actinospores were consistently present at low densities throughout the winter at the North Outlet and Hitching Post sites. The period of lowest spore densities during this study occurred from March through May 1998.

Comparison of microscope count results with PCR test results on parallel samples reveals that there is good agreement between these methods in identifying the presence of *M. cerebralis* (Table 2). Of the 32 samples in which no actinospores were observed, 22 (69%) of the parallel samples also tested negative by PCR. Of the 95 samples in which actinospores were observed, 86 (91%) of the parallel samples also tested positive by PCR. The other nine samples were ones in which one or two actinospores were observed by microscopy, so all discrepancies between the two diagnostic methods arose from samples in which microscope counts ranged from zero to two. All 12 blind negative control samples tested negative by PCR.

Discussion

We found the bucket method of filtering actinospores to be more utilitarian than was bilging or siphoning. This method was faster and did not require the transport of pumps, batteries, or long lengths of hose. Moreover, there was no time consumed by calculating flow rates or finding sites with a suitable gradient to accommodate siphoning.

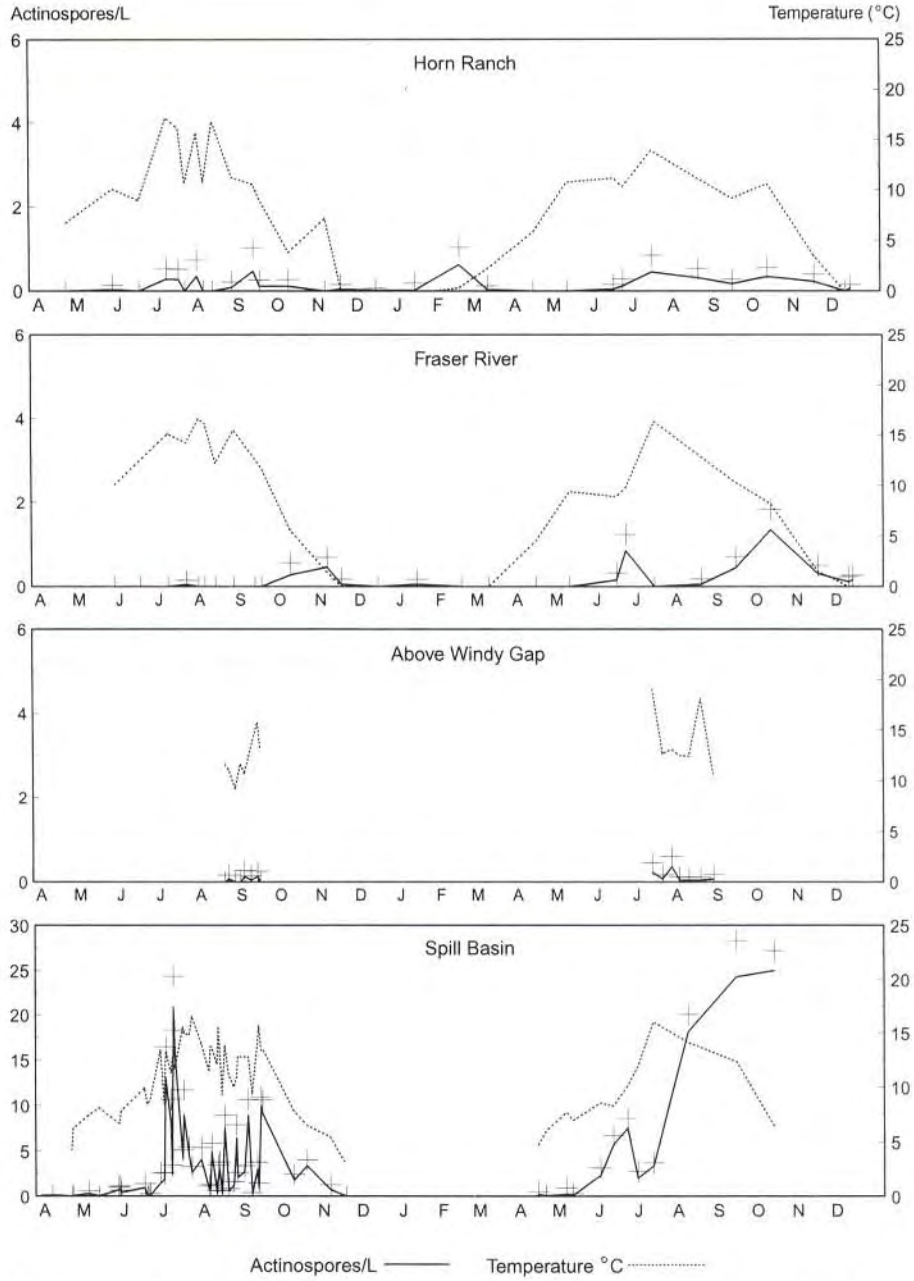

FIGURE 2.—Density of actinospores of *M. cerebralis* (number/L) and temperature (°C) of river water seen from April 1997 to December 1998 at field sampling sites in the upper Colorado River drainage. Crosshair markers indicate +2 SE of the density measurement for each sampling occasion. Sites are arranged upstream to downstream. Note the different scales on the *y*-axes. Months are indicated chronologically on the *x*-axis by single-letter abbreviations beginning with April (A).

After a field sample was obtained, it was possible to obtain an estimate of actinospore density in the sample; often little variation was seen among the 20 subsamples. However, it must be pointed out that we did not replicate field samples except on the occasions when we filtered different quantities sequentially at a single site over a short period. Other researchers, attempting to establish the

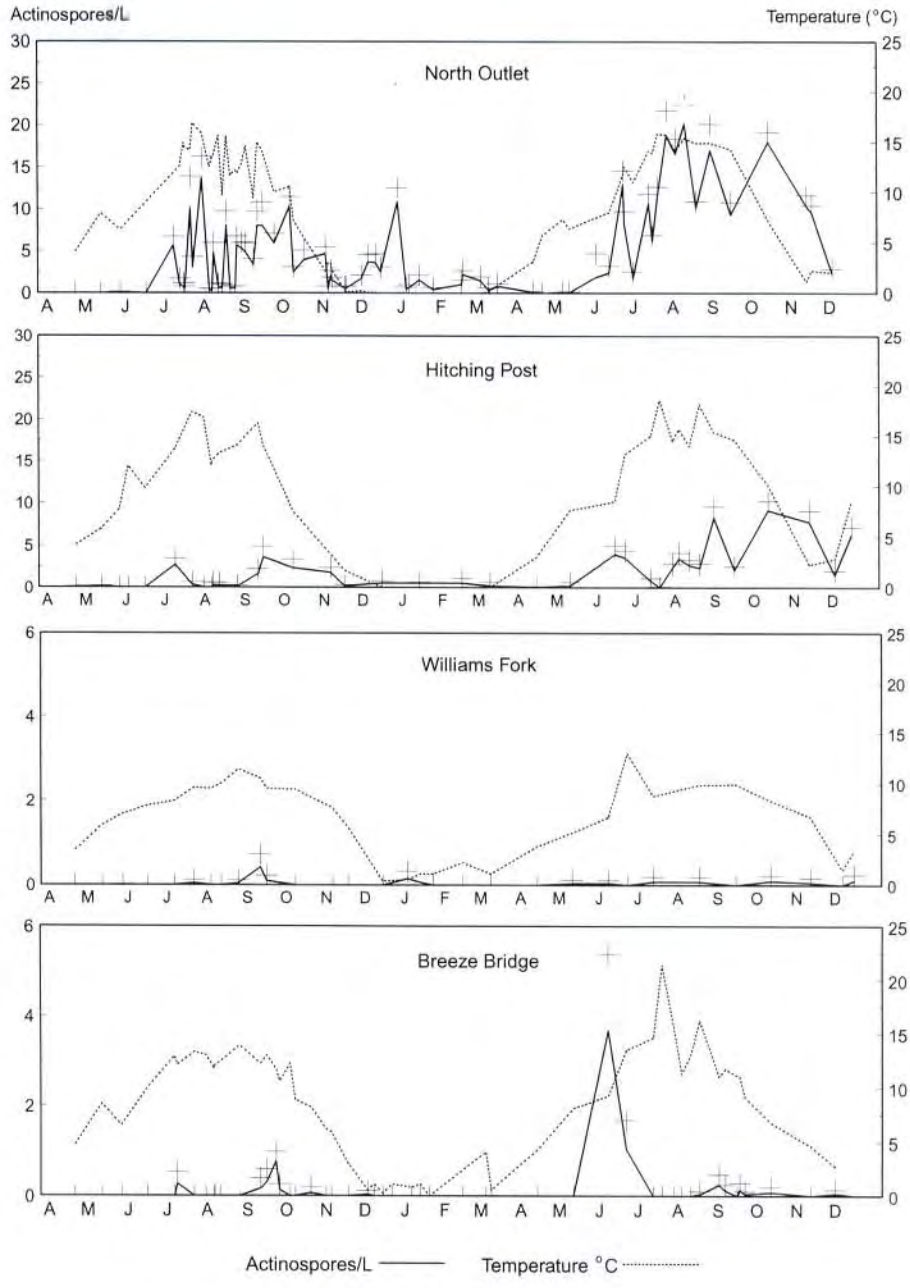

FIGURE 2.—Continued.

repeatability of screen filtration by retrieving known quantities of actinospores with the same mesh size used in this study, concluded that screen filtration was inaccurate and imprecise (F. T. Barrows, U.S. Fish and Wildlife Service, personal communication). Consequently, actinospore densities obtained by this method may be inconsistently underestimated and may be subject to variability that was not measured in this study.

Despite these potential problems, data from nearly 2 years of filtering revealed a strong seasonal pattern of actinospore release. However, there were exceptions, with relatively high densities observed during winter or spring at the Horn

TABLE 1.—Density estimates of actinospores of *Myxobolus cerebralis* and analysis of variance comparisons from four sequential water samples of differing quantities collected on two occasions.

Occasion	Density (actinospores number/L) in samples of 190 L	570 L	1,135 L	1,900 L	*F*	*P*-value
1	9.1	7.6	5.2	5.5	2.88	0.042
2	7.1	5.3	5.1	2.6	2.54	0.063

Ranch, North Outlet, and Williams Fork sites. Likewise, low-density estimates were not uncommon during seasons when average densities were high at the North Outlet and Spill Basin sites.

The reasons for many of these exceptions are unknown; however some are possibly explained by environmental variables. For instance, even though statistical analysis did not establish flow or temperature effects on actinospore density over the entire study period, in August 1997 there were several marked flow fluctuations that apparently had a profound influence on actinospore density at the North Outlet and Spill Basin sites. Nearly every marked flow increase during that month was accompanied by very low actinospore density estimates (Figure 3). However, there is perhaps more than a simple dilution effect associated with the spikes in flow. Each time the flows increased, water temperatures decreased. Water temperature can be an environmental cue for actinospore release, as was noted by El-Matbouli et al. (1999), who witnessed a large and rapid increase in the number of actinospores released from cultures of infected tubificids when an incubator door was inadvertently left open, allowing room temperature air to enter the 5°C chamber for a 2-d period. Higher estimates of actinospore density at the North Outlet site in August 1997 tended primarily to follow 2–3 d of receding flows and accompanying rises in water temperature. Conversely, because the decreases in actinospore density appeared to be greater in magnitude than simple dilution would account for, we think that falling temperatures may have suppressed actinospore releases. The tendency toward higher densities of actinospores observed in 1998 compared with 1997 was probably affected by discharge. In 1997, annual average discharge at the Hitching Post Bridge was 14.9 m^3/s, whereas in 1998 it was 6.9 m^3/s.

It was apparent that Windy Gap Reservoir was a source of actinospore production. The three sites below the reservoir showed densities that were consistently higher than those at sites above the reservoir

TABLE 2.—Comparison of the number of actinospores of *Myxobolus cerebralis* in water samples observed by microscopic examination of a total volume of 1.6 mL of each field sample filtrate with results from the polymerase chain reaction (PCR) on a parallel 1.6-mL sample of each filtrate.

Actinospore count	Frequency	PCR signal Negative	PCR signal Positive
Negative control[a]	12	12	
0	32	22	10
1	13	8	5
2	10	1	9
3–4	9		9
5–14	15		15
15–49	13		13
50–99	11		11
100–499	17		17
≥500	7		7

[a] Negative control samples were unknown to laboratory personnel.

or further downstream. Sentinel fish held at these sites also exhibited a higher severity of infection than did fish held at sites above the reservoir or at Breeze Bridge (Nehring and Thompson, unpublished data). The reservoir will likely continue to be a point source, because it provides a very good tubificid habitat. Estimates of the population of *Tubifex tubifex* in the reservoir exceed 34,000/m^2, with an infection rate of *M. cerebralis* of 1.2% (Zendt and Bergersen 2000). This reservoir exists for the sole purpose of providing a pumping station to move Fraser River water from the Colorado River to upstream reservoirs, from which it can be moved to the Front Range as part of the Colorado–Big Thompson project. The Colorado River and the trout populations therein

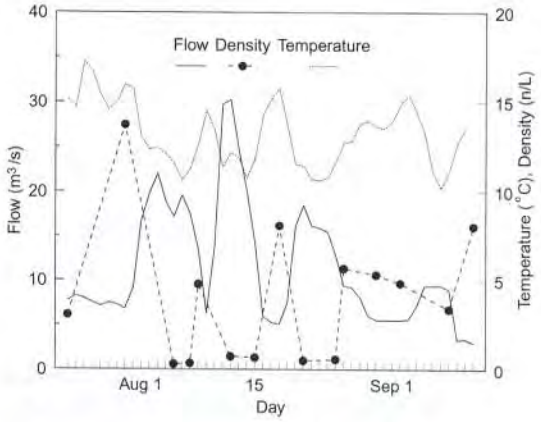

FIGURE 3.—Fluctuations in flow (m^3/s) and temperature (°C) below Windy Gap Reservoir from July 23 to September 11, 1997, and the apparent response in actinospore density (number of actinospores, n/L) at the North Outlet filtration site.

would likely benefit if Windy Gap Reservoir could be bypassed by an alternate channel during times of the year when pumping is not occurring.

The low actinospore densities observed at the Breeze Bridge and Williams Fork sites were unexpected. These areas, as well as the rest of the study reach, have experienced little or no recruitment of rainbow trout since 1992 because of losses of fry and fingerlings to whirling disease (Nehring and Walker 1996; Nehring et al. 1998). Consequently, we expected to observe a corresponding high actinospore density at these sites. That we did not suggests that chronic exposure of rainbow trout fry to relatively low densities of actinospores, as measured by this technique, is sufficient to induce a population effect among rainbow trout.

This filtration technique proved valuable in revealing effects of season and distribution on releases of actinospores of *M. cerebralis* in the upper Colorado River. The distribution of actinospores corroborated the intensity of infection observed in sentinel fish held at four of the filtration sites (Nehring and Thompson, unpublished data) and confirmed that localized zones of intense infectivity may be present in intermountain river systems. We believe the technique has great utility as an investigative tool in the field because it is light weight, simple, and quick and requires a minimal amount of inexpensive equipment. Moreover, it is a nonlethal sampling technique for determining whether *M. cerebralis* is enzootic in an ecosystem. Future work should investigate ways to improve actinospore capture efficiency.

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