Whirling Disease/Habitat Interactions

Federal Aid Project F-427-R5

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Federal Aid in Fish and Wildlife Restoration

FINAL Job Progress Report

Colorado Division of Wildlife

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Project Title: Whirling Disease / Habitat Interactions

Project No.: F-427-R5

Project Objective: To investigate the influence of aquatic habitat factors on the severity of *Myxobolus cerebralis* infections in free-ranging trout populations in selected stream ecosystems in Colorado, and whether aquatic habitat factors can be managed to reduce the impacts of the parasite.

Job No. 1: Identification and Reduction of *Tubifex tubifex* Habitat in Streams.

Job Objective: Develop and test strategies to reduce or eliminate *T. tubifex* habitat from areas of streams known to be foci of infectivity in order to reduce the production of actinospores of *Myxobolus cerebralis*.

Period Covered: Final summary for study period July 1, 2003 to June 30, 2008

INTRODUCTION

In the early 1990s major declines in wild rainbow trout *Oncorhynchus mykiss* were observed in certain rivers in Colorado. In most streams in Colorado where rainbow trout numbers declined significantly the effects persist to the present day. Research indicates that these declines are the result of whirling disease (Walker and Nehring 1995; Nehring 1996; Nehring and Walker 1996; Nehring et al. 1998; Nehring 1998; Nehring 1999), caused by the parasite *Myxobolus cerebralis*. More recently, it has been suggested that *M. cerebralis* may be contributing to the decline of Yellowstone cutthroat trout in Yellowstone National Park (Koel et al. 2006).

Sentinel fish studies in the Colorado River and studies on *M. cerebralis* actinospore filtration in numerous drainages suggest that some areas within streams act as foci of infection for the parasite (Thompson et al. 2002, Nehring and Thompson 2001; Thompson and Nehring 2000). For example, Windy Gap Reservoir proved to be such a focus of infection in one study (Thompson et al. 2002). Stocking Spring Creek Reservoir with catchable trout infected with *Myxobolus cerebralis* resulted in elevated infectivity in Spring Creek below the reservoir (Nehring et al. 2001), as measured by actinospore densities in the water column and myxospore concentrations in samples of brown trout.

Infectivity below reservoirs has been addressed by taking steps to insure that fish stocked in them are uninfected with the parasite. Capital improvements to enhance hatchery water supply security, changes in hatchery management, and changes in stocking policy have also played significant roles. The benefits to downstream fisheries from these management actions become more apparent as time passes.

Nevertheless, some sites of high infectivity that are not reservoir-related have been detected by actinospore filtration. Examples include some irrigation diversions, beaver ponds and other pond complexes. Such sites in some instances appeared to be discrete and isolated areas of preferred

oligochaete habitat, leading to the hypothesis that removal or reduction of such discrete habitat sites could reduce infectivity over a larger reach of stream. The objectives of this study were to determine whether it is possible to remove or greatly reduce these areas of infection by physical habitat manipulation or stream habitat improvement techniques, and to determine if such manipulations result in reduced prevalence and intensity of infection among resident trout downstream of modified sites.

METHODS and MATERIALS

Information at each study site (See Appendix Table A1.) was collected to describe the prevalence of infection in the fish and oligochaete populations, and the actinospore production dynamic.

Fish Sampling

Samples of age 1+ brown trout were obtained at each location and analyzed for *M. cerebralis* spore concentrations in individual heads by the pepsin-trypsin digest method (PTD, Markiw and Wolf 1974). In some locations young-of-the-year (YOY) trout were collected; they were examined by the polymerase chain reaction (PCR) technique described by Schisler et al. (2001) or a subsequent PCR technique using the HSP-70 gene to determine whether *M. cerebralis* was present. The resulting bands observed on agarose gels were graded independently by two reviewers and reported on a five-point scale ranging from '0' (negative, no band) to '4' (an intense band indicating a severe parasite infection), hence the results are qualitative but more informative than simple presence or absence.

Oligochaete Sampling

To establish baselines, oligochaete populations were characterized by sampling what was judged to be the best oligochaete habitat at each study site on three separate occasions. On each occasion, six separate samples were obtained by a kicknet technique. An area of about 0.5 m^2 was thoroughly disturbed with the sampler's feet for 60 seconds while holding a 250-μm mesh kicknet just downstream in the current to capture the organisms dislodged from the substrate. In a few cases, very fine substrates were sampled for only 30 seconds, and the results were standardized to match the 60-second samples. Each sample was placed in a 4-L pail and covered with water, labeled, and allowed to sit overnight. The following day, the overlying water was filtered through 20-μm Pecap® screen to concentrate any actinospores present, and the actinospore density was estimated using techniques described previously (Thompson and Nehring 2000, Nehring et al. 2001). Most of the samples were also tested by the polymerase chain reaction (PCR) technique described by Schisler et al. (2001) or a subsequent PCR technique using the HSP-70 gene to confirm the identity of actinospores observed as those of *M. cerebralis.* After removing as much water as possible, each sample was preserved separately with 10% buffered formalin, and the sample set was shipped to a private lab for further analysis by experts in oligochaete taxonomy.

At the laboratory, each sample was washed using a 250-μm mesh sieve to remove the formaldehyde fixative and fine sediments. Samples with a high volume of material were

subsampled using a modified Caton subsampler (Caton 1991). Two samples were randomly selected from each occasion and all the worms removed, counted and weighed. In the remaining four samples from each occasion, a minimum of 50 worms (if available) from each group were removed, counted and placed in petri dishes of water, while the remainder were left in the sample but enumerated. Groups consisted of tubificids with hair and pectinate chaetae, tubificids with bifid chaetae, enchytraeids, and lumbriculids. Naidids were not removed because they are very small, are not confused with *T. tubifex* and generally contribute little to total oligochaete biomass.

After the entire subsample was examined and the oligochaetes removed or counted, each group of worms that had been removed were weighed. The worms were blotted on a paper towel, placed into an aluminum weighing pan which had been weighed previously, and the difference was recorded as wet weight in grams. The balance was calibrated daily. The total weight was divided by the number of oligochaetes weighed, and the weight per oligochaete recorded. After weighing, the worms were placed in 70% ethyl alcohol for mounting and identification.

Approximately 50 tubificids with hair and pectinate chaetae were mounted in CMCP mounting medium for identification. In cases where there were obviously no mature oligochaetes, fewer were mounted. To identify tubificids with bifid chaetae, 5 to 10 mature specimens were removed and mounted. Oligochaetes with hair and pectinate chaetae were divided into five groups: mature *T. tubifex*, immature *T. tubifex*, mature *Ilyodrilus templetoni*, immature *I. templetoni*, and immature (unidentified). Worms were considered mature when penis sheaths were present. If no penis sheaths were present but other characters could be used to indicate that they were most likely one of the two species, they were labeled as immature of that species. Since only fully mature individuals may be positively identified, such tentative identifications of immature worms must be considered in that light.

Post-improvement oligochaete evaluations were accomplished with quantitative polymerase chain reaction (qPCR) analyses. Sampling continued at what was subjectively judged to be the best oligochaete habitat in each habitat type (riverine or backwater) at each study site on each occasion. Replicate samples from each individual sampling location were obtained on each occasion by a kicknet technique. A 0.5 m^2 area was selected by surrounding with a frame made of copper water pipe, and the total area was thoroughly disturbed with the sampler's feet for 60 seconds while holding a 250-µm mesh kicknet just downstream in the current to capture the organisms dislodged from the substrate. Each sample was placed in a 4-L pail and covered with water, labeled, and taken to the lab for processing. Two samples of 50 haired oligochaete worms (if available) were selected from each of the replicate substrate samples. The worm samples were tested by real-time quantitative PCR (qPCR) to estimate the percentage of DNA present from each *T. tubifex* lineage.

Actinospore Sampling

Replicate samples of water were filtered monthly at each study site through 20-um Pecap screen to concentrate actinospores, except when ice cover prevented access. Initially 1900 L of water were filtered for each replicate; after June 2004 the volume was reduced to 114 L (Thompson 2006). These concentrates were examined for the presence of *M. cerebralis* actinospores in the lab by established protocols (Thompson and Nehring 2000).

RESULTS

Beaver Creek (South Fork Rio Grande drainage)

Habitat modifications were accomplished at this site in October 2001 (see Nehring and Thompson 2003 for details). Monitoring below this modified site for actinospores ceased after June 2005. The fish population was sampled for the presence of the parasite among age 1+ brown trout and YOY brown and rainbow trout through 2006.

Myxospore monitoring in age 1+ brown trout suggests that prevalence and mean concentrations were not significantly reduced among the wild brown trout inhabiting the stream. Infection prevalence reached an apparent low point in 2002 (Table 1.01), the year after habitat modification. However, the 2003 sample represented the first year class of fish exposed to *M. cerebralis* as newly hatched fry under the new habitat conditions. Although prevalence was arguably lower in 2003 than in the baseline sampling, the average myxospore concentration was clearly not reduced. Moreover, four years of post-modification fish samples indicate that prevalence and average myxospore concentration were not dramatically changed from pre-modification values.

Table 1.01. Cranial *Myxobolus cerebralis* myxospore concentrations in brown trout sampled from Beaver Creek 1 km below Beaver Creek Reservoir.

Date Mm/Dd/Yy	Age	Sample Size N	Sample Size N+	Overall Mean Myxospore Concentration	Std Dev	Positive Fish Mean	Positive Fish Range
09/10/98	1+	10	8	24,200	47,302	30,200	1,350 – 74,700
08/31/99[a]	1+	10	6	20,900	35,636	34,800	2,220 – 100,000
09/22/00	1+	11	7	37,600	44,310	59,100	4,440 – 111,100
09/26/01	1+	20	14	28,200	94,741	40,300	8,100 – 230,400
09/13/02	1+	11	4	6,900	14,880	19,000	2,400 – 48,500
09/23/03	1+	19	9	35,000	84,813	74,000	7,200 – 353,500
09/19/04[b]	1+	20	18	26,800	44,913	29,800	600 – 183,300
09/22/05	1+	20	12	53,800	97,495	89,700	2,200 – 429,900
09/29/06	1+	20	11	27,100	60,730	49,300	1,700 – 234,000

a: Analyzed by a contracted lab using a plankton centrifuge technique rather than the usual pepsin-trypsin digest method.
b: Analyzed by a contracted lab that achieved smaller-than-usual volumes of PTD product.

Interpretation of the myxospore data is slightly complicated by the use of two labs and two techniques to evaluate the samples. A private lab in Maine was used in 1999 and the analysis was conducted using plankton centrifuge rather than the standard and more efficient pepsin-trypsin digest. The same private lab was used again in 2004 with pepsin-trypsin digest, but using this

technique tended to result in a higher probability of detection than the state lab due to lower volumes of PTD product (hence more concentrated spores). Prevalence was more affected than mean concentration as the additional detections occurred in fish exhibiting low spore concentrations, but it does help explain the highest recorded prevalence noted in 2004. It is encouraging to note that prevalence the last two years of monitoring was no higher than the long-term average even though the CDOW State Aquatic Animal Health lab also achieved lower volumes of PTD product during that time than in previous years. Clearly, the habitat project had no lasting effect on reducing the myxospore concentrations in age 1+ brown trout.

Table 1.02. Results of PCR tests on young-of-the-year brown and rainbow trout from Beaver Creek below Beaver Creek Reservoir from 2000 to 2006. Mean scores are based on a scale from '0' (negative, no PCR signal) to '4' (very strong positive signal).

Date	Sample size (N)	Positive fish	Mean PCR score	Sample size (N)	Positive fish	Mean PCR score
	Brown trout			Rainbow trout		
09/22/00	11	9	2.55	2	2	3.50
09/26/01	10	10	3.50	10	10	2.60
09/13/02	13	8	2.71	22	21	3.68
09/23/03	20	15	1.80	15	10	1.87
09/19/04	20	18	2.40	11	8	2.82
09/22/05	15	13	3.20	15	12	1.80
09/29/06	10	9	3.10	15	9	1.93

The PCR results (Table 1.02) continue to show high prevalence of infection among YOY brown and rainbow trout. In the case of PCR, 2002 was the first year of post-modification evaluations because young-of-the-year fish were used for evaluation. Although 2003 samples showed lower mean scores than during the baseline years, those lower scores were not maintained through other post-modification years for brown trout. In contrast, overall mean PCR scores for rainbow trout did remain generally lower in the post-modification evaluation period.

This tributary of the South Fork Rio Grande was a well-used rainbow trout spawning site during the 1990s, documented as a result of research conducted on establishing wild rainbow trout populations in a number of streams (Nehring 1998). If any evidence suggests that the habitat modifications had a positive effect for the fishery in Beaver Creek, it is the population statistics regarding the number of age 1+ rainbow trout in the fishery (Table 1.03). No rainbow trout appeared to be surviving through their second summer in the late 1990s, but from 2000 onward some wild age 1+ rainbow trout were captured each year of the study. The caveat with this observation is that age 1+ rainbow trout began showing up again before any habitat modifications occurred, so it is unlikely that habitat modification was the only factor influencing the survival of young rainbow trout. Moreover, 1998 is the earliest population data available; prior to that time, sampling targeted young-of-the-year only.

Table 1.03. Trout population biostatistics (fish ≥ 15 cm) for Beaver Creek 1 km below Beaver Creek Reservoir in September of 1998 through 2005.

	Brown Trout					Rainbow Trout				
Year	N	95% CI	Kg/ha	N/ha ≥ 15 cm	N/ha Age 1+	N	95% CI	Kg/ha	N/ha ≥ 15 cm	N/ha Age 1+
1998	103	± 3	190.2	1,704	1,014	2	± 0	2.3	33	0
1999	100	± 3	140.3	1,282	1,505	5	± 0	7.4	77	0
2000	232	± 6	344.5	2,828	891	5	± 0	6.7	61	24
2001	155	± 5	196.1	1,908	948	3	± 0	3.7	37	77
2002	152	± 4	244.1	1,852	811	4	± 0	5.3	49	49
2003	136	± 5	199.0	1,664	671	8	± 0	6.0	98	184
2004	138	± 7	185.0	1,695	974	10	± 1	9.0	123	89
2005	129	± 3	190.0	1,577	891	4	± 0	6.0	49	86

Cache la Poudre River

The Cache la Poudre River was added to the work schedule during the 2002-03 segment. Significant strides were previously made in reducing *M. cerebralis* actinospores emanating from the Poudre Rearing Unit (PRU) (Nehring and Thompson 2003, Schisler 2003), allowing additional attention to in-stream habitats near the PRU. Allen (1999) found that the main channel of the river in the low-gradient reach above PRU contained few oligochaetes, but that they were often numerous in side-pockets, alcoves, and side channels. While not detailed in Allen's thesis, one such site identified was at Kinikinik. In this stream segment there were two significant backwater areas that appeared to be excellent habitat for *T. tubifex*.

Berms designed to isolate both of the backwater areas at Kinikinik were constructed in September and October 2004, as described in Thompson (2005). The berms were designed to preclude 90% or more of all average daily flows in this reach from entering the backwater areas, based on historic data from a discontinued gage near Rustic. Flows that overtop the berms only occur during runoff, a time when actinospores are seldom encountered. Moreover, any actinospores produced during peak runoff would also be highly diluted if they entered the river. In 2006, the backwaters were connected to the river for six weeks or less (Figure 1.01). In 2007 the upper berm was inundated from May 20 to June 23, and the lower berm was inundated from May 15 to July 15.

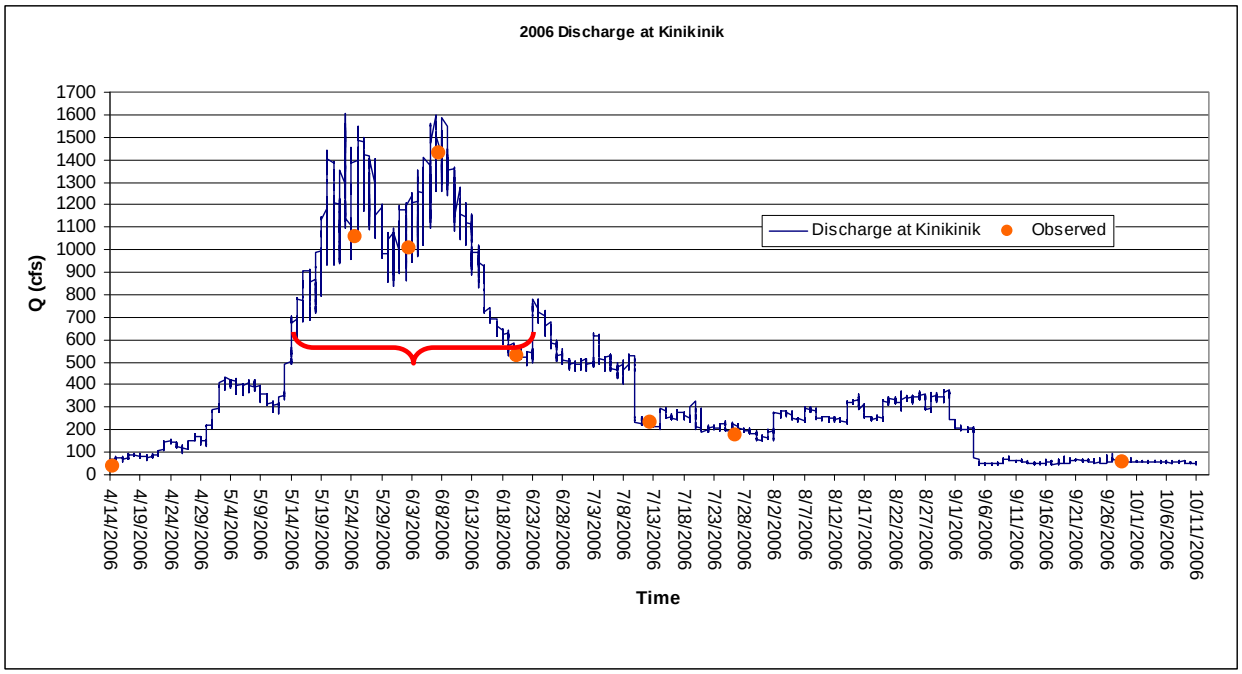

Figure 1.01. Discharge in the Poudre River at the Kinikinik study site, modeled from pressure sensors and observed flows. The approximate period of berm inundation is indicated by the bracket and extended from about May 14 to June 24, 2006. "Observed" data points are measured discharges.

Therefore, the berms functioned physically as anticipated during this study. The differences in inundation period arose from the necessity of having to build the upper berm about 20 cm higher than designed because of groundwater input within the backwater. A filter section was placed in the berm to allow water to pass through rather than run over the berm, and the additional height was needed to create sufficient head for the filter to operate correctly.

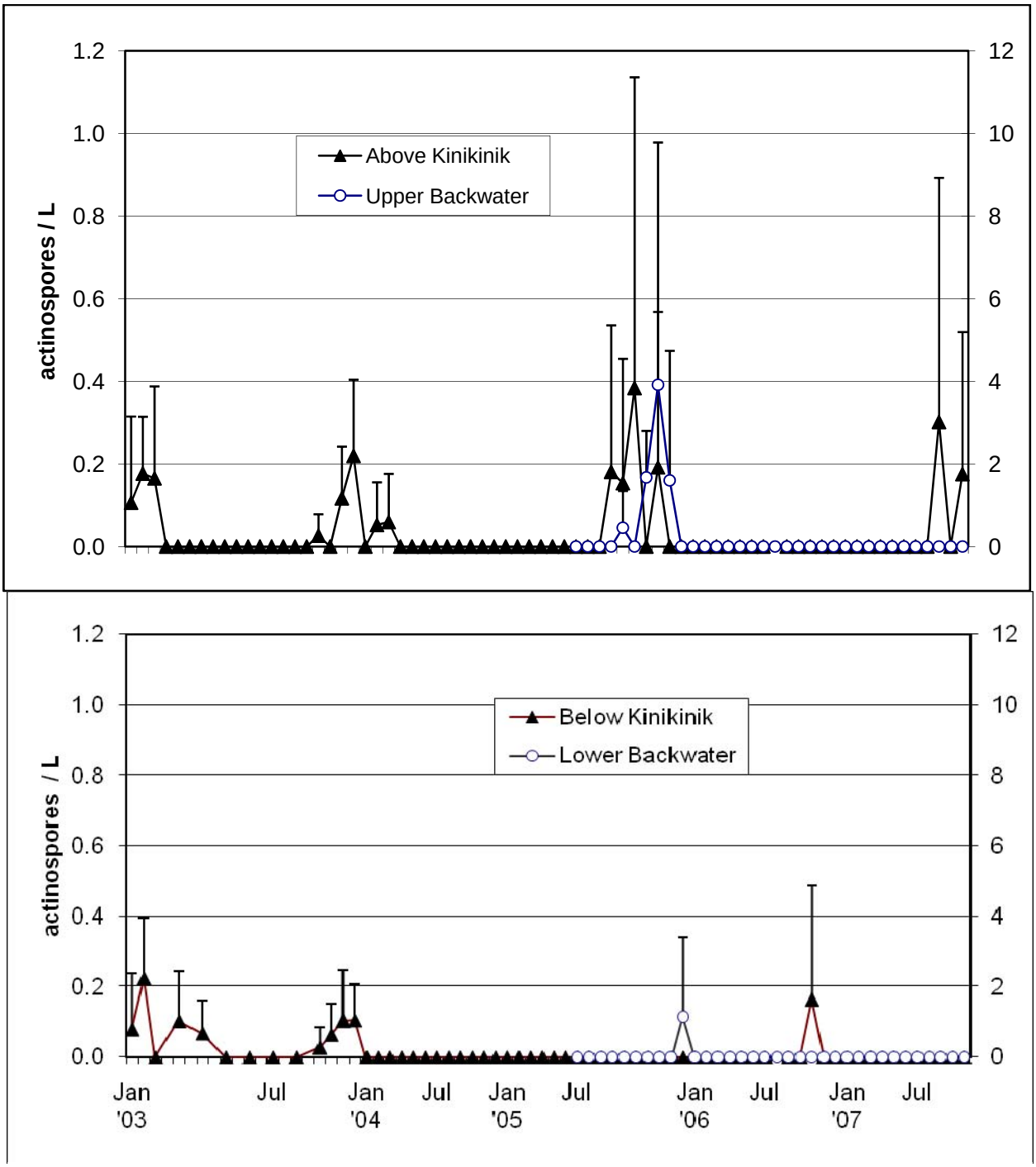

Figure 1.02. Estimates of actinospores/L in the Poudre River at above and below Kinikinik from January 2003 through May 2007. Error bars represent upper 95% confidence limit for the sample only based on subsample counts through June 2004. Samples taken in July 2004 and later were replicated so confidence limits apply to the stream. Sampling frequency was twice per month early in the monitoring and during late summer and fall 2005, hence the uneven x-axis. Backwater values are referenced to the 2nd y-axis.

Water samples were collected above and below the Kinikinik site beginning in January 2003 (Figure 1.02). Density estimates were low on all occasions when actinospores were actually observed in the river, but were higher in the backwaters when observed there. Actinospores were observed from the isolated upper backwater four times, all in 2006, and just once in the lower backwater. Such a frequency of detection in the backwaters was disappointing since the hypothesis was that the backwaters were sites of higher actinospore production and that production would be isolated there during non-runoff periods. However, the probability of detection in the backwaters was lower than in the river, given actinospore presence, because there was tremendous algae and plankton growth in both backwaters, resulting in large filtrates and a significant amount of organic matter on the slides examined for actinospores. Such organic content made it quite difficult to observe actinospores.

All oligochaete data from this site were analyzed by the qPCR methodology. Baseline sampling was completed in 2003 and 2004 (Table 1.04), and post-construction samples were collected in 2005 through 2007. Lineage V, a resistant lineage, was not represented in the oligochaete samples collected from this area. The susceptible lineage III (Beauchamp et al. 2002, DuBey et al. 2005) predominated at this site in early baseline sampling. Curiously, the proportion of lineage III DNA in the worm samples tested by qPCR showed a significant downward trend over the 13 month time span of the baseline sampling (ANOVA, $P = 0.0002$). This trend did not continue throughout the post-manipulation evaluation period.

The proportion of lineage III DNA in post construction samples has remained within the same range as the baseline data. All lineages have experienced wide fluctuations among sampling occasions; a dynamic no doubt influenced by the fact that it has been more difficult in post-construction monitoring to obtain replicate 50-worm samples from each location. Therefore the data are more sparse and the confidence intervals wider. Moreover, in many instances the qPCR results indicate low numbers of “worm equivalents”, a measure of how many copies of the target DNA were present in the sample. The low number of samples represented in Table 1.04 for some post-manipulation years was often the result of detecting no *T. tubifex* DNA despite submitting haired worm samples to the lab (in some riverine samples there were simply no worms present). This suggests that there are now haired worms in the backwaters that do not belong to any of the four lineages targeted by the qPCR test. Presumably, these oligochaetes were present in low numbers prior to the habitat modifications, but are now competing more effectively because of the habitat changes produced by isolating the backwaters. None of the worm samples collected in 2006 or 2007 tested positive for the presence of the parasite.

Myxospore analyses from samples of age 1+ brown trout collected over the last several years show highly variable mean concentrations and prevalence at both collection sites (Table 1.05). It is concerning that the mean concentration above Kinikinik increased over the last three years while prevalence did not. The mean in 2007 was affected considerably by a single large value, but even discounting that value the 2007 mean was consistent with the 2006 mean.

Table 1.04. Estimates of the proportion of each *Tubifex tubifex* lineage DNA found in oligochaete samples at the Kinikinik site. N refers to the number of the nine kicknet samples collected on each occasion that contained *T. tubifex*. The values in parentheses are 95% confidence intervals.

Date	N	Approximate percent DNA composition by *M. cerebralis* lineage			
		I	III	V	VI
		Pre-modification			
08/25/03	9	2.8 (1.7)	73.9 (11.2)	0.0 (0.0)	23.3 (10.2)
10/01/03	8	4.8 (2.6)	67.5 (10.8)	0.0 (0.0)	27.7 (9.9)
06/22/04	9	5.6 (6.1)	55.5 (25.1)	0.0 (0.0)	38.9 (23.3)
09/13/04	8	13.7 (6.6)	37.3 (21.8)	0.0 (0.0)	48.9 (20.1)
		Post-modification			
07/18/05	4	22.5 (54.6)	58.9 (62.7)	0.0 (0.0)	18.6 (22.3)
10/24/05	6	40.6 (18.7)	37.7 (19.3)	0.0 (0.0)	21.7 (9.7)
07/18/06	7	49.7 (46.7)	43.3 (44.5)	0.0 (0.0)	7.0 (13.2)
10/11/06	8	19.7 (11.1)	41.7 (20.5)	0.0 (0.0)	38.5 (19.8)
07/09/07	4	11.0 (26.9)	73.9 (14.3)	0.0 (0.0)	15.1 (8.7)
09/18/07	3	3.9 (10.5)	72.0 (42.8)	0.0 (0.0)	24.2 (32.5)

Table 1.05. Cranial *Myxobolus cerebralis* myxospore concentrations in age 1+ brown trout sampled from the Poudre River.

Date mm/dd/yy	N	Prevalence	Overall Mean Concentration	Std Dev	Positive Fish	
					Mean	Range
		Bliss State Wildlife Area – above Kinikinik				
10/19/99[a]	10	30.0%	2,700	6,933	8,900	2,200 – 22,200
09/19/00[a]	10	40.0%	900	1,279	2,400	1,700 – 3,200
09/30/02	10	10.0%	2,800	8,890	28,100	28,100
10/22/03	20	40.0%	4,400	8,595	11,000	2,300 – 31,600
10/28/04	10	20.0%	2,600	5,744	13,000	9,200 – 16,700
11/02/05	17	70.6%	2,000	3,351	2,800	560 – 13,300
10/16/06	7	85.7%	30,300	15,671	35,400	26,900 – 50,500
10/16/07	15	66.7%	79,700	185,729	119,600	5,600 – 726,600
		Big Bend – below Kinikinik				
09/19/00	10	50.0%	6,300	11,675	12,600	990 – 37,600
10/22/03	12	41.7%	3,900	6,029	9,400	920 – 16,000
10/28/04	15	40.0%	17,100	30,141	42,900	5,600 – 92,300
11/02/05	15	60.0%	3,600	7,139	6,000	560 – 27,200
10/16/06	10	80.0%	50,600	135,248	63,200	3,400 – 439,400
10/16/07	Not collected					

a: Samples obtained at Sleeping Elephant Campground, 3.6 km above Bliss SWA.

It is not possible to make the argument from these data that the habitat modifications have yet to have a substantial effect on these metrics. This project was the last one completed, so not as much evaluation opportunity has been realized, but results from other locations suggest that clear differences between upstream and downstream sites won't be realized based only on the habitat modifications in the Poudre River. It is possible that over time, decreases in prevalence and intensity of infections will occur as a result of changes in the oligochaete community. Certainly there appear to be legitimate changes occurring in the oligochaete community within the Poudre River study area.

Colorado River

No habitat manipulations were conducted in this stream reach. The oligochaete community was sampled in 2001 and the stream was monitored during this study for actinospores and myxospore concentrations in juvenile brown trout. It served as a control for sites where manipulations did occur. The results of the initial oligochaete sampling were previously reported (Nehring and Thompson 2003).

Samples of juvenile brown trout obtained since 1999 for analysis of cranial myxospore concentrations by PTD indicate that prevalence of infection is routinely 60% or greater (Table 1.06). The year-to-year variations in prevalence and myxospore concentration are not different than those being observed in treated stream sections. This suggests that the habitat manipulations implemented elsewhere may be no more responsible for changes in these metrics than random processes affecting stream habitat or host responses to enduring epizootic.

Table 1.06. Cranial *Myxobolus cerebralis* myxospore concentrations in age 1+ brown trout sampled from the Colorado River during the fall in 1999-2006.

Date	N	Prevalence	Overall Mean Concentration	Std Dev	Positive Fish	
					Mean	Range
	Hitching Post Bridge 1.9 km below Windy Gap Reservoir					
09/29/99	10	80.0%	6,330	6,330	7,920	1,110 – 15,550
10/12/00	10	100.0%	58,700	63,397	58,700	8,700 – 208,700
09/13/01	20	75.0%	20,300	28,569	27,500	4,000 – 96,000
09/27/02	10	60.0%	12,300	22,689	20,400	3,500 – 73,800
09/29/03	16	68.8%	11,700	16,125	17,000	2,500 – 43,700
09/27/04	22	95.5%	19,700	22,478	20,700	560 – 96,700
10/17/05	15	60.0%	4,900	7,367	8,200	560 – 24,400
10/23/06	20	55.0%	40,100	56,592	61,700	3,100 – 187,400
09/25/07	Not collected					
	Kemp/Breeze Wildlife Area 26 km below Windy Gap Reservoir					
09/29/99	10	60.0%	2,330	2,590	3,890	2,220 – 6,670
09/18/01	19	36.8%	13,800	37,473	37,300	1,900 – 160,600
10/08/02	13	84.6%	19,900	22,861	23,600	3,300 – 68,100
09/17/03	15	93.3%	14,400	18,192	15,400	3,300 – 70,100
09/30/04	21	76.2%	7,900	14,471	10,400	1,100 – 50,000
10/17/05	14	78.6%	9,800	21,602	12,500	560 – 25,600
10/23/06	20	65.0%	18,600	36,975	28,600	1,700 – 156,500
09/25/07	10	40.0%	19,800	36,303	49,600	8,400 – 92,200

Actinospore densities were monitored at the Breeze Bridge once each month throughout the study. *Myxobolus cerebralis* actinospores were observed with decreasing frequency over the course of the study (Figure 1.03). In addition, only two samples tested positive for DNA of the parasite among the 18 samples submitted for PCR since July 2005. These results suggest that factors other than oligochaete habitat quantity or quality may also affect *M. cerebralis* ecology, since no habitat manipulations were attempted in the Colorado River. Of course, it is also recognized that oligochaete habitat can change over time without intervention.

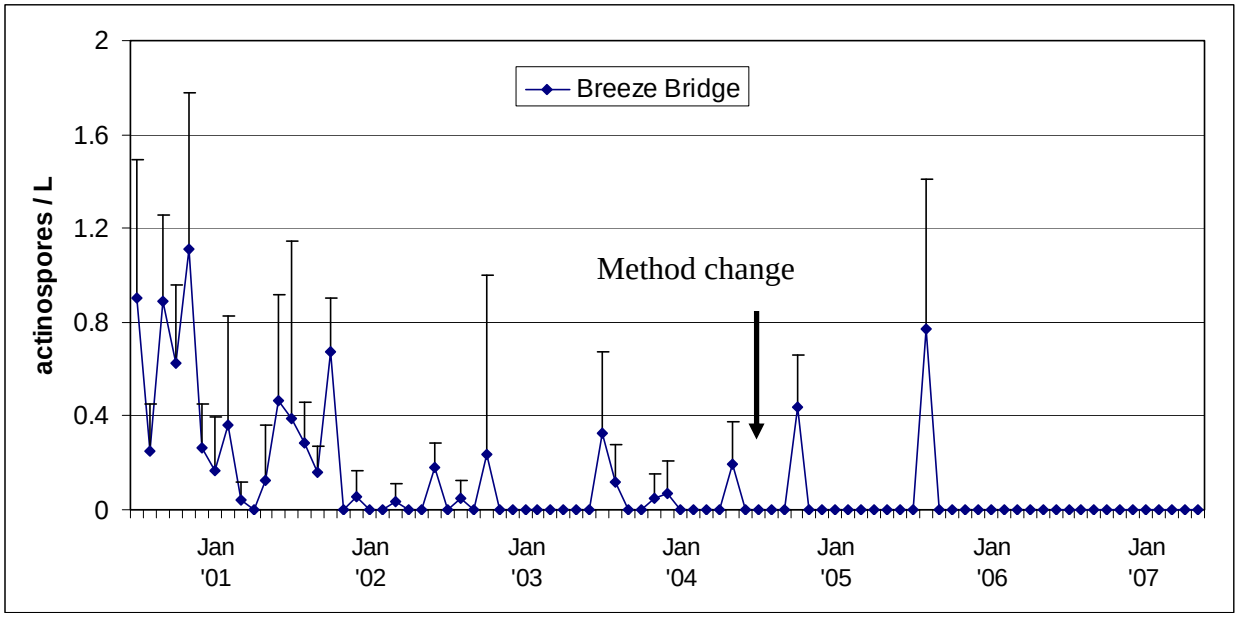

Figure 1.03. Results of water filtration to estimate ambient density of *M. cerebralis* actinospores (N/L) in the Colorado River at Breeze Bridge from July 2000 to May 2007. Error bars represent upper 95% confidence limit for the sample only based on subsample counts through June 2004. Samples taken in July 2004 and later were replicated so confidence limits apply to the stream.

Spring Creek (Taylor River drainage)

Habitat modifications occurred on this stream in October 2002. The brown trout population remains stable in this stream; by contrast, the rainbow trout population is sparse and consists largely of stocked catchable trout (Table 1.07). However, at the uppermost station below Spring Creek Reservoir we captured wild juvenile rainbow trout in 2006 and 2007. Rainbow trout of that age had not been seen in Spring Creek since 2001. The uppermost station is above the modified section of the stream.

Table 1.07. Trout population biostatistics for three sites upstream from, downstream from, and at Salsbury Gulch on Spring Creek, from fall electrofishing efforts. The treatment station 5 km below Spring Creek Reservoir was not sampled before 2002.

	Brown Trout					Rainbow Trout				
Year	N ≥ 15 cm	95% CI	Kg/ha	N/ha ≥ 15 cm	N/ha Age 1+	N ≥ 15 cm	95% CI	Kg/ha	N/ha ≥ 15 cm	N/ha Age 1+
	0.8 km downstream of Spring Creek Reservoir									
2000	284	± 3	419	4,742	4,264	0	---	0	0	0
2001	242	± 3	343	4,041	2,813	4	± 0	9	67	0
2002	265	± 2	506	5,725	4,571	0	---	0	0	0
2003	246	± 5	261	3,173	4,345	0	---	0	0	0
2004	231	± 1	258	2,967	2,323	0	---	0	0	0
2005	199	± 4	178	2,564	3,193	2	± 0	3	26	0
2006	190	± 1	253	2,450	2,809	6	± 2	18	77	13
2007	229	± 1	268	2,949	3,950	5	---	9	64	13
	5 km downstream of Spring Creek Reservoir at Salsbury Gulch (treatment)									
2002	393	± 1	329	2,861	1,182	0	---	0	0	0
2003	309	± 8	288	2,803	1,240	7	± 1	10	63	0
2004	363	± 4	326	3,290	1,875	53 [a]	± 0	83	480	0
2005	308	± 5	282	2,789	1,283	50 [a]	± 3	88	451	0
2006	273	± 7	305	2,473	853	63 [a]	± 1	114	589	36
2007	289	± 5	339	2,623	1,919	8	---	13	72	0
	19 km downstream of Spring Creek Reservoir (control)									
2000	172	± 3	180	1,532	1,448	2	± 0	6	18	0
2001	157	± 6	160	1,766	2,426	10	± 0	17	112	11
2002	175	± 5	207	2,105	1,814	207 [a]	± 1	427	2,435	24
2003	157	± 8	180	1,653	1,664	52 [a]	± 2	102	554	21
2004	146	± 5	124	1,538	1,245	71 [a]	± 4	124	748	0
2005	160	± 9	181	1,687	1,725	34 [a]	± 0	67	359	11
2006	169	± 9	171	1,781	2,104	21 [a]	± 1	39	223	0
2007	198	± 4	388	2,091	3,430	5	± 2	9	53	11

a: The majority of the rainbow trout comprising this population were stocked catchables.

The “post-treatment” samples of age 1+ brown trout collected in the last four segments show no meaningful reduction in prevalence or infection intensity compared to samples collected prior to habitat manipulation (Table 1.08). There is also no real evidence that there are any differences among the stations along the length of the study reach in prevalence and myxospore concentration. This circumstance makes it all the more surprising that there appear to be a few rainbow trout surviving into their second year and beyond in a stream exhibiting such high infectivity for *M. cerebralis*.

Table 1.08. Cranial *Myxobolus cerebralis* myxospore concentrations in age 1+ brown trout sampled from Spring Creek. Samples collected in 2004 and later comprise the post-manipulation data.

Date mm/dd/yy	N	Prevalence	Overall Mean Concentration	Std Dev	Positive Fish	
					Mean	Range
0.8 km downstream of Spring Creek Reservoir						
05/18/01	20	45%	6,500	14,351	14,400	1,400 – 56,000
08/01/01	20	80%	21,200	23,935	26,500	4,200 – 82,300
09/17/02	19	79%	43,900	55,638	55,700	2,000 – 195,000
09/22/03	23	78%	63,300	83,507	80,900	4,100 – 316,000
09/07/04	26	92%	50,700	58,484	54,900	4,400 – 56,700
09/21/05	20	65%	59,700	136,552	91,800	2,800 – 590,700
09/06/06	20	95%	80,400	132,678	84,600	1,700 – 500,000
09/25/07	20	70%	41,200	72,273	58,900	2,200 – 226,100
5 km downstream of Spring Creek Reservoir at Salsbury Gulch (treatment)						
05/18/01	20	90%	87,900	131,514	97,600	1,800 – 590,200
08/01/01	20	85%	67,300	97,912	79,200	3,900 – 401,000
09/17/02	20	85%	24,600	34,948	28,900	2,200 – 158,000
09/22/03	20	80%	39,600	50,202	49,600	2,700 – 151,600
09/07/04	20	100%	41,000	55,684	41,000	560 – 191,100
09/21/05	21	86%	64,900	100,682	76,900	7,100 – 422,500
09/06/06	20	95%	50,200	48,337	52,900	1,700 – 168,300
09/25/07	20	95%	43,300	49,042	45,600	4,500 – 197,000
19 km downstream of Spring Creek Reservoir (control)						
05/18/01	20	95%	57,000	37,174	60,000	15,200 – 173,200
08/01/01	20	90%	76,400	69,510	84,900	6,600 – 225,300
09/17/02	20	95%	13,200	11,484	13,900	1,300 – 30,300
09/23/03	20	90%	40,900	44,530	45,400	7,700 – 153,100
09/07/04	20	95%	53,300	64,998	56,100	4,400 – 212,200
09/21/05	20	90%	46,600	63,054	51,800	3,300 – 208,400
09/06/06	20	95%	52,300	49,850	55,000	1,700 – 178,400
09/25/07	20	75%	17,100	18,265	22,800	2,200 – 60,500

Samples of young-of-the-year (YOY) brown trout were collected at the same three sites in September of 2001 through 2006 (Table 1. 09). The YOY samples collected in 2003 were the first post-manipulation data. The heads were individually analyzed by the PCR technique and indicate

that there is a high prevalence of infection among YOY brown trout at all three sites for all years. The severity of infection among YOY at all stations for the last several years has been at or very near the top of the scale used to judge it. Young brown trout collected at the treatment site show as high a prevalence and severity as those collected at the un-manipulated sites.

Table 1.09. Results of polymerase chain reaction (PCR) tests of samples of young-of-the-year brown trout collected from Spring Creek. Samples collected in 2003 and later comprise the post-manipulation data. Mean PCR score is based on assigning numerical values to the qualitative score given to indicate strength of signal as follows: negative = 0, weak positive = 1, positive = 2, strong positive = 3, and very strong positive = 4.

Date	Sample size (N)	Positive fish	Mean PCR score
0.8 km downstream of Spring Creek Reservoir			
09/26/01	10	10	3.4
09/17/02	18	14	1.6
09/22/03	20	20	2.8
09/07/04	25	25	3.8
09/19/05	16	16	3.9
09/06/06	10	10	4.0
5 km downstream of Spring Creek Reservoir at Treatment site			
09/26/01	not sampled		
09/18/02	21	18	1.9
08/22/03	20	20	3.1
09/08/04	20	20	3.4
09/21/05	15	15	3.8
09/06/06	10	10	3.9
19 km downstream of Spring Creek Reservoir at Control site			
09/26/01	10	10	3.8
09/18/02	10	10	2.3
09/23/03	20	20	2.7
09/08/04	20	20	3.9
09/22/05	15	15	3.9
09/06/06	10	10	4.0

Water samples taken during the study indicated that habitat manipulation in Spring Creek did not result in reduced actinospore densities following construction. To the contrary, post-construction monitoring revealed a greater frequency of actinospore detection compared to pre-construction sampling for several years (Figure 1.04) at both the treatment and control sites. Only in 2006 and later did the frequency of detection and density fall to levels similar to those seen prior to construction.

One potential reason for the increased actinospore detections in the period after habitat manipulation could be the presence of infected rainbow trout catchables in the stream in 2003. Fourteen catchable trout captured at the control station in the fall of 2003 were tested for *M. cerebralis* by the PTD method. Eight fish were found to be positive, and the average for the

sample was 101,700 myxospores per head. Stocking records indicate that 1500 catchable rainbow trout were stocked into the stream on August 28, 2003. These very likely comprised the fish population that was sampled. Being stocked late in the summer, it is also possible that many of these fish were not creeled before fishing pressure dropped for the fall and winter. If so, a number of these rainbow trout perishing in the stream would have provided an extra infusion of myxospores to the environment in addition to what the resident brown trout population contributes. In contrast, little or no infectivity was found in stocked rainbow trout sampled in 2004 – 2006.

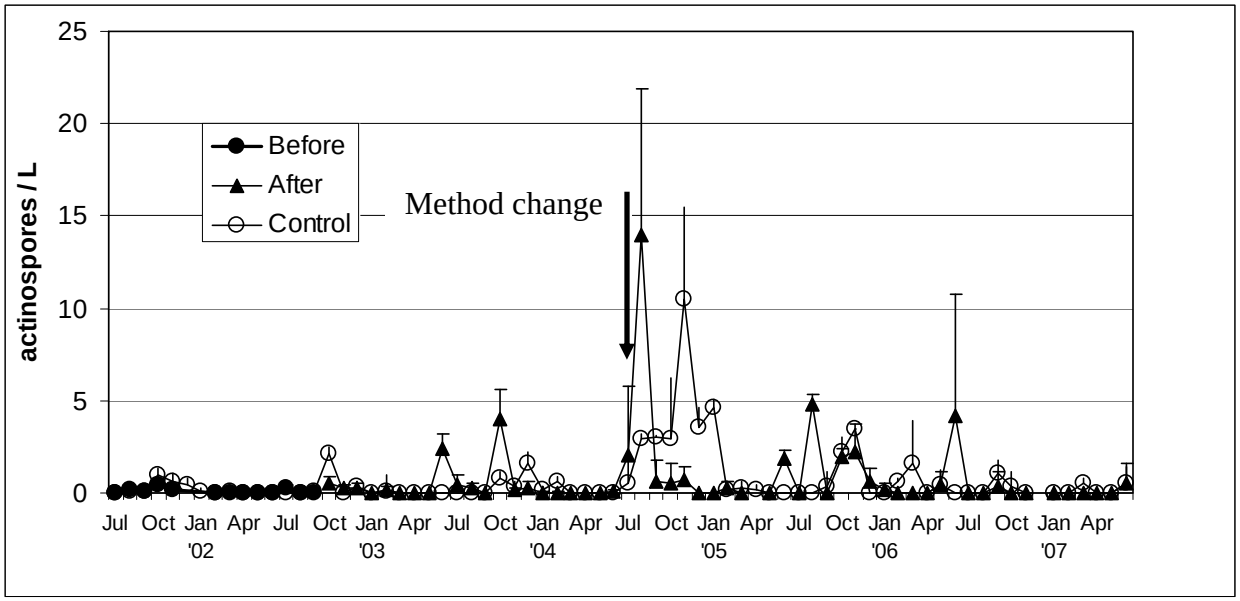

Figure 1.04. Density of actinospores observed in surface water samples collected at the Spring Creek treatment and lower control sites. "Before" designates the 15 months preceding construction at the treatment site.

Williams Fork River (Colorado River drainage)

Construction at the Williams Fork River site occurred during the first week of June 2002. Details of the habitat modifications and initial actinospore and oligochaete monitoring were presented previously (Nehring and Thompson 2003). Recent work on the Williams Fork River was limited to monitoring actinospore densities in surface water below the habitat modification site, collecting fish population information at two sites, and collecting age 1+ brown trout samples at three sites for myxospore information.

Trout population data were collected from the Williams Fork River for five years (Table 1.10). The rainbow trout population remains sparse. Biomass and overall density of rainbow trout remain consistently higher just below Williams Fork Dam versus below the habitat modification site. This is consistent with the hypothesis that the majority of present-day infectivity comes from within the river rather than Williams Fork Reservoir.

Table 1.10. Trout population biostatistics for two sites on the Williams Fork River below Williams Fork Reservoir.

	Brown Trout					Rainbow Trout				
Year	N	95% CI	Kg/ha	N/ha ≥ 15 cm	N/ha Age 1+	N	95% CI	Kg/ha	N/ha ≥ 15 cm	N/ha Age 1+
	0.3 km below Williams Fork Reservoir									
2002	269	± 6	279	1559	522	30	± 1	56	174	93
2003	999	± 9	816	5779	1003	24	± 3	45	138	74
2004	430	± 4	455	2490	213	33	± 2	70	188	54
2005	523	± 13	383	3028	666	24	± 5	55	137	71
2006	408	± 15	281	2361	456	25	± 2	40	147	29
	1.6 km below Williams Fork Reservoir, below habitat modification									
2002 [a]	593	± 15	651	2952	1600	25	± 1	56.8	125	55
2003	711	± 7	360	1811	1172	32	± 2	21	80	42
2004	472	± 8	373	1202	1336	21	± 2	21	54	3
2005	403	± 24	214	1026	796	33	± 7	13	83	79
2006	353	± 13	162	900	646	49	± 14	21	126	202 [b]

a: Station length 385 feet in 2002; 813 feet on all other occasions

b: The Williams Fork received nearly 10,000 rainbow trout fingerlings averaging 4.28 inches about 6 weeks before the electrofishing date; this circumstance is the explanation for the large estimate of age 1+ rainbow trout.

The high actinospore density observed 12 months post-construction still appears to be an aberration (Figure 1.05). Overall, we continue to detect actinospores less frequently than was the case before habitat modification. Although four of the eight highest actinospore densities observed have occurred after habitat modification, three of the samples were collected with the more sensitive technique used since July 2004 (Thompson 2005).

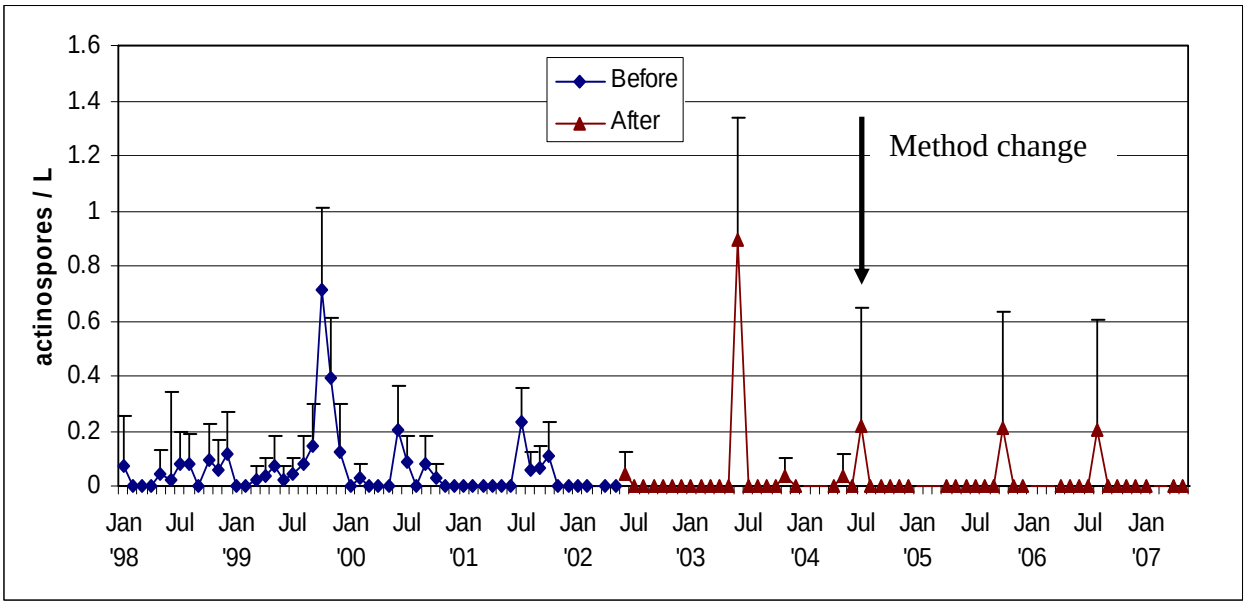

Figure 1.05. Density of actinospores observed in concentrates of surface water samples collected at the Williams Fork treatment site from January 1998 through May 2007. Error bars are 95% upper confidence limit for the sample only based on subsample counts through June 2004. Samples taken in July 2004 and later were replicated so confidence limits apply to the stream. "Before" designates samples collected prior to construction and "After" the samples collected following construction.

In recent years the prevalence and average myxospore concentrations have been moderate or low at all three sampling sites (Table 1.11). The brown trout collected in the fall of 2004 and later represent post-manipulation samples. Currently the trend below the treatment site suggests a decrease in prevalence of infection and an intensity of infection much lower than that seen before and just after habitat modifications. While this is encouraging, the data available from the upstream and downstream sites also hints that recent prevalence in those areas was decreased compared to previous years also, thereby once again casting doubt as to whether it was actually the habitat manipulation that was the primary cause of the observed trend.

Table 1.11. Cranial *Myxobolus cerebralis* myxospore concentrations in brown trout sampled from three sites in the Williams Fork River.

Date mm/dd/yy	N	Prevalence	Overall Mean Concentration	Std Dev	Positive Fish	
					Mean	Range
	0.3 km below Williams Fork Reservoir, above Treatment site					
09/13/01	15	13%	970	2,580	7,300	6,400 – 8,100
11/18/02	10	60%	6,200	12,982	10,400	2,000 – 42,400
11/18/03	20	35%	10,500	31,493	30,000	4,900 – 141,300
11/16/04	21	43%	710	1,219	1,700	560 – 4,400
11/15/05	20	55%	5,100	7,971	9,300	560 – 32,200
11/20/06	20	25%	3,300	9,898	13,100	1,700 – 43,800
11/14/07	Not collected					
	1.6 km below Williams Fork Reservoir, immediately below Treatment site					
08/06/01	20	45%	12,600	19,303	28,000	5,600 – 57,900
11/18/02	15	53%	26,900	87,813	50,500	1,900 – 342,700
11/18/03	20	80%	18,800	24,934	23,500	2,100 – 99,200
11/16/04	21	76%	12,200	20,689	16,100	560 – 66,700
11/15/05	20	55%	1,900	3,502	3,500	560 – 15,000
11/20/06	20	40%	2,500	4,930	6,300	1,700 – 20,200
11/14/07	20	25%	3,400	9,094	13,400	2,800 – 39,100
	2.6 km below Williams Fork Reservoir					
09/12/01	20	55%	21,600	30,693	39,200	4,300 – 113,700
11/18/02	15	53%	3,600	5,184	6,700	1,600 – 13,600
11/18/03	20	90%	14,300	16,537	15,800	2,900 – 61,500
11/16/04	20	60%	31,500	61,243	52,500	5,600 – 240,000
11/15/05	Not collected					
11/20/06	20	40%	6,000	11,449	14,900	3,400 – 33,700
11/14/07	Not collected					

Willow Creek (Colorado River drainage)

American beaver *Castor canadensis* activity in this study reach became so extensive in 2006 that the entire project area was inundated, rendering it useless for the purposes of this study. No oligochaete collections were made in 2006 or 2007 because it was no longer possible to evaluate the effect of the backwater isolation due to the flooding of the area by beaver ponds. It was also difficult to obtain adequate fish samples, and no YOY or age 1+ brown trout were encountered below the backwater in 2006, making it impossible to compare above versus below myxospore concentrations. No attempt was made to collect fish in 2007. The data collected previously are presented here (Table 1.12, Table 1.13). With habitat manipulation completed in 2003, brown trout samples would be considered post-manipulation beginning in 2005 for age 1+ and in 2004 for YOY.

Table 1.12. Cranial *Myxobolus cerebralis* myxospore concentrations in age 1+ brown trout sampled from Willow Creek.

Date mm/dd/yy	Age	N	Prevalence	Overall Mean Concentration	Positive Fish	
					Mean	Range
Above Willow Creek Gage						
09/30/03	1+	20	70%	21,400	30,600	2,600 – 194,700
09/29/04	1+	15	40%	8,100	20,100	5,000 – 64,500
10/17/05	1+	15	73%	3,800	5,200	560 – 12,200
09/26/06	1+	14	79%	13,900	17,800	1,700 – 47,100
Downstream of backwater site						
09/30/03	1+	20	60%	10,700	17,900	2,000 – 41,200
09/29/04	1+	10	30%	29,200	97,400	57,700 – 128,800
10/17/05	1+	13	38.5%	1,300	3,400	560 – 7,800
09/26/06	none encountered					

Table 1.13. Results of polymerase chain reaction (PCR) tests of samples of young-of-the-year brown trout collected from Willow Creek. Mean PCR score is based on assigning numerical values to the qualitative score given to indicate strength of signal as follows: negative = 0, weak positive = 1, positive = 2, strong positive = 3, and very strong positive = 4.

Date	Sample size (N)	Positive fish	Mean PCR score
Above Willow Creek Gage			
09/30/03	10	10	2.6
09/29/04	13	11	2.4
10/17/05	none encountered		
09/26/06	10	9	2.7
Downstream of backwater site			
09/30/03	11	7	1.7
09/29/04	20	16	2.9
10/17/05	1	0	0.0
09/26/06	none encountered		

DISCUSSION

The final project designed to isolate or remove discrete areas of preferred *T. tubifex* habitat from streams was constructed in the autumn of 2004 on the Poudre River near Kinikinik. Post-modification monitoring on all the sites has shown that the habitat modification strategy is unlikely to result in dramatic improvement of conditions for fish populations. Some indications have been positive, such as reduced actinospore detection in the Williams Fork River, reductions in the apparent amount of lineage III *T. tubifex* in Willow Creek and the Poudre River (the latter occurring before any habitat modifications were made), and the lower biomass of oligochaetes within the Spring Creek study site (Thompson 2004) following habitat improvements. The ultimate goal was evidence of reduced prevalence and severity of infection in the trout populations downstream of the project sites, and that goal has not been fully realized in a manner that supports habitat manipulations as the primary cause of change. This is evidenced not only by year-to-year comparisons in the study streams, but also in the fact that un-manipulated control sections exhibit the same sort of year-to-year variability in prevalence and concentration seen in the treatment sections. If improvement in the fish population could be asserted to have occurred on any study stream, it would be Beaver Creek, where somewhat higher age 1 rainbow trout densities have been observed in the last couple of years compared to prior years. But again, there is no assurance that habitat manipulation was a direct cause of the rainbow trout reappearance.

Over the last portion of this study the frequency of actinospore detection as well as estimated densities of actinospores fell at virtually all of the monitored study sites. This is certainly an encouraging development; however there has not been a concomitant notable drop in average prevalence or density of myxospores in individual brown trout heads, except possibly in the Williams Fork River. The reasons for the decrease in actinospores at monitoring sites are unclear but the phenomenon could very well be related to changes in oligochaete population composition. Increased proportions of resistant *T. tubifex* lineages in the study reaches would go far toward

explaining the phenomenon, and we suspect that such changes are occurring, but in most instances it is impossible to document because there is no baseline data to compare the recent data to with regard to worm lineage composition. The situation does suggest that low levels of actinospore density are sufficient to maintain moderate to high prevalence of infection in wild brown trout populations. Such low levels of actinospores are likely to be maintained by the many small areas of *Tubifex* habitat present in streams; the cumulative effect of many such small sites is capable of producing continued infection in wild trout populations even when isolated major habitats are removed or isolated.

At the annual Whirling Disease Symposium convened in Denver in February 2005, infectious disease authority and keynote speaker Dr. Paul Ewald (University of Louisville) noted that the two spores involved in the transmission of the parasite from host to host employ differing strategies. The myxospore is thought to be rather immobile once it is deposited, thus the transmission technique is to "sit and wait" for a suitable host to encounter it. Typically, disease agents characterized by this sort of transmission have a high impact on the host (Ewald 1994). In contrast, the actinospore is waterborne and disease agents characterized by this method of transmission generally have a lesser impact on the host than do "sit and wait" disease agents. Dr. Ewald asserted a focus on resistance to the parasite in the hosts would be the most productive avenue of research. For the trout host, this would suggest continued research into a resistant rainbow trout as a primary component of many important sport fisheries throughout North America.

An avenue of host resistance largely unexploited to date lies in the oligochaete hosts. Only recently has it become apparent that differences in susceptibility of *T. tubifex* to the parasite are lineage-related (Beauchamp et al. 2002, Hallett et al. 2008). This evidence, coupled with the knowledge that we have a number of sites where to date only the susceptible lineage III has been documented (Thompson 2005, Nehring 2005), leads to the conclusion that research into taking advantage of worm host resistance may be productive. While a resistant rainbow trout may be a suitable answer to the whirling disease problem in many waters, they would not be an acceptable solution in native cutthroat habitat. In such places it would be more desirable to displace susceptible worm hosts with non-susceptible ones. Additionally, new evidence is emerging that indicates lineages I and VI are much more resistant to infection than previously thought (Hallett et al. 2008). Currently, this avenue of research holds more promise than does small-scale habitat manipulation intended to remove oligochaete habitat.

Job No 2: Actinospore Hot Spot Abatement Studies.

Job Objective: Develop and test strategies to reduce, control, or eliminate the production of triactinomyxon actinospores of *Myxobolus cerebralis* from man-made ponds and settling ponds known to be focuses of infectivity.

Period Covered: Final summary for study period July 1, 2003 to June 30, 2008

INTRODUCTION

Whirling disease is a serious malady of some salmonid fishes that can result from exposure of susceptible salmonid fry or fingerlings to the waterborne actinospore of the myxosporean parasite *Myxobolus cerebralis* (Wolf and Markiw 1984; Markiw 1991). Phagocytic vegetative stages of the parasite feed on cartilage in young trout. A granulomatous inflammatory response usually develops in peripheral tissues adjacent to sites of infection. Destruction of the cartilage by the parasite interferes with normal bone development and can result in skeletal and cranial deformities. Young fish that are infected may display an erratic swimming behavior known as "whirling", hence the name whirling disease. Rose et al. (2000) suggested that the cause of the erratic swimming pattern is inflammatory response to parasite activity in the cranial and anterior spinal region, resulting in multiple compressions of the spinal cord.

Once considered an aggravating nuisance for salmonid aquaculture, it is now recognized that this disease can significantly impact wild trout populations (Walker 1997; Hedrick 1998). Nehring and Thompson (2001) found no substantive evidence that any environmental perturbation or stressor other than *M. cerebralis* adequately explained the recurring losses of young wild rainbow trout observed on nearly 600 km of Colorado's premiere trout streams. In some instances in Colorado, off-channel sources of infectivity have apparently influenced the rate and intensity of infection in trout. In the Fryingpan River, abundance of age 1 wild rainbow trout in the 15-km reach upstream from its confluence with the Roaring Fork River declined 90% between 1994 and 1998 (Nehring 1999). That trend continued in 1999, 2000, and 2001. A localized area of *Myxobolus cerebralis* infectivity emanating from a series of off-channel ponds was documented (Nehring et al. 2000). The most severe reduction in abundance of age 1 wild rainbow trout has occurred downstream of this focus of infection, suggesting that whirling disease induced the decline.

Fish rearing facilities may also contribute infectivity to waters receiving settling pond effluent. The number of State-owned rearing units experiencing parasite infestations peaked in 1998 at 11 facilities. Currently the number stands at five that actually stock fish; three of those are working toward *M. cerebralis*-free status. However, in some cases rearing units are free of the parasite but the settling ponds are not. In other cases there is no expectation of ever succeeding in freeing the rearing unit of the parasite.

The objective of this job was to document the changes in *M. cerebralis* infectivity that may occur in response to management actions on such off-channel sites, and to help develop best management practices for such sites.

METHODS and MATERIALS

Field Filtration and Sample Collection

Two 120-L volumes of water (1900 L prior to July 2004) were filtered monthly at each study site (see Appendix Table A2.) through 20-um Pecap screen to concentrate actinospores. These concentrates were examined for the presence of *M. cerebralis* actinospores in the lab by established protocols (Thompson and Nehring 2000). Actinospores of *M. cerebralis* were identified on the basis of general appearance, overall conformation, size and shape according to descriptive criteria in El-Matbouli and Hoffmann (1998). However, size was considered to a lesser degree than conformation because more recent evidence shows that there may be considerably more variability in the size of *M. cerebralis* triactinomyxons than previously thought (Hallett et al. 2004).

A single 1.6-mL sample (equal to the volume examined from 20 aliquots) of filtrate from some field samples was subjected to the polymerase chain reaction (PCR) test to determine whether samples contained the DNA of *M. cerebralis.* Since April 2001, we have used a PCR test developed by Pisces Molecular, Inc., that amplifies a segment from a heat shock protein gene of *M. cerebralis* designated as hsp70. Each sample tested by PCR was preserved in 70% ethyl alcohol in a 15-mL centrifuge tube, and was identified only by alphanumeric code when sent to the laboratory.

Fish Removal

Recognizing that *M.* cerebralis-free rearing units may still have settling ponds that are infected, in some cases fish were removed from these ponds in order to reduce the probability of continued infectivity contributed to receiving waters. Shore-based and backpack electrofishing equipment was used to accomplish fish removal from the Roaring Judy effluent channel. Pitkin Rearing Unit personnel accomplished fish removal in the Pitkin settling pond using gill-nets. Although not a rearing unit, fish were also removed from the Cap-K Ranch ponds. Here, brook trout were removed while rainbow trout and brown trout were not removed.

Oligochaete Sampling

Oligochaete populations were qualitatively assessed at some rearing stations in order to determine the relative proportions of the various lineages, and hence the danger posed by the possible infusion of myxospores. Oligochaete samples were collected from various sites in the effluent system of each unit by sampling suitable habitat in a number of locations within each pond or channel. These samples were combined for each location of interest (pond, portion of channel, side of pond), and then replicate samples of 50 haired oligochaetes were picked from each, provided the worms were available. These samples were subjected to the quantitative PCR test to determine

lineage composition of the DNA in the sample.

RESULTS and DISCUSSION

Cap-K Ranch Ponds (Fryingpan River drainage)

The Cap-K Ranch ponds are in a series designated by numbers 1 – 6, with pond 1 at the top and pond 6 at the terminus of the series. The effluent from pond 6 returns to the Fryingpan River, although the capability exists to divert pond water back to the Fryingpan River before it enters Pond 5. It is this pond complex that was identified by Nehring et al. (2000) as the only substantial contributor of actinospores to the Fryingpan River from off-channel sources after 12 months of monitoring. Filtration data obtained since July 2000 from ponds 1 and 2 indicates that pond 2 continued to be a consistent producer of actinospores throughout the study (Figure 2.01).

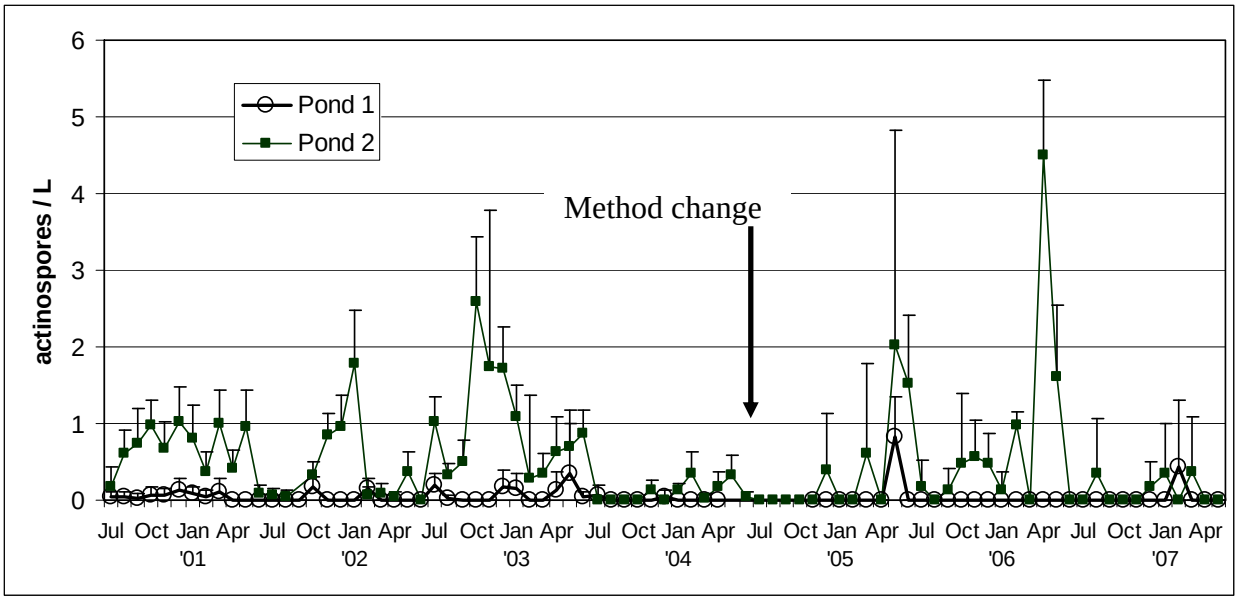

Figure 2.01. Estimates of *M. cerebralis* actinospore density in samples of water in the effluents of Cap-K Ranch ponds 1 and 2. Error bars are 95% upper confidence limit for the sample only based on subsample counts through June 2004. Samples taken in July 2004 and later were replicated so confidence limits apply to the body of water.

Pond 2 was electrofished during March and April of each year since 2002 to remove brook trout fry. Thousands of fry were removed from the system in this manner. By reducing the population of this susceptible species it was hoped that infectivity in the system would also be reduced, however actinospores were still commonly detected in pond 2 up until the conclusion of water monitoring (Figure 2.01).

Pond 6 was modified during February and March of 2003 to prevent the introduction of actinospores from the pond complex to the Fryingpan River. A description of the sand-based filter installed was previously provided (Nehring and Thompson 2003). The filter was short-lived in its

effectiveness and is no longer in service (Thompson 2006). The filter proved to be inadequate for the volume, prone to rapid plugging, and difficult and time-consuming to back-wash. Eventually actinospores were detected in the effluent. It is likely that these actinospores made it through the filter via cavities produced in the sand bed over time by water action.

Water filtration sampling occurred each month on the Fryingpan River from July 2000 through May 2007. Two locations 1.9 km upstream and 4.3 km downstream of the Cap-K Ranch served as evaluation sites for the manipulations that occurred on the Cap-K Ranch (Figure 2.02). Actinospores were detected infrequently at both locations prior to July 2004, and more frequently afterward. This change was likely due in large part to the implementation of the more sensitive detection technique in July 2004.

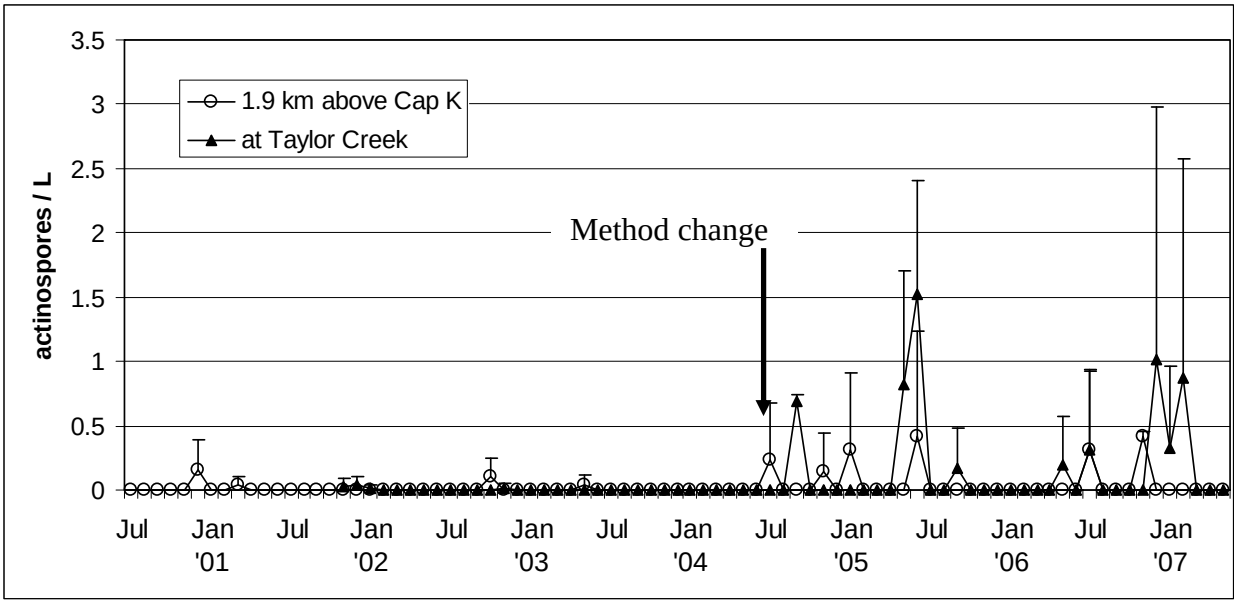

Figure 2.02. Results of water filtration to estimate ambient density of *M. cerebralis* actinospores (N/L) at three sites in the Fryingpan River from July 2001 to May 2007. Error bars are 95% upper confidence limit for the sample only based on subsample counts through June 2004. Samples taken in July 2004 and later were replicated so confidence limits apply to the stream.

Samples of age 1+ brown trout were obtained each year from sites in the Fryingpan River above and below the Cap-K Ranch (Table 2.01). The first three years of brown trout samples were pre-manipulation (construction of the filter in Pond 6), and the latter three years were post-manipulation. These samples indicate that prevalence of parasite infection remains high and fairly stable at the two lower collection sites. Once again, it is apparent that the manipulations at the Cap-K Ranch did not result in the dramatic reduction in parasite prevalence and infection intensity that was hoped for.

It appears from the myxospore data that the parasite invasion was still moving upstream during the early stages of this study. This conclusion is supported by the lack of infected fish at the uppermost station in 2000, and only one infected fish at the site 1.6 Km above the Cap-K Ranch. Both of these sites experienced increase in both prevalence and intensity over the first

couple years of the study.

Table 2.01. Cranial *Myxobolus cerebralis* myxospore concentrations in brown trout sampled from locations in the Fryingpan River above and below Cap-K Ranch from 2000 to 2006.

Date mm/dd/yy	N	Prevalence	Overall Mean Concentration	Std Dev	Positive Fish	
					Mean	Range
	1 Km below Ruedi Dam					
10/28/00	10	0%	---	---	---	---
10/30/01	11	36.4%	7,800	14,232	21,500	2,780 – 35,800
10/29/03	20	45.0%	38,500	120,098	85,600	4,880 – 541,300
10/26/04	23	82.6%	35,600	56,044	33,200	560 – 254,400
11/02/05	15	53.3%	3,700	6,403	6,900	560 – 20,600
11/02/06	20	50.0%	10,600	17,731	21,200	1,700 – 55,600
	1.6 Km above Cap-K Ranch					
10/28/00	10	10.0%	26,900	85,171	269,300	269,300
10/30/01	10	60.0%	15,700	24,947	26,100	2,670 – 71,000
10/29/03	20	55.0%	21,800	35,546	39,600	4,560 – 112,300
10/26/04	21	85.7%	45,600	53,243	55,200	1,670 – 197,800
11/02/05	20	90.0%	18,100	41,412	20,100	1,100 – 173,300
11/02/06	20	95.0%	71,800	67,683	75,600	3,400 – 244,100
	Taylor Creek confluence					
10/31/00	9	55.6%	37,700	62,282	67,800	9,300 – 181,900
10/30/01	11	63.6%	35,900	48,312	56,400	2,500 – 147,500
10/29/03	20	80.0%	29,600	63,280	37,000	1,500 – 189,900
10/27/04	20	85.0%	16,000	18,225	18,800	1,100 – 60,000
11/02/05	20	85.0%	10,600	18,403	12,400	1,100 – 82,200
11/02/06	19	78.9%	21,400	28,456	27,200	1,700 – 104,400

Chalk Cliffs Rearing Unit

The Chalk Cliffs unit is one of the rearing facilities that the Colorado Division of Wildlife recognizes will most likely never be *M. cerebralis*-free. Consequently minimizing any potential downstream impact has relied on the implementation of best management practices such as increasing the size at which young fish were moved from inside raceways to outside dirt rearing ponds. Such practices have been helpful both in reducing the spore levels in fish that are stocked from this unit (always in low elevation waters with no wild trout habitat downstream) and in keeping actinospores minimized in the effluent (Nehring and Thompson 2003).

With the advent of the capability to test oligochaete worms for lineage identity in recent years, several of the rearing ponds and the effluent pond at Chalk Cliffs were sampled later in the study to characterize the *Tubifex* populations in them. The oligochaete community has been widely sampled at Chalk Cliffs on three occasions, and archived DNA from two earlier occasions in the settling pond was also analyzed; from these samples, it is clear that lineage V worms are not present (Table 2.02). The other lineages vary considerably both between ponds and over time within a pond (see Figure 2.03 for the settling pond). Despite efforts to collect worms in each pond from a variety of locations, it is possible that the figures presented in Table 2.02 are distorted by the patchiness that characterizes oligochaete populations as well as the small sample size in each pond.

Table 2.02. *Tubifex* lineage composition estimated by qPCR on replicate 50-haired worm samples from each of four production ponds and the settling pond at Chalk Cliffs Rearing Unit.

Location	N	Approximate percent DNA composition by *M. cerebralis* lineage			
		I	III	V	VI
12/13/2005					
Pond 1	2	32.5	41.5	0.0	25.5
Pond 3	2	0.0	46.0	0.0	54.0
Pond 5	2	0.0	28.5	0.0	71.5
Pond 7	2	---	---	---	---
Settling Pond	2	6.0	1.0	0.0	93.0
04/26/2006					
Pond 1	2	17.5	39.0	0.0	43.5
Pond 3	2	1.5	75.0	0.0	23.0
Pond 5	2	0.0	84.0	0.0	16.0
Pond 7	2	0.0	41.0	0.0	59.0
Settling Pond	2	8.0	29.0	0.0	63.0
12/18/2006					
Pond 1	2	35.0	9.0	0.0	56.0
Pond 3	2	0.0	42.5	0.0	57.5
Pond 5	2	0.0	49.5	0.0	50.5
Pond 7	2	0.0	6.5	0.0	93.5
Settling Pond	2	40.5	11.0	0.0	48.5

One encouraging note on the sampling done over the last couple of years is the generally high proportion of non-susceptible worms occurring in the settling pond (Figure 2.03). This fact may explain why previous investigations failed to detect actinospores in the effluent of the settling pond despite the presence of numerous fish hosts in that pond. The settling pond was filtered from November 2000 through June 2002 (Nehring et al. 2001, Nehring and Thompson 2002). Actinospores were detected on seven of the first nine occasions, but none were detected on the last 11 occasions from August of 2001 through the end of the sampling. Nehring and Thompson (2003) credited certain best management practices for the reduction in actinospore detection and density. In retrospect, it seems also possible that changes in *Tubifex* lineage composition may have contributed to the observed reduction, as it appears certain that it has in Windy Gap Reservoir (Winkelman et al. 2007).

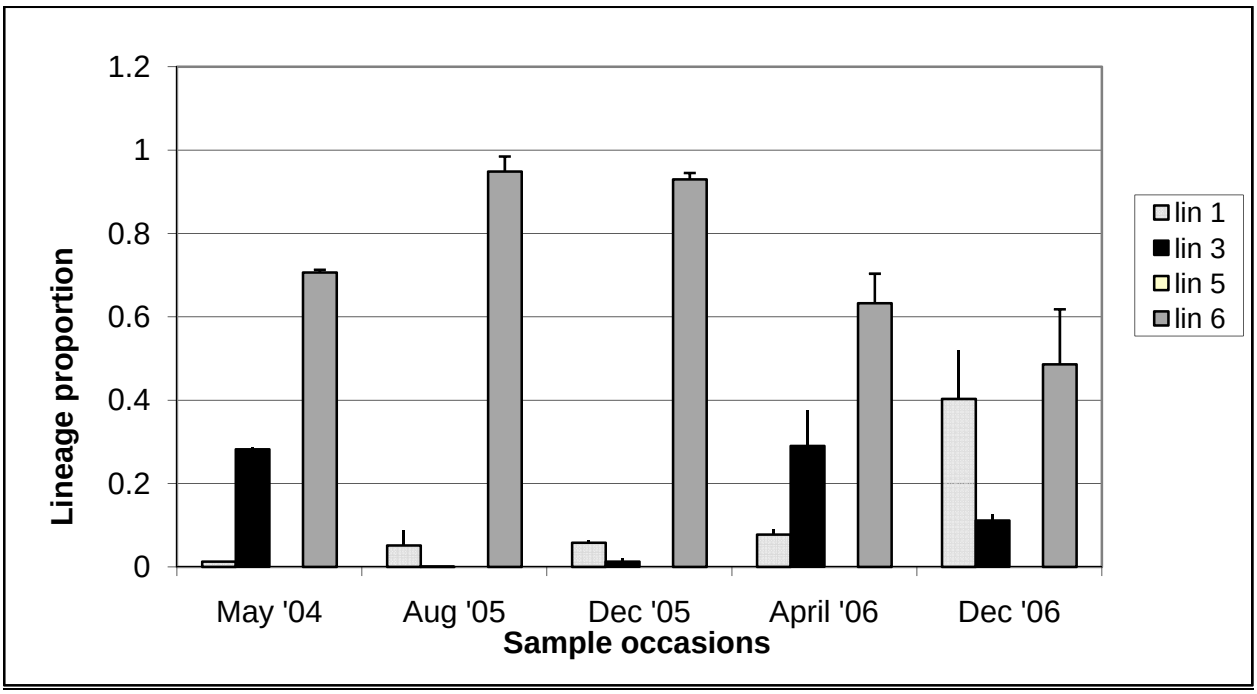

Figure 2.03. Estimates of the relative proportion of DNA specific to each lineage found in *T. tubifex* samples collected from the settling pond at Chalk Cliffs Rearing Unit.

Pitkin Rearing Unit

Trout reared at the Pitkin Rearing unit first tested positive for *M. cerebralis* in March 1997. The unit was taken out of production in 2001 and extensive renovation, modernization and securing of springs and well-water supplies was accomplished. The use of Quartz Creek surface water for rearing fish was discontinued upon re-start of the unit. The unit regained *M. cerebralis*-negative certification in January 2007.

Monitoring of actinospore densities began at the Pitkin Rearing Unit in November 2001 and continued during this segment. Actinospores of *M. cerebralis* were observed on two occasions in the effluent of the settling pond and on one occasion in Quartz Creek above the hatchery effluent (Figure 2.04). On all occasions the estimated densities were low.

Pitkin Unit personnel removed all feral fish from the unit's settling pond during unit renovation in 2001-02 (in May 2008 rising fish were observed in the pond, indicating a potential need for periodic removal efforts). The greatly diminished myxospore source available to the *T. tubifex* community residing in the settling pond has had a positive impact on the infectivity observed in the effluent. The oligochaete population in the settling pond is robust and the pond was sampled three times during last two segments of this project in order to determine the composition of the *Tubifex* community (Table 2.03). Oligochaetes are abundant in the pond, including those with hairs and pectinate chaetae which would initially indicate *T. tubifex*. Surprisingly, though, the samples of worms collected from the pond often indicated very low worm equivalent values, indicating that many of the worms may actually be something other than *T. tubifex*. Those that do test as *T. tubifex* overwhelmingly were lineage III. All samples were tested for the presence of *M. cerebralis* DNA and all were negative for each of the three sampling occasions, indicating a low prevalence of infection among worms inhabiting the Pitkin settling pond. Taken together, these two results bode well for continued low actinospore output from the Pitkin Rearing Unit settling pond.

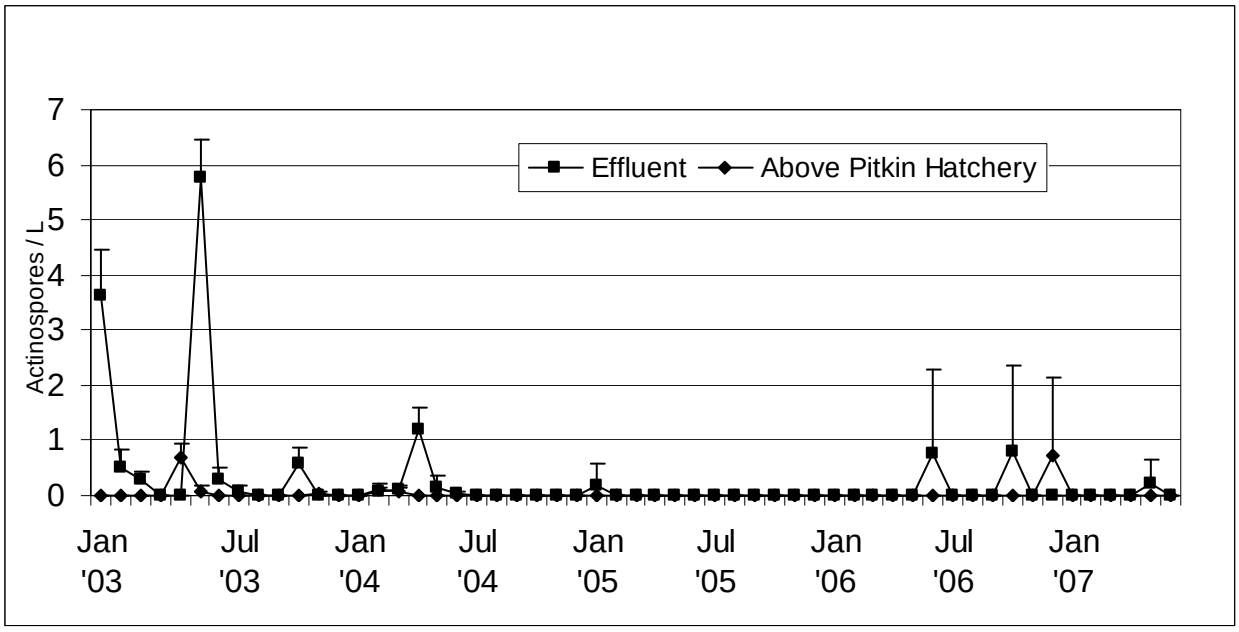

Figure 2.04. Results of water filtration to quantify actinospores of *M. cerebralis* in samples of water at Pitkin Hatchery, January 2003 through June 2007.

Table 2.03. *Tubifex* lineage composition estimated by qPCR on replicate 50-haired worm samples from three sectors of the settling pond at Pitkin Rearing Unit.

Location	N	Approximate percent DNA composition by *M. cerebralis* lineage				Worm equiv.
		I	III	V	VI	
			May 2007			
West	1[a]	0.0	100.0	0.0	0.0	1.3
South	1[a]	0.0	100.0	0.0	0.0	17.3
East	2	0.0	100.0	0.0	0.0	10.9
			July 2007			
West	0[a]	---	---	---	---	0.00
South	2	0.0	100.0	0.0	0.0	11.0
East	2	0.0	100.0	0.0	0.0	10.6
			May 2008			
West	3	0.0	100.0	0.0	0.0	11.8
South	2	0.0	100.0	0.0	0.0	6.2
East	3	0.0	100.0	0.0	0.0	16.4

a: One or both worm samples contained so little *T. tubifex* DNA that they were reported as "0.0 worm equivalents", consequently they contain no useful information on lineage composition.

Samples of brown trout were collected from Quartz Creek approximately one mile above and below Pitkin Rearing Unit each year of the study (Table 2.04). The prevalence and average concentrations at both sites have fluctuated considerably. Trends at the two sites have been similar, suggesting that factors other than Pitkin Rearing Unit effluent are at this juncture largely responsible for the parasite dynamic in this stream.

Table 2.04. Cranial *Myxobolus cerebralis* myxospore concentrations in age 1+ brown trout sampled from Quartz Creek above and below the Pitkin Fish Rearing Unit.

Date mm/dd/yy	N	Prevalence	Overall Mean Concentration	Positive Fish		
				Std Dev	Mean	Range
		Upstream of Pitkin Rearing Unit				
08/28/03	20	10.0%	2,900	9,153	29,40	25,300 – 33,500
08/09/04	20	85.0%	15,400	14,480	18,10	1,700 – 50,100
08/17/05	20	40.0%	17,400	42,768	43,60	2,500 – 151,500
09/05/06	20	70.0%	30,900	51,865	44,10	1,700 – 222,200
08/27/07	20	25.0%	2,900	5,849	11,80	5,900 – 19,600
		Downstream of Pitkin Rearing Unit				
08/28/03	20	45.0%	10,200	15,885	22,70	4,900 – 59,400
08/09/04	20	95.0%	67,200	111,812	70,70	1,500 – 489,300
08/17/05	20	60.0%	10,400	16,350	17,30	2,800 – 68,800
09/05/06	20	60.0%	22,500	48,460	37,50	1,700 – 200,300
08/27/07	20	25.0%	800	1,610	3,100	2,000 – 5,900

Poudre Rearing Unit

Actinospore monitoring began at several sites on the Poudre River in 1997. The data from 1997 through June 2001 indicated that the Poudre State Fish Rearing Unit (PRU) had become a major point source of *M. cerebralis* actinospore production. This resulted in severe infection in brown and rainbow trout downstream from the unit compared to upstream (Nehring et al. 2001; Schisler 2001).

The frequency that actinospores of *M. cerebralis* were encountered during the last portion of this study was greatly diminished compared to previous years (Figure 2.05). Estimated densities remained low in the PRU effluent compared to the historic high numbers seen in 1999-2000 when it was not uncommon to observe > 10 actinospores / L (Nehring et al. 2000, 2001).

The modification of the supply pipeline system at the Poudre Unit was completed in 2005. The unit now uses water directly from the Poudre River rather than from the supply pond except during critical months when warmer water from the supply pond is needed to prevent icing problems. Monitoring of the supply pond ceased after November 2005.

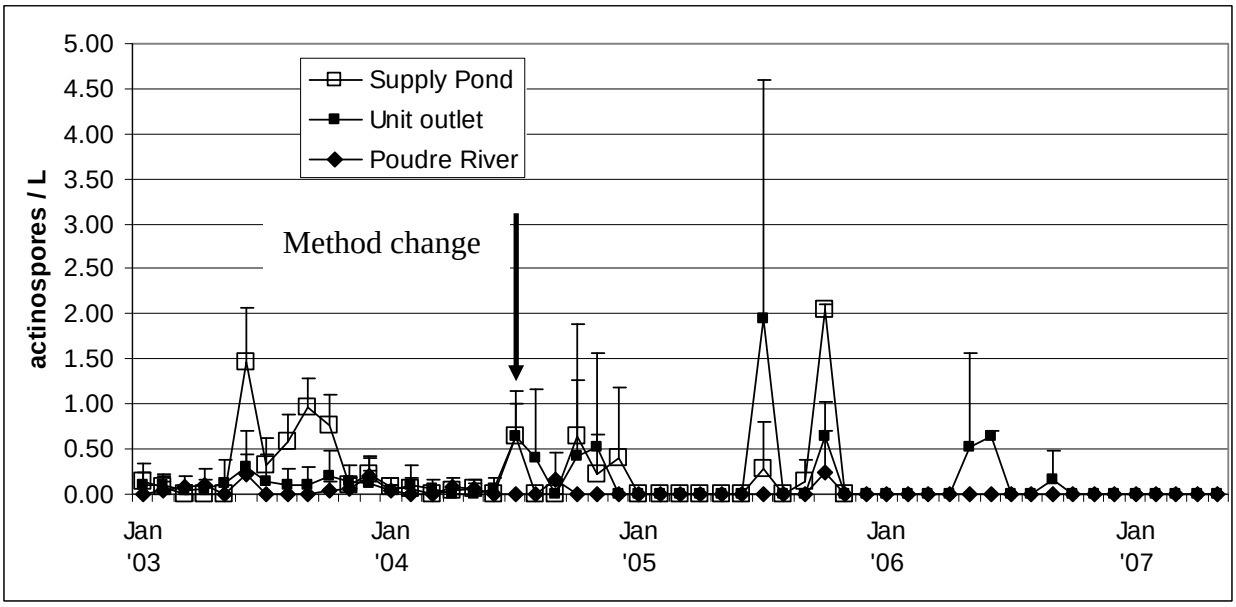

Figure 2.05. Comparison of actinospore densities from the Poudre River, the Supply pond, and the Unit effluent through May 2007. Error bars are 95% upper confidence limit for the sample only based on subsample counts through June 2004. Samples taken in July 2004 and later were replicated so confidence limits apply to the body of water.

Samples of brown trout were obtained above and below PRU throughout the study, although the site above the unit was not sampled in 2007 (Table 2.05). Prevalence of infection was higher at both sites late in the study, a result that appears at odds with the observed actinospore densities in the river and the rearing unit effluent. Acknowledging that sample sizes are generally smaller from this stream than from others, average myxospore concentrations were quite similar above and below PRU. An outlier at the Big Bend site in 2006 had considerable effect on the average; without it the sample average decreases to 12,900 myxospores. This figure is quite close to the average for the sample below PRU in that year.

Table 2.05. Cranial *Myxobolus cerebralis* myxospore concentrations in age 1+ brown trout sampled from the Poudre River above and below the Poudre Rearing Unit (PRU).

Date mm/dd/yy	N	Prevalence	Overall Mean Concentration	Std Dev	Positive Fish	
					Mean	Range
			Big Bend – above PRU			
09/19/00	10	50%	6,300	11,675	12,600	990 – 37,600
10/22/03	12	41.7%	3,900	6,029	9,400	920 – 16,000
10/28/04	15	40.0%	17,100	30,141	42,900	5,600 – 92,300
11/02/05	15	60.0%	3,600	7,139	6,000	560 – 27,200
10/16/06	10	80.0%	50,600	135,248	63,200	3,400 – 439,400
10/16/07	Not collected					
			Pasquinel's cabin – below PRU			
09/19/00	9	22.2%	4,300	11,602	21,000	3,900 – 35,100
10/22/03	21	14.3%	1,800	5,078	12,600	6,900 – 21,000
10/28/04	11	54.5%	10,700	17,865	19,500	1,700 – 59,000
11/02/05	15	60.0%	3,500	7,309	5,900	560 – 27,200
10/16/06	7	100.0%	13,500	21,248	13,500	1,700 – 60,600
10/16/07	15	73.0%	35,800	56,120	48,800	5,600 – 223,600

Roaring Judy Rearing Unit

Inspection records at the CDOW Aquatic Animal Health Laboratory show trout from the Roaring Judy State Fish Rearing Unit (ROJ) first tested positive for the presence of *M. cerebralis* in early 1992. Those same records indicate the parasite was detected in free-ranging rainbow trout collected from Meridian Lake in the Slate River drainage, tributary to the East River near Crested Butte, in 1988. Meridian Lake, about 25 km upstream of ROJ, was stocked with rainbow trout by a private aquaculturist whose facility tested positive for the parasite in late 1987.

While the Roaring Judy Unit regained certification as a *M. cerebralis*-free facility in the spring of 2005, that designation was lost in 2006. Despite that setback, research conducted on the facility over the course of this project resulted in methods and management strategies to minimize the number of actinospores in the settling pond effluent. Monthly monitoring throughout the study showed that detection of actinospores in the unit effluent decreased both in frequency and intensity over time (Figure 2.06). This is likely a response to at least two changes occurring in the ponds; first, the recent stringent stocking scheme to ensure that fewer catchable rainbow trout remain in the ponds after the end of the summer fishing season, and second, a likely response of the oligochaete population to the long lasting parasite presence there manifested as an increase in the proportion of non-susceptible oligochaetes.

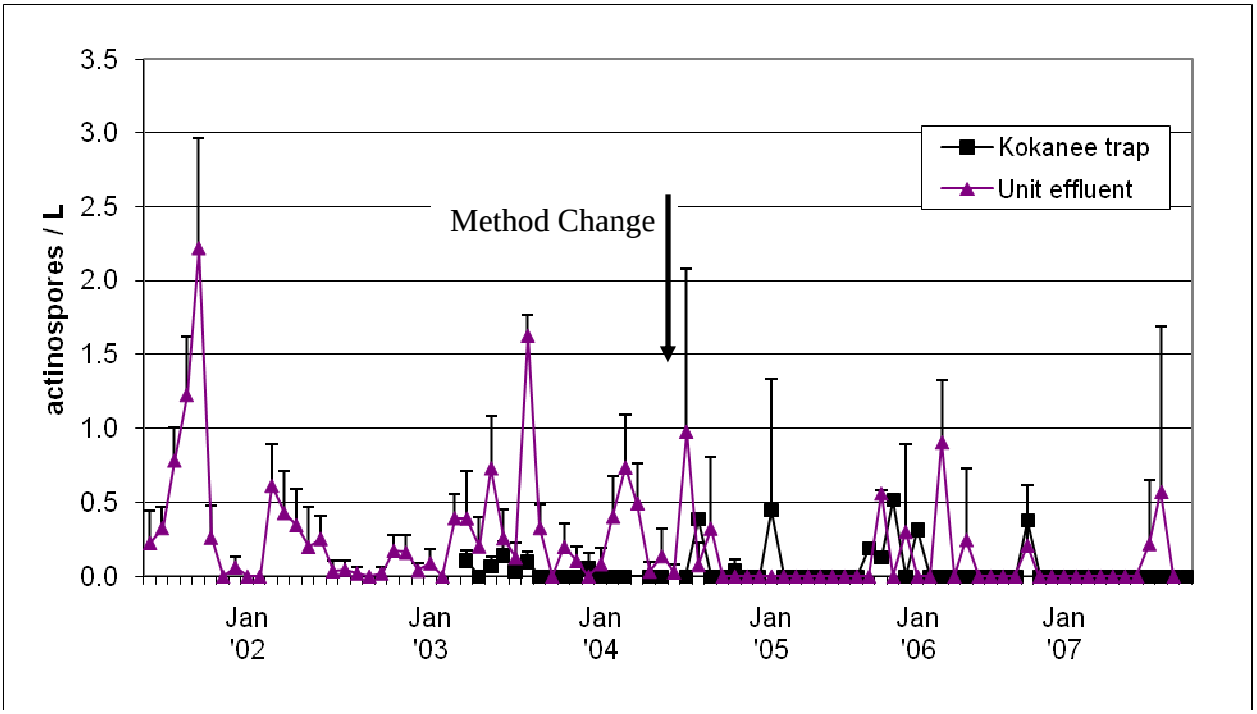

Figure 2.06. Comparison of actinospore densities from the ROJ kokanee trap and the Unit settling ponds effluent through May 2007. Error bars are 95% upper confidence limit for the sample only based on subsample counts through June 2004. Samples taken in July 2004 and later were replicated so confidence limits apply to the stream.

Over the years, samples of the trout removed from the effluent channel show that prevalence and intensity of *M. cerebralis* infection can be substantial (Table 2.06). However, the more recent samples of older rainbow trout show lower prevalence and intensity of infection after the removal efforts that occurred in 2003 and 2004. This population should continue to be monitored for myxospore concentration and parasite prevalence. If these metrics rise in the future, periodic fish removals may be prudent to eliminate heavily infected fish from the channel.

Table 2.06. Cranial *Myxobolus cerebralis* myxospore concentrations in trout sampled from the Roaring Judy State Fish Rearing Unit effluent channel.

Date mm/dd/yy	Age	Sample		Overall Mean Concentration	Std Dev	Positive Fish	
		N	prevalence			Mean	Range
		Brown trout					
05/16/03	1	12	75%	39,900	63,104	53,200	2,000 – 177,750
11/25/03	1+	20	70%	29,700	37,659	42,400	4,400 – 150,700
05/24/04	1	20	75%	23,500	44,594	31,300	300 – 161,900
11/30/04	1+	20	75%	25,700	47,209	34,300	1,100 – 193,300
11/22/05	1+	19	68%	37,500	78,772	54,700	2,900 – 335,800
11/30/06	1+	20	35%	25,500	34,616	39,200	3,100 – 101,400
10/24/07	1+	20	60%	18,300	28,849	30,500	2,800 – 111,800
		Rainbow trout					
05/16/03	2	21	100%	367,400	642,553	367,400	3,700 – 2,242,500
11/25/03	1+	22	50%	57,700	133,038	115,300	5,400 – 597,400
05/24/04	1	20	5%	100	414	1,850	1,850
05/24/04	2	20	80%	457,800	797,981	572,200	4,200 – 3,111,800
11/30/04	1+	20	20%	9,600	35,088	47,800	5,500 – 157,000
11/30/04	2+	25	12%	3,700	16,064	30,600	5,000 – 80,400
11/22/05	2+	25	36%	21,400	50,864	59,400	7,400 – 234,600
11/30/06	2+	25	20%	7,500	20,611	37,500	3,100 – 76,800
10/24/07	2+	25	28%	29,400	121,167	105,000	2,800 – 606,400

The two west settling ponds were stocked with 3600 fin-clipped catchable Tasmanian strain rainbow trout from the Roaring Judy Rearing Unit in May and June 2007, a slightly lower stocking rate than the previous two years. Samples of the remaining fish were collected in November during the kokanee spawn, having followed the kokanee into the trap. As in all years under this stocking scheme, the samples showed low prevalence and myxospore concentrations (Table 2.07). In contrast, feral rainbow trout captured in the kokanee trap showed higher average myxospore concentrations. This reflects perfectly the strategy that stocking rainbow trout for recreational opportunity in this situation should not result in fish at large carrying higher burdens than those represented in the feral population present.

It should also be noted that prevalence and intensity of infection among the feral rainbow trout collected from the settling ponds appeared to attenuate over the last several years of sampling compared to earlier years. From 2003 through 2005 the intensity of infection was considerable (noting the 2004 sample size was small), whereas in 2006 and 2007 intensity was much lower. Considering that in 2004 we began to see the dramatic decrease in actinospore presence and that the rainbow trout collected for the pond samples are generally two or three years old, it makes sense that decreases in intensity of infection among fish would be manifested in 2006 and later. This is encouraging, as it further suggests that changes in the *Tubifex* population there will complement the best management practices already implemented.

Table 2.07. Cranial *Myxobolus cerebralis* myxospore concentrations in trout sampled from the Roaring Judy State Fish Rearing Unit settling ponds.

Date mm/dd/yy	Species or Strain	Sample Size		Overall Mean Concentration	Std Dev	Positive Fish	
		N	prevalence			Mean	Range
				Settling Ponds			
11/04/03	Tas[a]	28	28.6%	5,100	11,394	17,800	3,300 – 40,000
11/04/03	Bel[a]	23	43.5%	17,200	33,748	39,600	3,300 –136,500
11/04/03	Rbt[b]	16	93.8%	365,700	460,290	390,100	7,200 – 1,387,400
11/30/04	Brown[c]	20	90%	22,600	33,208	25,100	560 – 142,200
11/30/04	Brown[d]	20	85%	11,800	15,949	14,100	1,100 – 64,400
11/30/04	Erw[e]	18	72.2%	35,800	60,418	49,500	4,400 – 199,500
11/30/04	Bel[e]	30	20%	34,700	171,705	173,400	6,300 – 942,200
11/30/04	Rbt[b]	6	50%	93,800	147,124	187,600	44,500 – 370,300
11/08/05	Tas[f]	25	8%	3,100	14,793	38,900	3,800 – 74,000
11/08/05	Bel[f]	25	4%	315	1,573	7,900	7,900
11/08/05	Rbt[b]	10	90%	232,100	342,569	257,900	15,100–1,101,200
11/07/06	Tas[f]	15	20%	8,600	8,691	10,800	1,700 – 26,900
11/07/06	Rbt[b]	16	38%	12,400	51,416	50,200	3,100 – 202,700
10/24/07	Tas[g]	25	16%	4,000	18,397	25,200	2,800 – 92,200
10/24/07	Rbt[b]	10	60%	24,300	39,957	40,500	2,800 – 114,600

a: Tasmanian strain rainbow trout were from the *M. cerebralis*-negative Crystal Rearing Unit, and the Bellaire strain rainbow trout were from the *M. cerebralis*-negative Rifle Rearing Unit.
b: Unmarked rainbow trout, presumed to be feral inhabitants of the ponds or immigrants from the East River.
c: Captured in upper pond.
d: Captured in lower pond.
e: Erwin strain rainbow trout were from the *M. cerebralis*-negative Rifle Rearing Unit, and the Bellaire strain rainbow trout were from the *M. cerebralis*-negative Durango Rearing Unit.
f: From the Roaring Judy Unit.
g: From the Pitkin Unit.

Brown trout were also sampled from the East River above and below the Roaring Judy Rearing Unit effluent during the study (Table 2.08). Prevalence of infection was similar between the two sites on all sampling occasions, but the data suggest that the intensity of infection may be slightly higher at the site downstream of Roaring Judy. These apparent differences are usually driven by one or two fish from the downstream site with large spore concentrations.

Table 2.08. Cranial *Myxobolus cerebralis* myxospore concentrations in age 1+ brown trout sampled from the East River above and below the Roaring Judy effluent.

Date mm/dd/yy	Age	Sample		Overall Mean Concentration	Std Dev	Positive Fish	
		N	prevalence			Mean	Range
		1.6 Km above Roaring Judy effluent					
08/26/03	1+	20	55%	10,700	14,011	19,400	2,500 – 43,000
08/16/05	1+	20	85%	25,400	26,428	29,900	2,100 – 79,800
08/28/06	1+	20	95%	49,400	54,114	52,000	2,200 – 205,200
08/20/07	1+	20	70%	24,200	39,000	34,600	2,000 – 125,500
		1.6 Km below Roaring Judy effluent					
08/26/03	1+	20	60%	13,200	14,569	21,900	3,200 – 43,300
08/16/05	1+	20	95%	37,800	56,946	39,800	2,300 – 248,500
08/28/06	1+	20	85%	77,900	127,343	91,700	2,300 – 537,800
08/20/07	1+	20	60%	28,500	63,993	47,600	2,000 – 225,500

Population estimates conducted during early December on the west settling ponds for four years indicated that usually very few stocked catchable rainbow trout remained in the ponds at the end of the fall fishing season (Table 2.09). Typically, many of the rainbow trout represented in the catch were unmarked, indicating they were feral fish or escapees from the rearing unit. Consequently it would appear that the annual stocking of 3000 - 4000 catchable rainbow trout into the settling ponds for the purpose of providing recreational fishing opportunity will not appreciably influence the density of actinospores in the pond effluent because most catchables are removed by anglers before they develop myxospores. Stocking in the future should continue to be completed prior to July to ensure that most catchable trout are removed from the system each year.

As with the other rearing unit study sites, the development of the qPCR test to distinguish lineages of *T. tubifex* allowed additional study at the Roaring Judy Unit to characterize the oligochaete population there. Results showed that the susceptible lineage III was generally not the predominant lineage in the effluent channel or settling ponds on three different sampling occasions in 2004 and 2007 (Table 2.10). In the ponds, lineage I dominated most sample areas. Although it is impossible to know what the lineage composition was prior to 2004, a reasonable hypothesis is that lineage III comprised a higher proportion of the population in earlier years, a situation that would make sense considering the higher actinospore densities frequently encountered in earlier years and the documented lineage composition changes elsewhere that also resulted in reduced frequency and intensity of actinospore detection (Winkelman and Nehring 2007).

Table 2.09. Trout population estimates from the Roaring Judy Fish Rearing Unit settling ponds for fish 15 cm and greater.

Date	Rainbow trout			Brown trout		
	N	95% CI	Kg/ha	N	95% CI	Kg/ha
			Upper pond			
12/03/03	30	23	8	1135	269	310
11/23/04	12	14	5	1132	249	315
11/22/05	39	---	15	944	169	234
11/27/06	34	40	18	605	225	166
			Lower pond			
12/03/03	8[a]	---	4	924	220	625
11/23/04	10[a]	---	6	1355	296	1098
11/22/05	31	65	53	620	101	459
11/27/06	131	163	161	584	355	491

a: No marked fish were recaptured, resulting in an infinite population estimate. These values represent the total numbers of rainbow trout captured in the lower pond. Biomass estimates were based upon actual and estimated (by regression) rainbow trout weights on the fish captured.

Table 2.10. *Tubifex* lineage composition estimated by qPCR on replicate 50-haired worm samples from the West Ponds and effluent ditch of the Roaring Judy Rearing Unit. Upper west pond = "UWP" and lower west pond = "LWP".

Location	N	Approximate percent DNA composition by lineage			
		I	III	V	VI
		11/18/2004			
West Ponds	4	43.9	14.4	23.2	18.5
Effluent Ditch	4	1.5	66.6	11.3	20.6
		3/28/2007			
UWP-East shore	2	5.5	46.0	8.5	40.0
UWP-West shore	2	33.5	26.0	0.0	40.5
LWP-All	4	51.3	9.4	2.6	38.0
LWP-Inlet	2	82.0	7.5	2.0	9.0
Upper Effluent Ditch	2	87.0	7.5	1.0	5.5
Lower Effluent Ditch	2	69.5	19.5	0.0	10.5
		6/11/2007			
UWP-East shore	1	54.0	0.0	46.0	0.0
UWP-West shore	2	90.0	7.5	0.0	2.5
UWP-Inlet	2	94.0	3.0	0.0	2.5
LWP-East shore	2	54.0	14.0	2.0	30.5
LWP-West shore	2	31.5	36.5	0.0	32.0
LWP-Inlet	2	85.0	6.0	2.5	6.5
Upper Effluent Ditch	2	48.0	34.0	8.5	9.5
Lower Effluent Ditch	2	83.5	3.0	3.0	11.0

RECOMMENDATIONS and CONCLUSIONS

Filtration studies at the CDOW's Pitkin, Poudre and Roaring Judy trout rearing units have identified earthen bottom settling ponds as major sources of actinospore production that likely contributed to the infection of wild trout stocks in the streams receiving the effluents of these units. Efforts to ameliorate the infectivity emanating from these ponds have been successful, with progress continuing to be made toward bringing effluent actinospore densities at these units into equilibrium with the adjacent streams. In addition to the efforts of the Division of Wildlife toward reducing actinospore densities in Unit effluents, selection of more resistant worm lineages appears to be occurring as well, further helping to reduce the contribution of actinospores to downstream trout fisheries. Nevertheless, the Division of Wildlife should continue to heed the best management practices enacted over the course of this and previous studies.

It is recommended that the settling pond at Pitkin continue to be kept as free of fish as possible. Since it appears impractical to depopulate the settling ponds at Roaring Judy, it is further recommended that catchable rainbow trout plants for these ponds continue to be stocked no later than the end of June. Such stocked fish should continue to be sampled and monitored following the kokanee spawning season to determine prevalence and intensity of infection. As rainbow trout incorporating resistance to *M. cerebralis* become available they should be increasingly used for

stocking the Roaring Judy ponds.

Encouragement of angling harvest in the effluent channel at Roaring Judy would result in beneficial use of the trout resources that occupy that area, and would seem preferable to removing them by electrofishing. Moreover, older fish would most likely be harvested, and these fish are likely to be carrying fully mature myxospores in larger numbers. Signs were posted in 2005 to encourage angler use; these should remain in place and made more prominent.

The Cap-K Ranch sand filter proved to be a disappointment in the loss of water capacity experienced over a short period of use. Now, it is clear that such filters will not effectively capture actinospores as a long-term solution to *M. cerebralis* infectivity. Any further efforts to construct sand filtration systems must include changes to filter design as recommended by the engineering proponent of the previous filter, namely, that the filter media be graded crushed glass, probably in a thinner layer than was used for the existing filter, and finally, that backwash air lines be laid in a much higher density than was the case with the existing filter. Even with such changes implemented there is still considerable question whether the life of the filter would be acceptable. Other strategies for reducing infectivity from the Cap-K Ranch ponds and similar habitats appear more appropriate at this time.

Job No. 3: Technical Assistance.

Job Objective: Provide information on impacts of whirling disease on wild trout populations to the Colorado Division of Wildlife Management and Hatchery Sections and to other interested agencies or publics.

Period Covered: July 1, 2007 to June 30, 2008

During this segment, requests for technical assistance were not limited to whirling disease information. Consultations included the following:

1) Responded to a request from Suzanne Keeling of MAF Biosecurity New Zealand to compare the efficacy of Plankton centrifuge and pepsin-trypsin digest methods of myxospore extraction for fish inspections.

2) Accommodated a number of internal requests from researchers, hatchery managers, and biologists for actinospore density, temperature, and myxospore concentration data.

3) Reviewed the Draft Statewide Zebra Mussel Response plan for the Aquatic section.

4) Assisted with the rotenone reclamation of Placer Creek to remove non-native brook trout and render the stream fishless for several years while resistant lineage *Tubifex* worms are introduced.

5) Reviewed the Wildlife Researcher brochure for Windi Padia, the CDOW Workforce Development / ADA coordinator.

6) Provided a reprint and additional comment to Professor Maria Santos in Portugal regarding the filtration of actinospores of *M. cerebralis*.

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APPENDIX A

SITE LOCATIONS for JOB1 and JOB2

Table A1. Locations of fish collection stations for Job 1 study sites. Coordinates were collected using hand-held GPS units. All are reported in WGS84 datum.

Stream	Name	Zone	UTMx	UTMy
Beaver Creek	Beaver Creek	13S	352228	4162975
Cache la Poudre	Bliss State Wildlife Area	13T	437265	4506599
Cache la Poudre	Big Bend	13T	438757	4506900
Colorado	Hitching Post	13T	414527	4440273
Colorado	Kemp/Breeze Wildlife Area	13T	398383	4435470
Spring Creek	0.8 Km below Spring Creek Res.	13S	352027	4302105
Spring Creek	5 Km below Spring Creek Res.	13S	349943	4298786
Spring Creek	19 Km below Spring Creek Res.	13S	346483	4290568
Williams Fork	0.3 Km below Williams Fork Res.	13T	397314	4432591
Williams Fork	1.6 Km below Williams Fork Res.	13T	397593	4433146
Williams Fork	2.6 Km below Williams Fork Res.	13T	399139	4434661
Willow Creek	Above Willow Creek Gage	13T	419796	4444403
Willow Creek	Downstream of backwater	13T	420185	4444247

Table A2. Locations of fish collection stations for Job 2 study sites. Coordinates were collected using hand-held GPS units. All are reported in WGS84 datum.

Stream	Name	Zone	UTMx	UTMy
Fryingpan River	1 Km below Ruedi Dam	13S	342365	4358637
Fryingpan River	1.6 Km above Cap-K Ranch	13S	339078	4359779
Fryingpan River	Taylor Creek confluence	13S	332903	4360473
Chalk Creek	Chalk Cliffs Rearing Unit[a]	13S	401963	4289195
Quartz Creek	Upstream of Pitkin Rearing Unit	13S	367258	4273612
Quartz Creek	Downstream of Pitkin Rearing Unit	13S	366134	4271724
Cache la Poudre	Big Bend – above PRU	13T	438757	4506900
Cache la Poudre	Pasquinel's Cabin – below PRU	13T	440446	4505432
East River	Roaring Judy effluent channel	13S	338950	4236008
East River	Roaring Judy settling ponds	13S	338970	4285823
East River	1.6 Km above Effluent	13S	339092	4287350
East River	1.6 Km below Effluent	13S	338853	4284876

a: No fish were collected from Chalk Creek; the location here is the Rearing Unit where the various ponds were sampled for oligochaetes.