Whirling Disease Investigations

Federal Aid Project F-237R-17

R. Barry Nehring
General Professional V

Tom Remington, Director

Federal Aid in Fish and Wildlife Restoration

Job Progress Report

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Bill Ritter, Governor

COLORADO DEPARTMENT OF NATURAL RESOURCES

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Nicole Vieira, Physical Scientist III, Toxicologist

Paula Nichols, Federal Aid Coordinator
Kay Knudsen, Librarian

Prepared by: _____
R. Barry Nehring, General Professional V, Aquatic Wildlife Researcher

Approved by: _____
Mark S. Jones, Aquatic Wildlife Research Leader

Date: _____

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State: Colorado Study No. F237 – R17

Title: Whirling Disease Investigations

Principal Investigator: R. Barry Nehring

Period Covered: July 1, 2009 to June 30, 2010

Project Objective: To determine, and then document through professional publication, the impacts of the myxosporean parasite *Myxobolus cerebralis (Mc)* on wild trout populations in selected stream ecosystems in Colorado with an overarching objective of developing risk assessment guidelines for the management of whirling disease.

Job No. 1: *Myxobolus cerebralis* in Colorado's Cutthroat Trout Populations

Job Objective: Determine the extent of occurrence and severity of impact of *Myxobolus cerebralis* on populations of greenback *Oncorhynchus clarki stomias*, Rio Grande *O. c. virginalis*, and Colorado River cutthroat trout *O. c. pleuriticus* throughout Colorado.

INTRODUCTION

"Whirling disease" (WD) is a debilitating malady of trout and salmon that was first observed in cultured rainbow trout in Germany in 1893 (Hofer 1903; Plehn 1905). The name comes from the abnormal swimming behavior of fry or fingerling salmonids that can occur after becoming infected by the myxosporean parasite *Myxobolus cerebralis.* When frightened the fish appear to be chasing their tail like a run-away boomerang. While it was considered a serious problem in aquaculture for much of the 20th century (Schäperclaus 1931; Uspenskaya 1957, 1982), it was not until the 1980s that the parasite life cycle was described (Markiw and Wolf 1983; Wolf and Markiw 1984).

Myxobolus cerebralis was first detected in Colorado in late 1987 (Walker and Nehring 1995). Population level declines observed among wild rainbow trout populations in major reaches of the upper Colorado, Cache la Poudre, Gunnison, Rio Grande, and South Platte rivers in Colorado in 1993 and 1994 were ultimately attributed to WD (Walker and Nehring 1995; Nehring and Walker 1996; Nehring et al. 1998; Nehring and Thompson 2001). Stocking of catchable size trout reared in waters enzootic for *Mc* exacerbated the spread of the parasite in the early 1990s through 2000 (Schisler 2001). By October 1997, *M. cerebralis* had been detected in feral salmonids at 118 different locations in lakes, reservoirs and major stream segments in Colorado and at 208 sites by spring 2000. Nehring and Thompson (2001) estimated that *Mc* infections had negatively impacted recruitment of wild rainbow and brook trout fry in 560 – 600 km (350-400 miles) of stream in Colorado at the end of the 20th century. A special technical report, Colorado's Cold Water Fisheries: Whirling Disease Case Histories and Insights for Risk

Management, summarized the effects of exposure to *M. cerebralis* upon Colorado's salmonid fisheries through 2005 (Nehring 2006a).

Beyond Colorado, the *Mc* parasite has been detected at one or more locations in almost all states west of the 100th meridian in the continental U.S. (Bartholomew and Reno 2002). Debilitating effects of the parasite were documented on wild rainbow trout in major reaches of the Madison River in Montana in the 1990s (Vincent 1996a,b). Research efforts between 1994 and 2004 revealed the parasite was enzootic in many coldwater habitats in Colorado (Nehring and Thompson 2003) and western Montana (Baldwin et al. 1998). Detected in Yellowstone cutthroat trout (*O. clarki bouvieri*) in 1998, *M. cerebralis* infections have had serious impacts on spawning runs in the Yellowstone River immediately downstream of Yellowstone Lake and in Pelican Creek and Clear Creek, major spawning tributaries that drain into the northeastern corner of the lake (Koel et al. 2005, Koel et al. 2006). Recent studies suggest that *M. cerebralis* may be enzootic in one or more locations in south central Alaska near Anchorage (Arsan 2006). There is increasing concern *M. cerebralis* infection may be affecting the mountain whitefish (*Prosopium williamsoni)* populations in some streams in the Rocky Mountain West.

Widely distributed in the mountainous regions of Colorado, the parasite has been detected in close proximity to areas designated as cutthroat trout recovery streams. In 2003, at the initiation of this study there were no known cases where the parasite had negatively impacted fry recruitment for any of Colorado's three sub-species of cutthroat trout. At that time, the parasite was enzootic among cutthroat trout in Trappers Lake in the Flat Tops Wilderness in western Colorado and in Zimmerman Lake in north central Colorado. Clinical signs of WD have been evident in collections of fry, fingerling, juvenile and adults of both Colorado River cutthroat trout and brook trout in Trappers Lake for at least five years or longer. At present, it is unclear whether or not recruitment of naturally spawned cutthroat trout is sufficient to sustain the population and the recreational fishery without supplementary stocking. However, it is noteworthy that long-term field exposure of young-of-the-year (YOY) of all three sub-species of Colorado's cutthroat trout to ambient levels of *M. cerebralis* in the Colorado River in the 1990s clearly demonstrated these fish are particularly vulnerable to developing a lethal infection after exposure (Thompson et al. 1999).

Total year class failure can occur among susceptible species of salmonids under the proper suite of environmental conditions once *M. cerebralis* becomes enzootic in an aquatic ecosystem. Increasingly, it is being shown in this study and others (Koel et al. 2005, Koel et al. 2006) that the proper suite of environmental conditions is not very restrictive and does not necessarily involve environmental degradation. The lack of a systematic effort to evaluate the distribution, establishment and spread of *M. cerebralis* into aquatic ecosystems capable of supporting native cutthroat trout was the primary impetus for the initiation of this project.

STUDY DESIGN

The primary study objective was to document the degree of spread of the parasite into habitats capable of supporting cutthroat trout populations. A three-pronged approach was used to determine if infection and/or establishment of *M. cerebralis* had occurred. First, multiple-pass electrofishing (Seber and LeCren 1967) was employed to derive population estimates on selected reaches of study streams to facilitate assessment of the species composition, size and age

structure of the fish population. Second, a representative sample of fish was preserved for disease screening for the WD parasite. At this level two testing procedures were applied. The polymerase chain-reaction (PCR) test was employed on young-of-the- year (YOY) trout to test for DNA of the parasite (Cavender et al. 2004). Also, the pepsin-trypsin digest (PTD) procedure was used on age 1 and older trout to detect myxospores of the *Mc* parasite (Markiw and Wolf 1974). Third, aquatic oligochaetes were collected, sorted, screened and enumerated, then tested for genetic markers to determine whether or not lineage III *Tubifex tubifex* worms were present in the aquatic habitats visited. More detailed descriptions of the study design and protocols employed can be seen in previous progress reports and will not be reiterated here in the interest of brevity (Nehring 2004, 2005, 2006b, 2007, 2008, 2009).

In most instances, cutthroat trout were euthanized for disease testing only when they occurred in allopatry. When other salmonids (usually brook trout) were sympatric with cutthroat trout the other fish were sacrificed for disease screening to avoid unnecessary depletion of the cutthroat trout population.

RESULTS and DISCUSSION

Historically, nine major river basins in Colorado have supported native cutthroat trout populations. These include the Arkansas, Colorado, Dolores, Gunnison, Rio Grande, San Juan, South Platte, White and Yampa river systems. At the beginning of this study in 2003, greenback cutthroat trout were considered native to the Arkansas and South Platte river basins. Rio Grande cutthroat trout were native to the Rio Grande basin. Colorado River cutthroat trout were considered native to the Colorado, Dolores, Gunnison, San Juan and White and Yampa river basins. No cutthroat trout were native to the North Platte drainage in Colorado. An overview of the number of streams and sites sampled each year for each of the three sub-species of native cutthroat trout is summarized in Table 1.

Table 1. Number of streams and sampling sites (stratified by year and cutthroat trout sub-species) between 2003 and 2007.

Year	Greenback cutthroat		Rio Grande cutthroat		Colorado River cutthroat	
	Streams	sites	Streams	Sites	streams	Sites
2003	9	12	9	13	22	29
2004	3	5	18	26	24	36
2005	9	12	18	24	10	12
2006	---	---	10	18	49	73
2007	1	2	3	5	48	61
Total	**22**	**31**	**58**	**86**	**153**	**201**

Detailed information regarding streams and sites sampled, results of the population assessments and disease testing from 2003 through 2007 can be seen in Nehring 2004, 2005, 2006b, 2007, 2008 and 2009. Data from the first five years of field study indicated that *M. cerebralis* was enzootic in numerous stream trout populations throughout Colorado. The primary reasons for this are three-fold. First, millions of *Mc*-exposed trout were stocked into salmonid habitats over a ten-year period from the mid-1980s to the mid-1990s. Second, the *M. cerebralis*-susceptible lineage III *T. tubifex* is the most cosmopolitan of the four lineages of this tubificid

worm in Colorado. And once infected, it is the only strain or lineage of *T. tubifex* that produces massive numbers of the fish-infective triactinomyxon (TAM) actinospores. Third, there are no apparent thermal or elevation barriers in aquatic habitats capable of supporting feral salmonid populations that limit the occurrence or establishment of *M. cerebralis* or the lineage III worm in Colorado. Over a five-year period beginning in 2005, free-ranging populations of lineage III *T. tubifex* collected from two lentic and 28 lotic aquatic habitats across ten major drainage basins at elevations ranging from 1,524 meters (5,000 feet) to 3,673 meters (12,046 feet) were exposed to *M. cerebralis* myxospores under controlled laboratory conditions. All 30 populations of lineage III *T. tubifex* exposed to 50 *Mc* myxospores/worm produced very large numbers of TAMs.

Stratification of the occurrence, relative abundance and distribution of the various lineages (I, III, V and VI) of *T. tubifex* into 1,000 foot elevation zones for the five-year data set revealed that the *M. cerebralis*-susceptible lineage III strain of *T. tubifex* was far more abundant and widely distributed at all elevation strata between 6,000 and 11,000 feet than the three non-susceptible *T. tubifex* lineages (I, V and VI) combined (Nehring 2008).

However, mitochondrial 16s rDNA for lineage III *T. tubifex* was detected in only 1 of 8 oligochaete samples collected at sites > 11,000 feet between 2003 and 2007. This was not an adequate sample size to determine whether or not harsh environmental conditions at elevations > 11,000 feet might be a factor limiting the distribution of lineage III *T. tubifex*. For these reasons the field study was extended an additional year to concentrate on collecting trout and aquatic oligochaete samples from aquatic habitats at sampling locations > 11,000 feet elevation.

During 2008, most of the sampling was concentrated in or near lakes at or above timberline. Salmonid samples were collected from 40 lakes, reservoirs, and beaver ponds, and five streams. The fish were euthanized and submitted for PTD testing for evidence of infection by *M. cerebralis.* There were 14 additional lakes and one stream visited where no fish samples were preserved for PTD testing. Seven of the 14 lakes were either known to be devoid of fish, no fish were observed, or we were unsuccessful in collecting trout for testing. In seven other lakes fish were observed and/or caught but not sacrificed for various reasons. When fish were caught and not sacrificed, they were too large, there were too few fish in the lake, or fish caught from the inlet or outlet streams were sacrificed for testing, making the lake sample redundant. Except for the collections from the Rio Grande River near Creede and Idaho Springs Reservoir all collection sites were at elevations ≥ 11,000 feet (3,354 m). Thirty-five collection sites were at elevations between 11,000 and 12,000 feet, and 22 were located at elevations > 12,000 feet (3,659 m).The results of six years of aquatic oligochaete sampling are summarized in Table 2.

These data suggest that the prevalence of non-*T. tubifex* aquatic oligochaetes appears to be greater at high elevations. However, at those sites where mt16s rDNA specific for *T. tubifex* was detected, the lineage III DNA was detected in 50% or more of the samples within all 1,000 foot elevation strata > 6,000 feet (Table 2).

Aquatic Oligochaete Sampling – Since the late 1990s, substantial research efforts have been directed at developing a better understanding the factors that affect the population dynamics and distribution of aquatic oligochaetes in the natural environment. In addition, much has been learned about the relative differences in susceptibility to *M. cerebralis* among the different

lineages of *T. tubifex* (Beauchamp et al. 2001, 2002, 2005, 2006; DuBey and Caldwell 2004; DuBey et al. 2005; Kaesar and Sharpe 2006; Kerans et al. 2004: Winkelman and Nehring 2007).

Table 2. Number and frequency of detection and percent occurrence (in parentheses) of mt16s rDNA specific for lineage I, III, V and VI *T. tubifex* and tubificid oligochaetes with "haired" chaetae stratified within 1,000 foot elevation zones, and within and among strains or lineages across all elevation strata (2003 – 2008).

Elevation (ft.)	**Number of Sites where each Lineage of *Tubifex tubifex* was present**				
	Lineage I	**Lineage III**	**Lineage V**	**Lineage VI**	**No Lineage**
5,000 – 6,000	0	3 (43)	0	4 (57)	0
6,001 – 7,000	6 (11)	28 (54)	3 (6)	15 (29)	3
7,001 – 8,000	1 (3)	29 (74)	2 (5)	7 (18)	6
8,001 – 9,000	8 (11)	46 (61)	5 (7)	16 (31)	19
9,001- 10,000	3 (7)	33 (73)	2 (4)	7 (16)	25
10,001- 11,000	1	17 (81)	1 (5)	2 (9)	14
11,001 – 12,000	0	4 (57)	0	3 (43)	24
> 12,001	0	2 (50)	0	2 (50)	13
Total	**19 (5)**	**162 (46)**	**13 (4)**	**56 (16)**	**104 (29)**

As more and more research investigations have been directed at the aquatic oligochaete side of the life cycle of *M. cerebralis* it has become increasingly clear that the presence of the lineage III *T. tubifex* in an aquatic environment is often the primary determining factor governing whether or not *M. cerebralis* becomes established after the initial introduction occurs. In the San Juan River below Navajo Dam in New Mexico, DuBey and Caldwell (2004) found that only lineage III *T. tubifex* were infected with *M. cerebralis*, even though *T. tubifex* belonging to lineages I and VI were also present in the stream. Moreover, in a follow-up laboratory study where worms from lineages I, III and VI were exposed to myxospores of *M. cerebralis*, evidence of infection was only detected in lineage III worms (DuBey et al. 2005). Similar outcomes have emerged from laboratory tests where lineage I, III, IV, V and VI *T. tubifex* have been exposed to varying concentrations of *M. cerebralis* myxospores in Colorado (Nehring, unpublished data), Oregon (Dr. Jerri Bartholomew, personal communication), California (Baxa and Hedrick 2008) and in states in the eastern U.S. (Dr. Vicki Blazer, personal communication). Although variations in sediment type or quality, (i.e., sand, mud or organically rich muck) can enhance the severity of infection among *T. tubifex* worms that are susceptible to *M. cerebralis*, parasite development and infectivity is not altered in lineages of worms that are refractory (V and VI) or highly resistant (I) to infection, regardless of sediment type (Baxa and Hedrick 2008). For these reasons, ascertaining the distribution and relative abundance of the various lineages of *T. tubifex* in Colorado's cutthroat trout streams is a critically important component in assessing the risk of establishment and spread of *M. cerebralis* in Colorado.

Cutthroat Trout Population Status - More often than not, Colorado's self-sustaining populations of cutthroat trout persist at high elevations in stream reaches where the water temperatures are cold and the growing season can be quite short. In some cases these populations exist in habitats near the upper thermal limits of the species (Coleman and Fausch 2006). At lower elevations where the thermal regime may be more conducive to successful

reproduction, growth and survival, cutthroat trout face extirpation due to competition from nonnative brown and brook trout (Peterson and Fausch 2003a; Peterson and Fausch 2003b; Peterson et al. 2004). Hybridization with nonnative rainbow trout can dilute the genetic purity of native cutthroat trout. These negative selection pressures, coupled with the fact that Colorado's cutthroat trout often suffer high mortality rates after infection by *M. cerebralis* (Thompson et al. 1999), present daunting obstacles for resource managers charged with recovery efforts.

Table 3. *Myxobolus cerebralis* cranial myxospore concentrations in salmonids collected from various sites in high elevation lakes and streams in Colorado during the 2008 field season.

Date Mmddyy	Collection Site (Water Name/Water Code)	Species	Sample Size		Myxospores	
			№	№ +	Mean	Range in Positive Fish
08/14/08	Ptarmigan Lake/ 91861	GBN	16	0	0	0
08/21/08	Lower Copper Lake/ 89121	CRN	34	0	0	0
07/14/08	Lower Hancock Lake/80527	GBN	10	0	0	0
07/15/08	Upper Pomeroy Lake/81074	GBN	10	0	0	0
07/18/08	North Fork Reservoir/79891	Rainbow	10	6	23,833	1,389 – 196,389
10/13/08	Rio Grande near Creede/42539	Brown	10	2	2,434	2,989 – 21,350
09/24/08	Emma Lake/80414	GBN	10	0	0	0
09/24/98	Linkins Lake/67783	CRN/BRK	11	0	0	0
09/24/08	Independence Lake/67416	CRN/BRK	17	0	0	0
07/16/08	Hartenstein Lake/80565	GBN/BRK	13	0	0	0
07/17/08	Ptarmigan Lake/81086	GBN	13	0	0	0
07/08/08	Henry Lake/90439	CRN	30	0	0	0
07/12/08	Upper Lamphier Lake/90871	CRN	10	0	0	0
07/12/08	Lower Lamphier Lake//90869	CRN	20	0	0	0
07/30/08	Middle Fork Saguache Creek/42806	RGN	17	1	187	2,811
08/20/08	Big Verde Lake/92926	Brook	20	0	0	0
08/07/08	Poage Lake/91760	SRN/BRK	17	0	0	0
08/13/08	Lower Deadman Lake/89397	RGN	10	0	0	0
08/21/08	Little Highland Mary Lake/90491	CRN	1	0	0	0
08/31/08	Big Highland Mary Lake/90489	Brook	5	0	0	0
07/23/08	Mill Lake/91265	CRN	10	0	0	0
07/25/08	Upper Lottis Lake/91087	CRN	13	0	0	0
07/27/08	Lake Fork Cochetopa Creek/39215	CRN	12	0	0	0
07/29/08	Wallace Lake	CRN	7	0	0	0
07/02/08	Texas Creek (Rio Grande)/43620	Brook	10	0	0	0
06/30/08	East Willow Creek/44076	Brook	10	0	0	0
08/27/08	Murray Lake Beaver ponds/55788	GBN	10	0	0	0
07/24/08	Lower Powderhorn Lake/91823	Brook	20	0	0	0
09/08/08	Silver Dollar Lake/56487	GBN	6	0	0	0
08/28/08	Naylor Lake	RBT/BRN	10	0	0	0
08/28/08	W. Summit Lake (Mt. Evans)/56641	GBN	8	0	0	0
09/19/08	Murray Lake/55788	GBN	12	0	0	0
09/10/08	Chinns Lake/54368	BRK/SPL	6	0	0	0
09/10/08	Sherwin Lake/56451	BRK/GBN	8	0	0	0
09/09/08	Slater Lake/56514	GBN	10	0	0	0
09/05/08	Idaho Springs Reservoir/54320	RBT/GBN	11	0	0	0
09/11/08	Lost Man Lake/68064	Brook	10	0	0	0
08/19/09	Cunningham Creek/39506	Brook	9	0	0	0
07/24/08	Powderhorn L. Beaver ponds/91823	Brook	6	0	0	0
09/03/08	Lower Chicago Lake/54332	GBN/BRK	28	2	469	11,244
09/16/08	Upper Chicago Lake/54344	GBN	23	21	63,879	2,811 – 368,256
09/08/08	Kite Lake (S. Platte Basin)/80717	GBN	20	4	8,293	14,056 – 70,278
09/17/08	Lower Square Top Lake/56576	GBN	4	2	22,256	22,256 – 66,767
09/17/08	Upper Square Top Lake/56588	GBN	11	0	0	0

Table 4. Summary of *Myxobolus cerebralis* testing in wilderness areas of Colorado (2003-2008).

Wilderness Area	Location (Lake or Stream Name)	Elevation (feet)	Species	Infection Severity
Collegiate Peaks Wilderness	Hartenstein Lake	11,451	GBN	None
Collegiate Peaks Wilderness	Ptarmigan Lake	11,758	GBN	None
Eagles Nest Wilderness	Piney River ↓ Piney Lake	9,315	BKT/LOC	Moderate-Severe
Eagles Nest Wilderness	Piney River ↑ Piney Lake	9,405	BKT/LOC	Moderate-Severe
Flat Tops Wilderness	Cabin Creek ↑ Trappers Lake	9,642	BKT/CRN	Moderate-Severe
Flat Tops Wilderness	Fraser Creek ↑ Trappers Lake	9,650	BKT/CRN	Moderate-Severe
Flat Tops Wilderness	Heberton Creek ↑ Trappers Lake	9,650	BKT/CRN	Moderate-Severe
Flat Tops Wilderness	Upper Marvine Lake	9,324	BKT/CRN	Moderate-Severe
Flat Tops Wilderness	Lower Marvine Lake	9,314	BKT/CRN	Moderate-Severe
Flat Tops Wilderness	Slide Lake	8,654	BKT/RBT	Moderate-Severe
Flat Tops Wilderness	Marvine Creek	9,299	BKT/RBT	Moderate-Severe
Flat Tops Wilderness	Trappers Lake	9,635	BKT/CRN	Moderate-Severe
Fossil Ridge Wilderness	Henry Lake	11,757	CRN	None
Fossil Ridge Wilderness	Upper Lamphier Lake	11,703	CRN	None
Fossil Ridge Wilderness	Lower Lamphier Lake	11,227	CRN	None
Fossil Ridge Wilderness	Mill Lake	11,457	CRN	None
Gunnison Gorge Wilderness	Gunnison River	5,300	LOC/RBT	Moderate-Severe
Hunter-Fryingpan Wilderness	Lost Man Lake	12,450	BKT	None
Hunter-Fryingpan Wilderness	Independence Lake	12,490	BKT/CRN	None
Hunter-Fryingpan Wilderness	Linkins Lake	12,008	BKT/CRN	None
La Garita Wilderness	Middle Fork Saguache Creek	11,956	RGN	Very light
La Garita Wilderness	Cochetopa Creek↑Stewart Creek	10,289	BKT/BNT	Moderate-Severe
La Garita Wilderness	Cochetopa Creek↑Canon Diablo	10,921	CRN	None
La Garita Wilderness	Lake Fork Cochetopa Creek	11,231	CRN	None
La Garita Wilderness	Machin Lake	12,480	RGN	No Sample
La Garita Wilderness	Wallace Lake	12,621	CRN	None
Mt. Evans Wilderness	Upper Chicago Lake	11,755	GBN	Severe
Mt. Evans Wilderness	Lower Chicago Lake	11,420	GBN	Very light
Mt. Evans Wilderness	West Summit Lake	12,841	GBN	None
Powderhorn Wilderness	Upper Powderhorn Lake	11,877	No fish	No Sample
Powderhorn Wilderness	Lower Powderhorn Lake	11,663	BKT/CRN	None
Powderhorn Wilderness	Beaver ponds ↓Powderhorn Lakes	11,600	BKT	None
Sangre de Cristo Wilderness	West Deadman Lake	11,772	No Fish	No Sample
Sangre de Cristo Wilderness	Upper Deadman Lake	11,709	RGN	None
Sangre de Cristo Wilderness	Lower Deadman Lake	11,661	RGN	None
Sangre de Cristo Wilderness	Deadman Creek	11,740	RGN	None
Maroon Bells-Snowmass	Lower Copper Lake	11,321	CRN	None
South San Juan Wilderness	East Fork Piedra River	7,992	BKT/BNT	Mild
South San Juan Wilderness	North Fork Conejos River	10,286	BKT	None
South San Juan Wilderness	Middle Fork Conejos River	10,249	BKT	None
South San Juan Wilderness	Rio de los Pinos	10,365	RGN	None
Uncompahgre Wilderness	Fall Creek	11,344	CRN	None
Weminuche Wilderness	Lower Flint Lake	11,620	CRN	None
Weminuche Wilderness	Highland Mary Lakes	12,096	BKT/CRN	None
Weminuche Wilderness	Big Verde Lake	12,186	BKT	None
Weminuche Wilderness	Lost Lake	12,190	CRN	No Sample
Weminuche Wilderness	Weminuche Crk.↑ Rio Grande Rsvr.	10,356	BKT	Very Light
Weminuche Wilderness	Ute Creek ↑ Rio Grande Reservoir	9,473	BKT/RBT	Moderate
West Elk Wilderness	North Golden Lake	11,028	No Fish	No Sample
West Elk Wilderness	South Golden Lake	11,066	GOLDEN	No Sample

Results of the collections and testing of salmonids from high elevation habitats (≥ 3,354 m or 11,000 feet) and in wilderness areas of Colorado are summarized in Tables 3 and 4. A review of the fish sampling and aquatic oligochaete collections from 2008, together with similar results from the previous studies (Nehring 2004, 2005, 2006b, 2007, 2008; Schisler 2000, 2001) indicate that *M. cerebralis* has spread into high elevation lakes and streams in Colorado that either already support cutthroat trout, or are capable of supporting cutthroat trout. The Wilderness Institute website (Wilderness.net) lists 41 wilderness areas in Colorado. Samples of fish and/or aquatic oligochaetes have been collected from at least 50 sites in 14 of the 41 wilderness areas in the state. *Myxobolus cerebralis* infections have been detected in salmonids at 18 sites in one or more aquatic habitats in 6 of the 14 wilderness areas visited. Detections occurred in 11 streams and six lakes. The *Mc* parasite was detected in 41% (18 of 44) of the fish samples collected in wilderness areas.

The data collected during this study over the past six field seasons demonstrate that within aquatic habitats capable of supporting salmonids there is no elevation or thermal barrier prohibiting the establishment of the parasite in Colorado. *Myxobolus cerebralis* infections were documented in cutthroat trout in four lakes at elevations > 11,400 feet, including 2 lakes over 12,000 feet. The infections in two of the four lakes (Upper Chicago and Lower Square Top lakes) would be considered moderate to severe (≥ 50% prevalence). The presence of lineage III *T. tubifex* oligochaetes and a salmonid fish appear to be all that is required for establishment of the life cycle of the *Mc* parasite. Lineage III *T. tubifex* oligochaetes were collected in all 4 lakes with infected cutthroat trout located at elevations ≥ 11,400 feet.

All the foregoing seems to suggest there is little hope for stopping the spread of *M. cerebralis* or better yet breaking the life cycle of the parasite once it becomes enzootic in a specific aquatic habitat. This would certainly seem to be the case given the results of studies from the 20th century suggesting that *Mc* myxospores remain viable for periods of time measured in years or even decades (Uspenskaya 1957; Bauer 1959; Hoffman et al. 1962; Funk 1968) However, recently published results of laboratory tests are tantalizingly suggestive that myxospores of the *Mc* parasite might not be near as resistant to degradation in the natural environment as previously thought. In one study (Hedrick et al. 2008), TAM production among lineage III *T. tubifex* inoculated with *Mc* myxospores held in water suspension for seven days at 5 °C and 22 °C was reduced 64% in the replicates held at the higher temperature prior to the exposure to the oligochaetes. In a second study (Hedrick et al. 2008), replicate groups of *Mc* myxospores were held in water suspension at 4 °C, 10 °C and 20 °C for 60 days prior to inoculating lineage III *T. tubifex.* Mean TAM production among the 4, 10 and 20 °C treatments was 20,550, 17,450 and 1.5, respectively. Similarly, mean TAM production among lineage III *T. tubifex* exposed to myxospores held in 5 °C water for seven days and 4 °C water for 60 days prior to inoculation was 71,735 and 20,550 for the 7 day and 60 day holding periods, respectively. Mean TAM production was reduced 71% in the 60-day pre-inoculation treatment group! In all cases, the worms were held in 15 °C water for the 200-230 day post-inoculation/TAM-enumeration test period. These findings suggest that *Mc* myxospores may degrade and become non-viable in a much shorter period of time under environmental conditions that would support salmonids than previously believed (Uspenskaya 1957; Bauer 1959; Hoffman et al. 1962; Funk 1968).

Freezing, exposure to sunlight, and simple desiccation for less than 24 hours were also shown to render *Mc* myxospores non-viable. Hedrick et al. (2008) found *Mc* myxospores were rendered non-viable by freezing at -20 °C and -80 °C for seven days. *Mc* myxospores in a 1-mL suspension held in an uncovered petri dish were rendered non-viable by exposure to direct sunlight for 105 minutes. Temperature at the petri dish was 18 °C at the start and 42 °C at the end of the time period. Similarly, 1- mL suspensions of *Mc* myxospores were rendered non-viable when exposed to the air for 18.5 hours at a temperature of 22 °C. In all of these experiments, the myxospores were used to inoculate lineage III *T. tubifex* that were subsequently screened for TAM production for 200-230 days post-exposure.

In summarizing the results of their multi-faceted study, Hedrick et al. (2008) conclude (in part), "These studies reveal that the *M. cerebralis* myxospore stage's reputation for resistance to a broad set of chemical and physical treatments may not be completely deserved". The 71% reduction in TAM production among replicates where *Mc* myxospores were held in sediment and water for 60 days prior to inoculation with lineage III *T. tubifex*, compared to similarly treated replicates inoculated with worms after seven days is very intriguing. Those results suggest that *Mc* myxospores do not remain viable for periods of time measured in years or even decades as suggested in mid-20th century literature (Bauer 1959; Hoffman et al. 1962; Funk 1968) published long before the 2-host life cycle of the parasite was described (Markiw and Wolf 1983; Wolf and Markiw 1984).

These inconsistencies and incongruities in the scientific literature led to a second extension of this study in an attempt to develop a more thorough understanding of the viability of *Mc* myxospores when held in sediment and water under environmental conditions reflective of spring to fall conditions in streams and lakes supporting salmonids on a year-round basis. Moreover, studies have never been done that clearly prove whether or not the non-susceptible lineages (I, V, and VI) truly function as biological filters in the natural environment and deactivate *Mc* myxospores. As a result, a series of laboratory tests requiring a multi-faceted complex study design were completed over a five-year period to elucidate answers to these questions. Three separate hypotheses were set up and tested as follows:

1. Are lineage III *T. tubifex* oligochaetes from Colorado, across all major drainages and a wide range of elevations, susceptible to infection by *M. cerebralis?*
2. How long do *Mc* myxospores remain viable when held in sand and water at temperatures $\geq$ 5 °C and $\leq$ 15 °C, the temperature range at which most salmonid fishes in Colorado thrive?
3. Do *T. tubifex* lineages (I, V and VI) that are not susceptible to infection by *M. cerebralis* actually function as true biological filters and deactivate or destroy *Mc* myxospores or do the myxospores merely pass through the alimentary tract of these worms in a viable state and produce TAMs when subsequently consumed by lineage III *T. tubifex*?

To test the first hypothesis, aquatic oligochaetes were collected from lake and stream habitats supporting salmonids from numerous sites in every major drainage in the state and across a wide range of elevations over the course of seven field seasons. These worms were screened by stereozoom microscopy for the presence of haired and pectinate chaetae on the anterior portion of the body, phenotypic traits that usually indicate the oligochaetes most likely

belong to the species complex *Tubifex tubifex* (Kathman and Brinkhurst 1998) . When present in the collection, two aliquots of 50 worms were preserved in 70% ethanol and screened for DNA markers using qPCR techniques designed to distinguish between the various lineages (I, III, V and VI) of *T. tubifex* known to occur in Colorado (Beauchamp et al. 2001; Beauchamp et al. 2002; Beauchamp et al. 2005; Beauchamp et al. 2006). Among pure populations of lineage III worms collected during the field seasons from 2005 through 2009, representative groups across a wide range of elevations from all major drainages in the state were held in aquaria, cultured, and subsequently tested under controlled exposure conditions. In most cases, two or more exposure replicates of 250 *T. tubifex* were held in 1 L. containers with 240 g of sterilized playground sand (≤ 1 mm diameter) and de-chlorinated tap water at temperatures ranging from 5 °C to 15°C over the seven-month long test period. Each replicate was inoculated with 12,500 *Mc* myxospores, a dosage of 50 myxospores/oligochaete. Once TAM release began (usually 75 – 90 days post inoculation), TAM production was estimated 1-2 times per week in all replicates for 120 -150 days, and total estimated TAM production was determined for each population of lineage III *T. tubifex*. This study was completed over a five-year period. The results are summarized in Table 5.

Among the 25 separate populations of 100% pure lineage III *T. tubifex* tested, there was only one population where estimated average TAM production did not exceed one million. That was the Poose Creek population in the headwaters of the Yampa River (Table 5). However, it is noteworthy that the highest level of TAM production (over 22 million) among 250 lineage III worms was documented in Silver Dollar Lake at an elevation of just under 12,000 feet. Moreover, TAM production averaged more than four million among the two replicates of lineage III worms at Lower Square Top Lake at an elevation of 12,054 feet. These test data refute any contention that elevation and the associated cold temperatures and long periods of ice cover might provide refuge habitats where cutthroat trout might escape infection by the *Mc* parasite. Indeed, the cutthroat trout population in Lower Square Top Lake has been severely infected with *M. cerebralis* at least since 1998 (Schisler 1999).

Other tests of mixed lineage *T. tubifex* populations were run concurrently with the pure lineage III treatment groups; however, the lineage III worms were always the sub-dominant lineage in these mixed cultures. In addition, pure populations of the non-susceptible lineage worms (lineages I, V and VI) were also exposed and tested along with the lineage III replicates summarized in Table 5. Results for the mixed lineage replicates and non-susceptible lineage tests are summarized in Table 6. These data support the hypothesis that the non-susceptible lineages (I, V and VI) occurring in Colorado may function as biological filters that are capable of deactivating Mc myxospores.

In an attempt to evaluate the long-term viability of *Mc* myxospores held in sediment and water under environmental conditions reflective of spring to fall conditions in streams and lakes supporting salmonids on a year-round basis (hypothesis #2) the following experiment was carried out. Aliquots of 12,500 myxospores were mixed into each replicate with 240 g of sterilized white sand (≤ 1 mm grain size) and 750 ml de-chlorinated tap water in 1 L containers. The water was continuously aerated and the temperature was held in the range of 5 °C to 15 °C

Table 5. Total triactinomyxon (TAM) production among replicates of 250 lineage III *T. tubifex* oligochaetes from various streams, lakes, major drainages and elevations across Colorado. All replicates except where noted were exposed to 12,500 myxospores of *Myxobolus cerebralis*, a dosage of 50 myxospores per worm.

River Basin	Population Source	Elevation (feet)	Replicate 1 Total TAMs	Replicate 2 Total TAMs
Arkansas River	S. Fk. Huerfano River	9,065	1,923,652	1,046,871
Arkansas River	Trout Creek	8,813	3,429,249	3,164,659
Blue River	Swan River	9,738	4,753,376	5,008,264
Colorado River	East Parachute Creek	8,376	9,954,088	Worms died
Colorado River	East Parachute Creek	8,376	17,052,617[a]	8,333,344[a]
Colorado River	East Parachute Creek	8,376	17,528,270[b]	7,993,803[b]
Colorado River	Sheephorn Creek	6,678	6,496,757	267,535
Colorado River	West Fk. Big Creek	9,587	8,283,238	1,003,750
Dolores River	Buckeye Creek	7,626	1,783,664	912,181
Eagle River	Cross Creek	8,075	5,404,034	7,185,392
Gunnison River	Cochetopa Creek	8,735	1,751,477	3,667,138
Gunnison River	Spring Creek	9,924	5,027,510	4,544,211
Rio Grande	San Francisco Creek	8,543	9,207,382	2,018,349
Rio Grande	Rio de los Pinos	9,643	552,346	1,641,635
San Juan River	Piedra River	7,633	3,060,112	2,865,711
South Platte River	Middle Boulder Creek	8,987	5,935,675	7,797,478
South Platte River	M. Fk. South Platte R.	10,506	2,508,129	1,352,841
South Platte River	South Clear Creek	10,637	6,161,611	6,653,738
South Platte River	Silver Dollar Lake	11,950	22,576,325	3,902,420
South Platte River	Lower Square Top Lake	12,054	5,918,810	2,750,844
White River	Fawn Creek	9,105	2,021,278	19,427,147
White River	North Elk Creek	6,819	5,738,530	2,528,912
Yampa River	Indian Run Creek	7,075	2,977,157	58,272
Yampa River	Poose Creek (2007)	9,250	320,412	400,471
Yampa River	Poose Creek (2008)	9,250	565,255	432,006
Yampa River	Smith Creek	7,869	2,146,303	6,128,487
Yampa River	Trout Creek	9,935	5,076,628	2,821,814
Little Snake River	S. Fk. Little Snake R.	7,797	3,928,476	1,534,984

[a]: Worms in these replicates were dosed at 250 myxospores/worm.
[b]: Worms in these replicates were dosed at 500 myxospores/worm.

throughout the entire test period. The water was gently decanted off and changed once each week for the duration of the experiment. Care was taken not to disturb the sand containing the myxospores during the decanting process to avoid decanting off myxospores. There were two replicate containers for each time-delay treatment. The time-delay treatments were 0, 15, 30, 60, 90, 120, 180 and 365 days. All replicates up to and including 180 days were seeded with 12,500 *Mc* myxospores at day 0. Two additional replicates for an additional 180 day and 365 day delay- time treatments were also inoculated at day zero with one million myxospores. Beginning at day zero and every time-delay treatment thereafter, 250 lineage III *T. tubifex* from a culture of East

Table 6. Total triactinomyxon (TAM) production among replicates of 250 *T. tubifex* oligochaetes from various streams, lakes, major drainages and elevations across Colorado where the populations were of mixed lineages (I, III, V and VI) in varying proportion(s) containing some lineage III worms, or from populations that were pure populations of non-susceptible lineages (I, V or VI) . Lineage III worms from Mt. Whitney, CA were included as laboratory standard controls for comparative purposes. All replicates except where noted were exposed to 12,500 myxospores of *Myxobolus cerebralis*, a dosage of 50 myxospores per worm.

Lineage(s) Tested	Population Source	Elevation (feet)	Replicate 1 Total TAMs	Replicate 2 Total TAMs
III	Mt. Whitney, CA – Lab controls [a]	na	36,078,064	22,082,301
III	Mt. Whitney, CA – Lab controls [c]	na	10,949,383	15,619,950
III	Mt. Whitney, CA – Lab controls [d]	na	4,710,302	3,761,807
III	Mt. Whitney, CA – Lab controls [b]	na	2,625,737	2,268,858
III	East Parachute Creek (Colorado R.) [a]	8,376	9,594,088	Worms died
III	East Parachute Creek (Colorado R.) [b]	8,376	2,935,463	4,316,683
VI, III	Cap K Ranch (Fryingpan River) [c]	7,192	607,170	98,672
I, III	Coal Creek (East River) [d]	9,764	118,123	247,904
V, III	Williams Fork River (Colorado R.)[a]	7,684	165,604	900
I, VI,III	Mt. Massive Lakes (Arkansas R.) [c]	9,472	52,768	2,251
I, V, III	Eagle River @ Wolcott[d]	6,939	21,193	100,639
I, ,V,III	Eagle River @ Wolcott[b]	6,939	21,001	2,231
I, V, VI, III	Windy Gap Reservoir (Colorado R.)[a]	7,808	100,781	66,754
V	Williams Fork River (Colorado R.)[b]	7,684	0	0
VI	Windy Gap Reservoir (Colorado R.)[a]	7,808	0	0
VI	Windy Gap Reservoir (Colorado R.)[b]	7,808	0	0
VI	Upper Square Top Lake [f]	12,264	0	0

[a]: Tested in 2006
[b]: Tested in 2009
[c]: Tested in 2007
[d]: Tested in 2008
[f]: Tested in 2010

Parachute Creek worms were introduced to the containers. After introduction, the worms were fed weekly with a ration of 0.2 g of dehydrated spirulena discs, tetramin tropical fish granules and ALGAMAC 2000 in a ratio of 6:3:1 by weight. The ration was ground to a fine powder with a small commercial coffee bean grinder. The working hypothesis was that if the *Mc* myxospores remained 100% viable for long period of time, then there should be no attenuation in TAM production among any replicates across all time-delay treatments. Alternatively, there should be decreases in TAM production with longer time delay treatments if *Mc* myxospores become less viable with increasing time-delays prior to being consumed by *Mc*-susceptible lineage III *T. tubifex*. The results of these tests are summarized in Table 7. These data are congruent with the findings of Hedrick et al. (2008) indicating that myxospores of the *Mc* parasite decrease in viability at an exponential rate and do not remain viable for long periods of time, up to years or even decades as suggested by studies from the mid-20th century (Bauer 1959; Hoffman et al. 1962; Funk 1968). The degradation processes begins to affect *Mc* myxospores almost

immediately after liberation from fish tissues and exposed to sand, water and the elements given that TAM production among the two 15-day delay replicates was reduced by 74.7% compared to the day-0 replicates (Table 7). TAM production was reduced by more than 99% after being suspended in sand and water for 120 and 180 days. When the number of myxospores inoculated into the sand at 180 days was increased by 80 fold, significant TAM production did occur, but it was still less than 50% of the production among the day-0 replicates. No TAM production was observed among either replicate of 250 lineage III *T. tubifex* exposed to one million myxospores that had been held in an aqueous suspension at temperatures ≤ 5 °C for 365 days prior to inoculation. At the end of 180 days post exposure all lineage III worms in both replicates from the 365 days treatment were tested by PCR (Cavender et al. 2004) and found to be negative for any presence of *Mc* DNA. These test results provide convincing evidence that the long-term viability of *M. cerebralis* myxospores is less than one year, and is severely reduced after 120 to 180 days. Tables summarizing the onset, duration and amplitude of TAMs on a weekly basis are shown in the Appendix.

Table 7. Estimated TAM production among time-delay treatments of myxospores of *Myxobolus cerebralis* held in sterilized sand and continuously aerated with de-chlorinated tap water prior to inoculation with 250 lineage III *Tubifex tubifex* oligochaetes that were then held in the same container for an additional 210 days or longer until weekly TAM production dropped below 0.5% of the total TAM production elaborated during the entire exposure period. Approximately 99% of the water in each replicate was decanted off, sub-sampled and screened for TAM production once each week commencing 75-90 days after exposure.

Time-Delay (Days)	**Replicate 1**	**Replicate 2**	**Average**	**% Decrease Vs. Day 0**
0 days	2,935,463	4,316,683	3,626,073	0
15 days	793,844	1,042,255	918,050	74.7%
30 days	705,357	612,627	658,992	81.8%
60 days	3,944,264	2,053,561	2,998,913	17.3%
90 days	378,838	17,385	198,112	94.5%
120 days	22,612	18,855	20,734	99.4%
180 days	1,698	5,572	3,635	99.9%
180 days[a]	192,982	3,883,931	2,038,456	43.8%
365 days[b]	0	0	0	100%

[a]: These replicates were inoculated with one million *Mc* myxospores at day zero.
[b]: Myxospores for these replicates were held in an aqueous suspension for 365 days at temperatures ≤ 5 °C prior to inoculating 250 lineage III *T. tubifex* that were subsequently held for an additional 180 days.

To determine whether or not non-susceptible lineages (I, V and VI) of *T. tubifex* function as true biological filters that deactivate and destroy myxospores of the *M. cerebralis* parasite a two-phase experimental design was employed. For Phase One, multiple replicates of *Mc*-susceptible (lineage III), mixed lineage (I, III and V) and non-susceptible lineage (V and VI) worms were placed in 1 L containers with 240 g of sterilized playground sand (≤ 1 mm grain size) and aerated, de-chlorinated tap water. Each replicate was seeded with 250 worms. Water temperatures ranged from 5 °C to 15°C over the nine-month long test period. Each Phase One

replicate was inoculated with 12,500 *Mc* myxospores, a dosage of 50 myxospores/oligochaete. All Phase Two replicates contained clean, sterilized playground sand and aerated, de-chlorinated tap water that had not been inoculated with myxospores. The Phase Two replicates were seeded with the worms removed from the Phase One replicates after the proscribed exposure period. After removal of all of the exposed worms at the end of the prescribed exposure period for the Phase One replicates, 250 unexposed lineage III worms from Parachute Creek were stocked into the Phase One replicate containers, still containing all of the sand/substrate from the original exposure. Once TAM release began (usually 75 – 90 days post inoculation or exposure), TAM production was estimated once each week in all replicates for 120 -150 days, and estimated total TAM production was determined for each population of *T. tubifex*. This study was completed over a nine-month period. Results are summarized in Table 8. Tables summarizing the onset, duration and amplitude of TAMs on a weekly basis for these experiments are in the Appendix.

Table 8. All replicates for Phase One were stocked with 250 *Tubifex tubifex* oligochaetes of various lineages and inoculated with 12,500 myxospores of *Myxobolus cerebralis* (50 spores/worm). At intervals of 15, 45, 90 and 135 days these worms were removed from the respective Phase One replicate and placed into a new replicates containing sterile sand and water and no *Mc* myxospores. For Phase Two, 250 lineage III *Tubifex tubifex* oligochaetes **not previously exposed to *Mc* myxospores** were placed back into the replicates containing the original substrate (and any remaining myxospores) from which the Phase One worms were removed. Water in all replicates was decanted off once weekly, filtered and concentrated through a 20 micron mesh screen and TAM production was estimated.

Worm Source	**Lineage(s)**	**Time of Initial Exposure in Days**			
		15 Days	**45 Days**	**90 Days**	**135 Days**
Phase One					
Mt. Whitney	Pure III	930,518	10,276,062	2,625,737	2,268,858
Parachute Crk	Pure III	1,250,159	2,935,463	4,316,683	877,347
Cross Creek	Pure III	108,391	69,713	59,061	711,947
Eagle River	I, III, V	1,092	34,238	2,231	21,001
Williams Fork	Pure V	0	8,439[a]	0	0
Windy Gap	Pure VI	0	0	0	0
Phase Two					
Mt. Whitney/Parachute Crk	Pure III	2,809,274	0	3,386	2,514
Parachute Crk/Parachute Crk	Pure III	173,594	66	260	54
Cross Creek/Parachute Crk	Pure III	452,283	220	506	334
Eagle River/Parachute Crk	Pure III	1,070,609	18,281	0	0
Williams Fork/Parachute Crk	Pure III	252,942	29,413	1,264	0
Windy Gap/Parachute Crk	Pure III	129,630	3,623	1,574	270

[a]: At the end of the experiment, qPCR testing of all of the worms in this replicate revealed a very small percentage of lineage III DNA, indicating that apparently the 250 oligochaetes from the original stocking contained a very small number of lineage III worms, even though multiple previous qPCR screenings of the worms from this culture were shown to be a pure lineage V population.

These data indicate that 15-day exposure during Phase One was an inadequate period of time for any of the exposed worms to consume a substantial portion of the myxospores, given that the estimated total TAM production among the unexposed lineage III worms seeded into the original substrates during Phase Two produced between 129,630 and 2,809,274 TAMs (Table 8). However, unexposed pure lineage III worms for Phase Two seeded into the original substrate(s) from the 45-day Phase One exposure produced an average of 8,600 TAMs, compared to an average of 814,722 TAMs produced by the lineage III oligochaetes for the 15-day replicates, a reduction of 98.9%! Similarly, estimated reductions in TAM production among the unexposed lineage III oligochaetes seeded into original substrates for the 90-day and 135-day replicates (compared to the average for the 15-day replicates) were reduced 99.86% and 99.94%, respectively (Table 8).

It is especially noteworthy that no TAM production was ever observed among 7 of 8 replicate groups for the lineage V and lineage VI treatment groups for the Phase One exposures! The minimal amount of TAM production observed in the 45-day Phase One replicate of Williams Fork River lineage V worms was due to a very small number of lineage III worms (perhaps 1 to 3) that were in the original culture. Moreover, the fact that TAM production among all Phase Two lineage III oligochaete replicates was dramatically lower across all replicates for the 45, 90 and 135-day treatment groups is ***prima facie*** evidence that the lineage I, V and VI *Tubifex tubifex* worms truly function as living biological filters, deactivating and destroying *M. cerebralis* myxospores.

All of the foregoing, together with the findings of Hedrick et al. (2008) study suggest that it may be possible to break the life cycle of the *Mc* parasite in smaller high altitude lakes without a source of Mc-contaminated water by removing fish and suspending fish stocking for only a year or two.

CONCLUSIONS

Recent developments in the DNA typing and testing of the various lineages of *T. tubifex* for susceptibility or resistance to *M. cerebralis* infection are very encouraging. Studies conducted in California and Colorado (Beauchamp et al. 2002, 2006; Winkelman and Nehring 2007), Montana (Rasmussen et al. 2004; Kerans et al. 2004), Oregon (Arsan et al. 2007), New Mexico (DuBey and Caldwell 2004; DuBey et al. 2005), Utah and Argentina (Rasmussen et al. 2008) and West Virginia (Blazer et al. 2003; Dr. Vicki Blazer' personal communication) have repeatedly shown that only lineage III *T. tubifex* consistently produces TAMs of *M. cerebralis.* Oligochaetes belonging to lineages I, IV, V and VI are either refractory or highly resistant to infection by the parasite and do not produce fish-infective TAMs (Arsan et al. 2007). These results offer hope that non-susceptible *T. tubifex* lineages can act as "biofilters" to consume and deactivate *M. cerebralis* myxospores in habitats where the parasite is already enzootic, and dramatically reduce ambient levels of infection. Indeed, this appears to have been occurring at Windy Gap Reservoir in Colorado beginning in 2001 (Winkelman and Nehring 2007).

Aquatic oligochaete sampling and testing over the past six field seasons reveal that the *Mc*-susceptible lineage III *T. tubifex* is the most widely distributed of the four lineages of *T. tubifex* occurring in Colorado. Mitochondrial DNA specific to the lineage III oligochaete has

been detected in far more worm samples at all elevation zones between 6,000 and 12,000 feet in Colorado than for worms belonging to lineages I, V and VI. These findings indicate that the risk of establishment of *M. cerebralis* is quite high, once introduction into a previously unexposed aquatic ecosystem occurs. Moreover, there is no indication that high elevation and/or cold thermal conditions will prohibit establishment of *M. cerebralis* even above 12,000 feet. Indeed, detection of significant *M. cerebralis* infections in cutthroat trout and brook trout in wilderness area oligotrophic lakes such as Trappers, Piney and Marvine lakes in Colorado and Yellowstone Lake in Yellowstone National Park (Koel et al. 2005, 2006) do not support the hypothesis that habitat degradation and/or organic enrichment are requisite parameters for development of whirling disease epizootics among salmonid fish populations (Kaesar and Sharpe (2006).

After six field seasons, it is evident that *M. cerebralis* has become established in numerous aquatic habitats that support native cutthroat trout populations. The degree of spread of the parasite into high elevation habitats in the White and Yampa River basins is especially disconcerting. Many locations where the parasite is enzootic have direct connectivity to streams and lakes that support excellent populations of Colorado River cutthroat trout. It is critical that efforts be increased to construct barriers to isolate these populations and prevent invasion by non-native salmonids carrying the parasite from other areas where it is already enzootic.

Trappers Lake and Upper and Lower Marvine Lakes lie within the Flattops Wilderness Area in the White River National Forest. The headwaters of Piney Creek (above Piney Lake) north of Vail, Colorado lie within the Eagles Nest Wilderness Area. Upper and Lower Chicago Lakes lie at elevations over 11,400 feet in the Mt. Evans Wilderness. Lower Square Top Lake (west of Guanella Pass) and Kite Lake in the Mosquito Mountain Range west of Alma are located at elevations above 12,000 feet. Like Yellowstone Lake in Yellowstone National Park, these aquatic habitats are in pristine areas, located at relatively high elevation, and have no habitat degradation problems. Yet the cutthroat trout populations in all of these aquatic ecosystems are heavily infected with *M. cerebralis.* These cases belie the conventional "wisdom" that this parasite can only thrive in degraded or organically enriched environments. Rather, there are two important factors common to all of these ecosystems. Those factors are 1) the presence of a highly susceptible salmonid host, and 2) the presence of lineage III *T. tubifex.* It has been a proven fact for a decade that brook trout and Colorado's three sub-species of native cutthroat trout are more prone to develop a lethal infection after exposure to *M. cerebralis* than either brown trout or rainbow trout exposed under identical conditions (Thompson et al. 1999).

After six years of sampling and testing, we know that lineage III *T. tubifex* are highly abundant and the most widely distributed of the four lineages of *T. tubifex* known to occur in Colorado. Moreover, lineage III worms have been readily collected at all elevations in the state $\geq$ 3,659 meters (12,000 feet). The majority of core conservation populations of Colorado's native cutthroat trout occur in lakes and streams at elevations between 2,439 and 3,354 meters (8,000 – 11,000 feet). The empirical evidence collected over the past six years reveal there is a very high degree of congruence between aquatic habitats that 1) either sustain or are capable of supporting core conservation populations of native cutthroat trout, and 2) support dense populations of lineage III *T. tubifex.* Given these realities, it would be foolhardy for fisheries resource managers to assume that threat or risk of exposure of Colorado's native cutthroat trout to *M. cerebralis* is minimal. On the contrary, the risk is high once the parasite is introduced.

Finally, results of the exposure of various lineages (I, III, V and VI) of *T. tubifex* to *M. cerebralis* conducted under controlled laboratory conditions summarized in this report have significant management implications for managing risk of exposure for vulnerable salmonid species, as well as ameliorating if not eliminating (in some circumstances) *Myxobolus cerebralis* in some aquatic habitats. The important highlights and insights of these efforts are as follows:

1. Only lineage III strain *T. tubifex* in Colorado produce TAM actinospores that are infective to trout. However, this strain is the most cosmopolitan of the four strains currently know to occur in Colorado. Sites where it was detected outnumber all other sites combined where the other three lineages have been observed. The wide distribution of this lineage across all elevation zones from 5,000 feet to over 12,000 feet make it imperative that fishery resource managers remain fully aware of the risks that this pernicious parasite poses to salmonid fish populations in Colorado. This is particularly true for cutthroat trout.
2. In Colorado, lineage I *T. tubifex* have never been shown to produce TAMs. This strain is highly resistant to infection. In-situ DNA hybridization histology and qPCR testing has repeatedly demonstrated that only 5% of lineage I worms exposed to *M. cerebralis* become infected and develop early vegetative stages of the parasite. Exposed worms belonging to this lineage have never been shown to produce a single TAM when held in 1-ml well plates for up to 72 hours while identically exposed lineage III worms were producing large numbers of TAMs when placed in well plates. These highly resistant lineage I worms do function as true biological filters. They consume and destroy *M. cerebralis* myxospores and dramatically reduce the number of TAMs produced in replicates exposures where they are sympatric with lineage III worms, compared to identically exposed allopatric lineage III replicates.
3. In Colorado, lineage V and VI *T. tubifex* are refractory to infection by *M. cerebralis.* They do not produce TAMs when exposed to this parasite in allopatry. In-situ DNA hybridization histology and qPCR testing has repeatedly demonstrated that infection does not develop at all in these lineages. Moreover, the results of the experimental exposures summarized in this report are ***prima facie*** evidence that they do function as biological filters, consuming and destroying *M. cerebralis* myxospores, dramatically reducing the number of TAMs produced in replicate exposures where they are sympatric with lineage III worms, compared to identically exposed allopatric lineage III replicates.
4. The lineage V and VI *T. tubifex* are very easy to rear in aquaria. They have a very high rate of reproduction. Replicates of 250 worms exposed to *M. cerebralis* have been shown to produced 1,000s of offspring during a six-month holding period when fed a nutritious diet. They should be easy to rear in the earthen settling ponds at public and private fish culture facilities and could be employed to reduce ambient levels of *Mc* infection in the natural environment.
5. Myxospores of *Myxobolus cerebralis* do not remain viable in the environment for years or even decades. Research findings in this study are congruent with those of Hedrick et al. (2008) in demonstrating that the myxospores rapidly lose viability and are (in all likelihood) rendered 100% non-viable after six months to one year when held in sediment and water within the range of temperatures (5 °C to 15°C) conducive to long-term survival of trout in their natural habitat.

After almost two decades of intense research efforts on a multiplicity of fronts, the number of powerful tools and techniques that can be brought to bear on the problem of whirling disease and its potential for significant negative impacts on wild trout populations are considerable. These include the development of powerful diagnostic tools such as in-situ DNA hybridization histology (Antonio et al. 1998), PCR (Andree et al. 1998) and qPCR (Cavender et al. 2004) techniques for detection of DNA of the *Mc* parasite in fish, oligochaetes and water. It includes a field technique for concentration, detection and quantification of TAMs of the parasite from water samples in the field that is cheap to use, can be applied in all seasons of the year, and portable to almost any site where water occurs (Thompson and Nehring 2000). Development of DNA markers used to separate and identify the various lineages of the oligochaete host from field collections makes it possible to assess the potential threat for establishment of the parasite in the natural environment if introduction were to occur (Beauchamp et al. 2001, 2002, 2005, 2006). Finally, the discovery of high resistance to *Mc* infection in the HOFER (or German) strain of rainbow trout early in the 21st century was of paramount importance. These fish were thoroughly tested and screened for numerous pathogens of salmonid fishes in the research laboratories of Drs. Mansour El-Matbouli in Munich, Germany and Ron Hedrick at the U. of Ca-Davis (Hedrick et al. 2003). The subsequent development, cross-breeding and testing of various stocks of wild strain rainbow trout with the Hofer strain that demonstrate significant resistance to infection by the *Mc* parasite and that this resistance is heritable from one generation to another (Schisler et al. 2006) is one of the capstones of this two-decades long research effort.

RECOMMENDATIONS

The lineage III strain *T. tubifex* is highly susceptible to infection by *the M. cerebralis* parasite. It is also far and away the most cosmopolitan, widely distributed lineage of this species among the 4 lineages (I, III, V and VI) currently known to occur in Colorado. It is important that fishery management biologists continue disease testing for this parasite among salmonid populations in high altitude areas of the state where cutthroat trout are either naturally endemic or stocked for recreational purposes. While this effort need not be regimented nor regularly conducted, it should be done as a standard part of any fisheries survey or gill netting operation in lakes and streams in wilderness areas.

The lineage V and VI strain *T. tubifex* can easily be cultured in aquaria, and probably in sediment laden ponds almost any place in the state where salmonids are reared in aquaculture facilities. Introduction of these oligochaetes into ponds where the lineage III strain is either allopatric or dominant will significantly reduce the number of *M. cerebralis* myxospores available for consumption by the highly susceptible lineage III worm in habitats where *M. cerebralis* is enzootic. The net effect will be a dramatic decline in the production of trout-infective TAMs being produced in settling ponds and released into receiving waters. Over time this may result in the near elimination of the parasite. A recently published study has clearly demonstrated that exposure of lineage III worms to 500 *M. cerebralis* myxospores results in parasitic emasculation, rendering all infected lineage III worms incapable of reproduction (Shirakashi and El-Matbouli 2009). This puts populations of lineage III *T. tubifex* at a severe competitive disadvantage when occurring in sympatry with lineage V and VI worms in aquatic habitats where *M. cerebralis* is enzootic.

LITERATURE CITED

Arsan, E. L. 2006. Potential for dispersal of the non-native parasite *Myxobolus cerebralis*: qualitative risk assessments for the state of Alaska and the Williamette River basin, Oregon. Master of Science thesis. Oregon State University, Corvallis.

Arsan, E. L., S. L. Hallett and J. L. Bartholomew. 2007. *Tubifex tubifex* from Alaska and their susceptibility to *Myxobolus cerebralis*. Journal of Parasitology 93(6):1332-1342.

Baldwin, T. J., J. E. Peterson, G. C. McGhee, K. D. Staigmiller, E. S. Motteram, C. C. Downs, and D. R. Stanek. 1998. Distribution of *Myxobolus cerebralis* in salmonid fishes in Montana. Journal of Aquatic Animal Health 10:361-371.

Bartholomew, J. L., and P. W. Reno. 2002. The history and dissemination of whirling disease. In Bartholomew, J. L., and J. C. Wilson (editors). 2002. Whirling disease: reviews and current topics. American Fisheries Society, Symposium 29, Bethesda, Maryland USA.

Bauer, O. N. 1959. Parasites of freshwater fish and the biological basis for their control. Bulletin of State Scientific Research Institute of Lake and River Fisheries 49. Israel Programme for Scientific Translations. Jerusalem 1962. 236 pp.

Baxa, D. V., and R. P. Hedrick. 2008. Effect of substratum on the development and release of the triactinomyxon stage of *Myxobolus cerebralis* in mitochondrial DNA 16S *Tubifex tubifex* Lineages. Final Report to the Whirling Disease Initiative. University of California-Davis. 17 pages.

Beauchamp, K. A., M. El-Matbouli, M. Gay, M. P. Georgiadis, R. B. Nehring, and R. P. Hedrick. 2006. The effect of cohabitation of *Tubifex tubifex* (Oligochaeta:Tubificidae) populations on infections to *Myxobolus cerebralis* (Myxozoa:Myxobolidae). Journal of Invertebrate Pathology 91:1-8.

Beauchamp, K. A., G. O. Kelley, R. B. Nehring, and R. P. Hedrick. 2005. The severity of whirling disease among wild trout populations corresponds to differences in genetic composition of *Tubifex tubifex* populations in central Colorado. Journal of Parasitology 91(1):53-60.

Beauchamp, K. A., M. Gay, G. O. Kelley, M. El-Matbouli, R. D. Kathman, R. B. Nehring, and R. P. Hedrick. 2002. Prevalence and susceptibility of infection of *Myxobolus cerebralis*, and genetic differences among populations of *Tubifex tubifex*. Diseases of Aquatic Organisms 51:113-131.

Beauchamp, K. A., R. D. Kathman, T. S. McDowell, and R. P. Hedrick. 2001. Molecular phylogeny of tubificid oligochaetes with special emphasis on *Tubifex tubifex*. Molecular Phylogenetics and Evolution 19 (2):216-224.

Cavender, W. P., J. S. Wood, M. S. Powell, K. Overturf, and K. D. Cain. 2004. Real-time quantitative polymerase chain reaction (QPCR) to identify *Myxobolus cerebralis* in rainbow trout *Oncorhynchus mykiss*. Diseases of Aquatic Organisms 60:205-213.

Coleman, M. A., and K. D. Fausch 2006. Causes of recruitment bottlenecks in translocated cutthroat trout populations: investigations of low temperature effects. Final Report to Colorado Division of Wildlife, USDI Central Utah Project Completion Act Office, USDA Forest Service, and National Park Service. Department of Fishery and Wildlife Biology, Colorado StateUniversity, Fort Collins.

DuBey, R., and C. A. Caldwell. 2004. Distribution of *Tubifex tubifex* lineages and *Myxobolus cerebralis* infection in the tailwater of the San Juan River, New Mexico. Journal of Aquatic Animal Health 16:179-185.

DuBey, R., C. A. Caldwell, and W.R. Gould. 2005. Effects of temperature, photoperiod and *Myxobolus cerebralis* infection on growth, reproduction, and survival of *Tubifex tubifex* lineages. Journal of Aquatic Animal Health 17:338-344.

Funk, F. 1968. Trout diseases and injuries. Schweiz. Fisch Zeitung 76:10-13.

Hedrick, R.P., T. S. McDowell, G. D. Marty, G. T. Fosgate, K. Mukkatira, K. Myklebust, and M. El-Matbouli. 2003. Susceptibility of two strains of rainbow trout (one with suspected resistance to whirling disease) to *Myxobolus cerebralis* infection. Diseases of Aquatic Organisms 55:37-44.

Hedrick, R.P., T. S. McDowell, K. Mukkatira, E. McConnell. And B. Petri. 2008. Effects of freezing, drying, ultraviolet irradiation, chlorine, and quaternary ammonia treatments on the infectivity of myxospores of *Myxobolus cerebralis* for *Tubifex tubifex*. Journal of Aquatic Animal Health 20:116-125.

Hofer, B. 1903. Uber die Drehkrankheit der Regenbogenforelle. Allgemeine Fischerei Zeitschrift 28:7-8.

Hoffman, G. L., C. E. Dunbar and A. Bradford. 1962. Whirling disease of trouts caused by *Myxosoma cerebralis* in the United States. U. S. Fish and Wildlife Service Special Scientific Report Fisheries 427.

Kaesar, A. J., and W. E. Sharpe. 2006. Patterns of distribution and abundance of *Tubifex tubifex* and other aquatic oligochaetes in *Myxobolus cerebralis* enzootic areas in Pennsylvania, Journal of Aquatic Animal Health 18:64-78.

Kathman, R. D., and R. O. Brinkhurst. 1998. Guide to the freshwater oligochaetes of North America. Aquatic Resources Center. Thompson Station, Tennessee.

Kerans, B.L., C. Rasmussen, R. Stevens, A.E.L. Colwell, and J. R. Winton. 2004. Differential propagation of the metazoan parasite *Myxobolus cerebralis* by *Limnodrilus hoffmeisteri, Ilyodrilus templetoni* and genetically distinct strains of *Tubifex tubifex*. Journal of Parasitology 90(6):1366-1373.

Koel, T. M., P. E. Bigelow, P. D. Doepke, B. D. Ertal, and D. L. Mahoney. 2005. Nonnative lake trout in Yellowstone cutthroat trout decline and impacts to bears and anglers. Fisheries 30(11): 10-17.

Koel, T.M., D. L. Mahoney, K.L. Kinnan, C. Rasmussen, C.J. Hudson, S. Murcia, and B. L. Kerans. 2006. *Myxobolus cerebralis in native* cutthroat trout of the Yellowstone Lake ecosystem. Journal of Aquatic Animal Health 18:157-175.

Markiw, M. E. and K. Wolf. 1983. *Myxosoma cerebralis* (Myxozoa: Myxosporea) etiologic agent of salmonid whirling disease requires tubificid worm (Annelida: Oligochaeta) in its life cycle. Journal of Protozoology 30(3):561-564.

Markiw, M. E. and K. Wolf. 1974. *Myxosoma cerebralis*: isolation and concentration from fish skeletal elements - sequential enzymatic digestions and purification by differential centrifugation. Journal of the Fisheries Research Board of Canada. 31:15-20.

Nehring, R. B. 2009. Whirling disease investigations. *Myxobolus cerebralis* in Colorado's cutthroat trout populations. Colorado Division of Wildlife Job 1 Progress Report, Federal Aid Project F-237-R16. Fort Collins.

Nehring, R. B. 2008. Whirling disease investigations. *Myxobolus cerebralis* in Colorado's cutthroat trout populations. Colorado Division of Wildlife Job 1 Progress Report, Federal Aid Project F-237-R15. Fort Collins.

Nehring, R. B. 2007. Whirling disease investigations. *Myxobolus cerebralis* in Colorado's cutthroat trout populations. Colorado Division of Wildlife Job 1 Progress Report, Federal Aid Project F-237-R14. Fort Collins.

Nehring, R. B. 2006a. Colorado's cold water fisheries: whirling disease case histories and insights for risk management. Colorado Division of Wildlife Special Technical Report Number 79. DOW-S-79-06. 46 pages.

Nehring, R. B. 2006b . Whirling disease investigations. *Myxobolus cerebralis* in Colorado's cutthroat trout populations. Colorado Division of Wildlife Job 1 Progress Report, Federal Aid Project F-237-R13. Fort Collins.

Nehring, R. B. 2005 . Whirling disease investigations. *Myxobolus cerebralis* in Colorado's cutthroat trout populations. Colorado Division of Wildlife Job 1 Progress Report, Federal Aid Project F-237-R12. Fort Collins.

Nehring, R. B. 2004. Whirling disease investigations. *Myxobolus cerebralis* in Colorado's cutthroat trout populations. Colorado Division of Wildlife Job 1 Progress Report, Federal Aid Project F-237-R11. Fort Collins.

Nehring, R. B., and K. G. Thompson. 2003. Whirling disease investigations. Colorado Division of Wildlife Final Report, Federal Aid Project F-237-R-10. Fort Collins.

Nehring, R. B., and K. G. Thompson. 2001. Impact assessment of some physical and biological factors in the whirling disease epizootic among wild trout in Colorado. Colorado Division of Wildlife Special Report Number 76. Fort Collins.

Nehring, R. B., K. G. Thompson, and S. Hebein. 1998. Impacts of whirling disease on wild trout populations in Colorado. Pages 82 – 94 In K. G. Wadsworth (editor) Transactions of the 63rd North American Wildlife and Natural Resources Conference. Wildlife Management Institute. Washington, D.C.

Nehring, R. B. and P. G. Walker. 1996. Whirling disease in the wild: the new reality in the intermountain west. Fisheries. 21(6):28-30.

Peterson, D. P., and K. D. Fausch. 2003a. Upstream movement by nonnative brook trout (*Salvelinus fontinalis*) promotes invasion of native cutthroat trout (*Oncorhynchus clarki*) habitat. Canadian Journal of Fisheries and Aquatic Sciences 60:1502-1516.

Peterson, D. P., and K. D. Fausch. 2003b. Testing population-level mechanisms of invasion by a mobile vertebrate: a simple conceptual framework for salmonids in streams. Biological Invasions 5:239-259.

Peterson, D. P., K. D. Fausch, and G. C. White. 2004. Population ecology of an invasion: effects of brook trout on native cutthroat trout. Ecological Applications 14:754-772.

Plehn, M. 1905. Uber die Drehkrankheit der salmoniden [(*Lentospora cerebralis*) (Höfer) Plehn]. Archiv Protistenkunde 5:145-166.

Rasmussen, C., J. Zickovich, J. R. Winton, and B. L. Kerans. 2008. Variability in triactinomyxon production from *Tubifex tubifex* populations from the same mitochondrial DNA lineage infected with *Myxobolus cerebralis*, the causative agent of whirling disease in salmonids. Journal of Parasitology 94(3):700-708.

Schäperclaus, W. 1931. XXI Die Drehkrankheit in der Forrellenzucht und ihre Bekämpfung. Zeitschrift Fischerei 29:521-567.

Schisler, G. J. 2001. Whirling disease investigations. Rapid assessment technique evaluation. Colorado Division of Wildlife Federal Aid Job Number 5 Progress Report. Federal Aid Project F-237-R-8. Fort Collins.

Schisler, G. J., K. A. Myklebust and R.P. Hedrick. 2006. Inheritance of *Myxobolus cerebralis* resistance among F_1-generation crosses of whirling disease resistant and susceptible rainbow trout strains. Journal of Aquatic Animal Health 18:109-115.

Seber, G.A.F., and E. D. LeCren. 1967. Estimating population parameters from catches large relative to the population. Journal of Animal Ecology 36:631-643.

Shirakashi, S., and M. El-Matbouli. 2009. *Myxobolus cerebralis* (Myxozoa), the causative agent of whilring disease, reduces fecundity and feeding activity of *Tubifex tubifex* (Oligochaeta). Parasitology 136:603-613.

Thompson, K. G., R. B. Nehring, D. C. Bowdin, and T. Wygant. 1999. Field exposure of seven species or subspecies of salmonids to *Myxobolus cerebralis* in the Colorado River, Middle Park, Colorado. Journal of Aquatic Animal Health 11:312-329.

Uspenskaya, A.V. 1957. The ecology and spreading of the pathogen of trout whirling disease – *Myxosoma cerebralis* (Hofer, 1903, Plehn, 1905) in the fish ponds of the Soviet Union. Pages 47-55 *In* G. K. Petrushevskii, editor. Parasites and diseases of fish. Bulletin All-Union Institute of Freshwater Fisheries. Leningrad.

Uspenskaya, A.V. 1982. New data on the life cycle and biology of Myxosporidia. Archiv fur Protistenkunde 126:309-338.

Vincent, E. R. 1996a. Whirling disease - the Montana experience, Madison River. Page 159 *In* E. P. Bergersen and B. A. Knopf, editors. Proceedings: Whirling disease workshop-where do we go from here? Colorado Cooperative Fish and Wildlife Research Unit. Fort Collins, Colorado.

Vincent, E. R. 1996b. Whirling disease and wild trout: the Montana experience. Fisheries. 21(6):32-33.

Walker, P. G. and R. B. Nehring. 1995. An investigation to determine the cause(s) of the disappearance of young wild rainbow trout in the upper Colorado River, in Middle Park, Colorado. Colorado Division of Wildlife Report. Denver.

Winkelman, D., and R. B. Nehring. 2007. Assessing the density and distribution of *Tubifex tubifex* lineages in Windy Gap Reservoir, Colorado. Final Project Report to the Whirling Disease Initiative, Montana Water Center, Bozeman, Montana.

Wolf, K., and M. E. Markiw. 1984. Biology contravenes taxonomy in the Myxozoa: new discoveries show alternation of invertebrate and vertebrate hosts. Science 225:1449-1452.

APPENDIX

Table 1A. Phase 1 Parachute Creek IIIs; Phase 2 Parachute Creek IIIs. All replicates for Phase One were stocked with 250 *Tubifex tubifex* oligochaetes and exposed to 12,500 myxospores of *Myxobolus cerebralis* (50 spores/worm). At intervals of 15, 45, 90 and 135 days these worms were removed from replicates 1,2,3, and 25 and placed into replicates 1A, 2A, 3A and 25 A, respectively. For Phase Two, 250 unexposed lineage III *Tubifex tubifex* oligochaetes from Parachute Creek were placed back into replicates 1, 2, 3 and 25 (with the original substrate) at the time the Phase One worms were removed.

Date	**Phase One – Parachute Creek IIIs**				**Neg.**	**Phase Two – Parachute Creek IIIs**			
Mmddyy	**15 d**	**45 d**	**90 d**	**135 d**	**Control**	**15 d**	**45 d**	**90 d**	**135 d**
	1A	**2A**	**3 A**	**25A**	**22**	**1**	**2**	**3**	**25**
2/27/09	**Start**	**Start**	**Start**	**Start**	**Start**				
3/06/09	Ns	Ns	Ns	Ns	Ns	**Start**			
4/06/09	Ns	Ns	Ns	Ns	Ns		**Start**		
4/13/09	0	0	0	0	0	0	0		
4/20/09	Ns	Ns	Ns	Ns	Ns	Ns	Ns		
4/27/09	0	0	0	0	Ns	Ns	Ns		
5/04/09	0	0	0	0	Ns	Ns	Ns		
5/07/09	0	0	0	0	Ns	Ns	0		
5/11/09	0	0	0	0	Ns	Ns	Ns		
5/18/09	0	124,110	55,825	0		Ns	Ns		
5/21/09	Ns[a]	Ns	191,625	Ns		Ns	Ns	**Start**	
5/25/09	120,345	180,960	381,550	120		0	Ns	Ns	
6/01/09	17,038	394,583	1,303,999	7,956		4,056	Ns	Ns	
6/08/09	437,500	500,000	291,250	5,000		8,093	Ns	Ns	
6/11/09	140,000	495,000	425,000	136,625		20,625	Ns	Ns	
6/18/09	5,000	420,000	80,000	177,500		77,500	Ns	Ns	
6/22/09	1,325	457,500	572,500	77,500		6,153	0	Ns	
6/29/09	3,179	100,000	90,000	223,750	0	14,963	0	Ns	
7/06/09	1,568	30,282	16,250	56,250	Ns	9,281	0	Ns	**Start**
7/13/09	110	518	548	26,250	Ns	0	0	Ns	Ns
7/20/09	89	13,230	21,063	41,113	Ns	3,225	0	Ns	Ns
7/27/09	5,250	67,620	104,558	51,150	0	3,124	0	70	Ns
8/3/09	92,588	27,500	179,215	1,838	Ns	1,024	0	0	Ns
8/10/09	51,700	15,625	115,000	17,938	Ns	5,655	0	0	Ns
8/17/09	213,380	66,728	312,320	17,438	Ns	7,203	0	0	Ns
8/24/09	154,980	12,968	132,435	10,638	0	11,558	66	0	Ns
8/31/09	5,510	25,755	37,500	22,605	Ns	975	0	0	Ns
9/08/09	184	486	454	713	Ns	0	0	0	Ns
9/14/09	413	1,473	5,591	2,963	Ns	159	0	0	Ns
9/21/09	0	1,125	0	0	0	0	0	0	0
9/28/09	[b]	[b]	[b]	[b]	[b]	0	0	0	0
10/04/09	[b]	[b]	[b]	[b]	[b]	0	0	0	54
10/12/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	0
10/19/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	0
10/27/09	[b]	[b]	[b]	[b]	[b]	[c]	0	190	0
11/02/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	0
11/09/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	0
11/16/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
11/23/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
11/30/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
12/07/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
12/14/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	[f]
12/21/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0 [g]	[f]
Total	**1,250,159**	**2,935,463**	**4,316,683**	**877,347**	**0**	**173,594**	**66**	**260**	**54**

[a]**:** Ns indicates no sample taken on that date in that replicate. [b]: Terminated on 9/21/09.
[c]: Terminated on 10/4/09. [d] :Terminated on 11/10/09. [f]: Terminated on 12/07/09
[g] :Terminated on 12/21/09

Table 1B. Phase 1 Windy Gap VIs; Phase 2 Parachute Creek IIIs; All replicates for Phase One were stocked with 250 *Tubifex tubifex* oligochaetes from Windy Gap Reservoir and exposed to 12,500 *Myxobolus cerebralis* myxospores (50 spores/worm). At intervals of 15, 45, 90 and 135 days these worms were removed from replicates 4,5,6, and 26 and placed into replicates 4A, 5A, 6A and 26A, respectively. For Phase Two, 250 unexposed lineage III *Tubifex tubifex* oligochaetes from Parachute Creek were placed back into replicates 4, 5, 6 and 26 (with the original substrate).

Date	**Phase One – Windy Gap VIs**				**Neg.**	**Phase Two – Parachute Creek IIIs**			
Mmddyy	**15 d**	**45 d**	**90 d**	**135 d**	**Control**	**15 d**	**45 d**	**90 d**	**135 d**
	4A	**5A**	**6A**	**26A**		**4**	**5**	**6**	**26**
2/27/09	**Start**	**Start**	**Start**	**Start**	**Start**				
3/06/09	Ns	Ns	Ns	Ns	Ns	**Start**			
4/06/09	Ns	Ns	Ns	Ns	Ns	Ns	**Start**		
4/13/09	0	0	0	0	Ns	0	0		
4/20/09	0	0	0	0	Ns	Ns	Ns		
4/27/09	0	0	0	0	Ns	Ns	Ns		
5/04/09	0	0	0	0	Ns	Ns	Ns		
5/07/09	Ns[a]	Ns	0	0	Ns	Ns	Ns		
5/11/09	Ns	Ns	0	0	Ns	Ns	Ns		
5/18/09	Ns	Ns	0	0	Ns	Ns	Ns		
5/21/09	Ns	Ns	0	0	Ns	Ns	Ns	**Start**	Ns
5/25/09	Ns	Ns	0	0	Ns	Ns	Ns	Ns	Ns
6/01/09	0	0	0	0	Ns	0	Ns	Ns	Ns
6/08/09	Ns	Ns	Ns	Ns	Ns	90	Ns	Ns	Ns
6/11/09	Ns	Ns	Ns	Ns	Ns	270	Ns	Ns	Ns
6/18/09	Ns	Ns	Ns	Ns	Ns	10,408	Ns	Ns	Ns
6/22/09	Ns	Ns	Ns	Ns	Ns	5,625	68	Ns	Ns
6/29/09	0	0	0	0	0	29,900	0	Ns	0
7/06/09	Ns	Ns	Ns	Ns	Ns	19,375	113	Ns	**Start**
7/13/09	Ns	Ns	Ns	Ns	0	625	0	Ns	Ns
7/20/09	Ns	Ns	Ns	Ns	Ns	1,760	0	Ns	Ns
7/27/09	0	0	0	0	Ns	1,650	0	0	Ns
8/03/09	Ns	Ns	Ns	Ns	0	2,763	0	0	Ns
8/10/09	Ns	Ns	Ns	Ns	Ns	7,200	188	84	Ns
8/17/09	Ns	Ns	Ns	Ns	Ns	4,440	170	1,211	Ns
8/24/09	0	0	0	0	0	24,890	2,350	188	Ns
8/31/09	Ns	Ns	Ns	Ns	Ns	2,625	0	0	Ns
9/08/09	Ns	Ns	Ns	Ns	Ns	3,460	325	0	Ns
9/14/09	Ns	Ns	Ns	Ns	Ns	14,288	325	0	Ns
9/21/09	0	0	0	0	0	0	0	0	0
9/28/09	[b]	[b]	[b]	[b]	[b]	0	0	0	0
10/04/09	[b]	[b]	[b]	[b]	[b]	261	176	0	0
10/12/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	0
10/19/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	0
10/27/09	[b]	[b]	[b]	[b]	[b]	[c]	150	0	0
11/02/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	270
11/09/09	[b]	[b]	[b]	[b]	[b]	[c]	83	0	0
11/16/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	91	0
11/23/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
11/30/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
12/07/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
12/14/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	[f]
12/21/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0 [g]	[f]
Total	**0**	**0**	**0**	**0**	**0**	**129,630**	**3,623**	**1,574**	**270**

[a]: Ns indicates no sample taken on that date in that replicate.
[b]: Terminated on 9/21/09
[c]: Terminated on 10/4/09
[d] Terminated on 11/10/09
[f] Terminated on 12/07/09
[g] Terminated on 12/21/09

Table 1C. Phase 1 Mt. Whitney IIIs; Phase 2 Parachute Creek IIIs. Phase One replicates were stocked with 250 Mt. Whitney lineage III *Tubifex tubifex* oligochaetes and exposed to 12,500 myxospores of *Myxobolus cerebralis* (50 spores/worm). At intervals of 15, 45, 90 and 135 days these worms were removed from replicates 7, 8, 9 and 27 and placed into replicates 7A, 8A, 9A and 27A, respectively. For Phase Two, 250 unexposed lineage III *Tubifex tubifex* oligochaetes from Parachute Creek were placed back into replicates 7, 8, 9 and 27 (with the original substrate) when the Phase 1 worms were removed.

Date mmddyy	**Phase One – Mt. Whitney, CA IIIs**				**Neg. Control**	**Phase Two – Parachute Creek IIIs**			
	15 d	**45 d**	**90 d**	**135 d**		**15 d**	**45 d**	**90 d**	**135 d**
	7A	**8 A**	**9A**	**27A**	**23**	**7**	**8**	**9**	**27**
2/27/09	**Start**	**Start**	**Start**	**Start**	**Start**				
3/06/09	Ns	Ns	Ns	Ns	Ns	**Start**			
4/06/09	Ns	Ns	Ns	Ns	Ns	Ns	**Start**		
4/13/09	0	0	0	0	0	Ns	0		
4/20/09	Ns[a]	Ns	Ns	Ns	Ns	Ns	Ns		
4/27/09	Ns	Ns	Ns	Ns	Ns	Ns	Ns		
5/04/09	0	0	0	0	Ns	Ns	Ns		
5/07/09	0	0	0	Ns	Ns	Ns	Ns		
5/11/09	0	0	0	0	Ns	Ns	Ns		
5/18/09	40,273	4,561	30,700	0	Ns	NS	Ns		
5/21/09	Ns	Ns	54,145	Ns	Ns	Ns	Ns	**Start**	
5/25/09	259,200	41,688	117,115	195	Ns	Ns	Ns	Ns	
6/01/09	103,750	431,250	371,250	162,540	0	1,740	Ns	Ns	
6/08/09	367,500	623,750	190,000	290,000	Ns	93,450	Ns	Ns	
6/11/09	37,500	397,500	155,000	427,000	Ns	120,000	Ns	Ns	
6/18/09	30,000	965,000	42,500	772,500	Ns	382,500	Ns	Ns	
6/22/09	20,000	4,025,000	18,750	177,500	Ns	632,500	0	Ns	
6/29/09	21,250	275,000	48,125	45,000	0	477,500	0	Ns	
7/06/29	581	335,625	41,269	247,500	Ns	166,875	0	Ns	**Start**
7/13/09	0	76,875	18,125	16,250	Ns	42,500	0	Ns	Ns
7/20/09	155	271,250	77,859	35,100	Ns	54,250	0	Ns	Ns
7/27/09	6,824	516,250	66,875	25,200	0	90,960	0	0	Ns
8/03/09	1,095	348,205	386,719	13,454	Ns	336,473	0	0	Ns
8/10/09	7,778	425,000	200,000	4,988	Ns	110,000	0	0	Ns
8/17/09	11,900	39,400	167,195	25,223	Ns	92,111	0	235	Ns
8/24/09	10,124	1,081,084	462,500	14,080	0	117,500	0	0	Ns
8/31/09	11,700	380,000	105,000	2,228	Ns	57,928	0	0	Ns
9/08/09	550	27,225	47,175	0	Ns	14,531	0	0	Ns
9/14/09	338	6,013	23,875	10,105	Ns	9,455	0	810	Ns
9/21/09	0	5,386	1,560	0	0	1,919	0	0	0
9/28/09	[b]	[b]	[b]	[b]	[b]	1,148	0	0	198
10/04/09	[b]	[b]	[b]	[b]	[b]	5,940	0	200	1,170
10/12/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	910
10/19/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	65
10/27/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	38
11/02/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	0
11/09/09	[b]	[b]	[b]	[b]	[b]	[c]	0	83	133
11/16/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	1,320	0
11/23/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	150	0
11/30/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	488	0
12/07/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
12/14/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	[f]
12/21/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0[g]	[f]
Total	**930,518**	**10,276,062**	**2,625,737**	**2,268,858**	**0**	**2,809,274**	**0**	**3,386**	**2,514**

[a]**:** Ns indicates no sample taken on that date in that replicate.
[b]: Terminated on 9/21/09
[c]: Terminated on 10/4/09
[d] :Terminated on 11/10/09
[f] :Terminated on 12/07/09
[g] :Terminated on 12/21/09

Table 1D. Phase 1 Williams Fork River V's; Phase 2 Parachute Creek IIIs. . Phase One replicates were stocked with 250 *Tubifex tubifex* oligochaetes from the Williams Fork River and exposed 12,500 *Myxobolus cerebralis* myxospores (50 spores/worm). At intervals of 15, 45, 90 and 135 days these worms were removed from replicates 10, 11, 12 and 28 and placed into replicates 10A, 11A, 12A and 28A, respectively. For Phase Two, 250 unexposed Parachute Creek lineage III *T. tubifex* oligochaetes were placed in replicates 10, 11, 12 and 28 with the original substrate after removal of Phase I worms.

Date	**Phase One – Williams Fork R. V's**				**Neg.**	**Phase Two – Parachute Creek IIIs**			
mmddyy	**15 d**	**45 d**	**90 d**	**135 d**	**Control**	**15 d**	**45 d**	**90 d**	**135 d**
	10A	**11A**	**12 A**	**28A**		**10**	**11**	**12**	**28**
2/27/09	**Start**	**Start**	**Start**	**Start**	**Start**				
3/06/09	Ns	Ns	Ns	Ns	Ns	**Start**			
4/06/09	Ns	Ns	Ns	Ns	Ns	Ns	**Start**		
4/13/09	0	0	0	0	Ns	0	0	Ns	Ns
4/20/09	0	1,485	0	0	Ns	Ns	Ns	Ns	Ns
4/27/09	0	428	0	0	Ns	Ns	Ns	Ns	Ns
5/04/09	0	563	0	0	Ns	Ns	Ns	Ns	Ns
5/07/09	Ns	798	Ns	Ns	Ns	Ns	Ns	Ns	Ns
5/11/09	0	398	0	0	Ns	Ns	Ns	Ns	Ns
5/18/09	0	118	0	0	Ns	Ns	Ns	Ns	Ns
5/21/09	Ns	Ns	Ns	Ns	Ns	Ns	Ns	**Start**	Ns
5/25/09	0	575	0	0	Ns	Ns	Ns	Ns	Ns
6/01/09	0	0	0	0	Ns	0	Ns	Ns	Ns
6/08/09	0	2,000	0	Ns	Ns	73	Ns	Ns	Ns
6/11/09	0	0	0	Ns	Ns	3,780	Ns	Ns	Ns
6/18/09	0	0	0	Ns	Ns	68,125	Ns	Ns	Ns
6/22/09	0	100	0	Ns	Ns	27,500	23,265	Ns	Ns
6/29/09	0	900	0	0	Ns	46,903	625	Ns	Ns
7/06/09	0	0	0	Ns	Ns	35,625	2,325	Ns	**Start**
7/13/09	0	325	0	Ns	0	4,375	538	Ns	Ns
7/20/09	0	0	0	Ns	Ns	21,600	0	Ns	Ns
7/27/09	0	710	0	0	Ns	11,180	65	128	Ns
8/03/09	0	0	0	Ns	0	1,734	488	36	Ns
8/10/09	0	89	0	Ns	Ns	1,215	130	0	Ns
8/17/09	0	0	0	Ns	0	2,956	1,398	225	Ns
8/24/09	0	0	0	0	0	17,238	473	765	Ns
8/31/09	0	0	0	Ns	Ns	4,650	0	70	Ns
9/08/09	0	0	0	Ns	Ns	2,738	0	70	Ns
9/14/09	0	0	0	Ns	Ns	2,550	0	0	Ns
9/21/09	0	0	0	0	0	0	0	0	0
9/28/09	[b]	[b]	[b]	[b]	[b]	0	0	0	0
10/04/09	[b]	[b]	[b]	[b]	[b]	700	0	40	0
10/12/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	0
10/19/09	[b]	[b]	[b]	[b]	[b]	[c]	101	0	0
10/27/09	[b]	[b]	[b]	[b]	[b]	[c]	70	0	0
11/02/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	0
11/09/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	0
11/16/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
11/23/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
11/30/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
12/07/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
12/14/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	[f]
12/21/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0[g]	[f]
Total	**0**	**8,489**	**0**	**0**	**0**	**252,942**	**29,413**	**1,264**	**0**

[a]**:** Ns indicates no sample taken on that date in that replicate.
[b]: Terminated on 9/21/09
[c]: Terminated on 10/4/09
[d] :Terminated on 11/10/09
[f] :Terminated on 12/07/09
[g] :Terminated on 12/21/09

Table 1E. Phase 1 Cross Creek IIIs; Phase 2 Parachute Creek IIIs. Phase One replicates were stocked with 250 Cross Creek Lineage III *Tubifex tubifex* and exposed 12,500 myxospores of *Myxobolus cerebralis* (50 spores/worm). At intervals of 15, 45, 90 and 135 days these worms were removed from replicates 13, 14, 15 and 29 and placed into replicates 13A, 14A, 15A and 29A, respectively. For Phase Two, 250 unexposed Parachute Creek lineage III *Tubifex tubifex* oligochaetes were placed in replicates 13, 14, 15 and 28 (with the original substrate) after the Phase One worms were removed. Individual TAM counts in **BOLD** numbers are the result of pre-exposure to myxospores in Cross Creek. Individual TAM numbers not in bold numbers are most likely the result of myxospore exposure beginning on 2/20/2009 or thereafter.

Date Mmddyy	**Phase One – Cross Creek IIIs**				**Neg. Control**	**Phase Two – Parachute Creek IIIs**			
	15 d	**45 d**	**90 d**	**135 d**		**15 d**	**45 d**	**90 d**	**135 d**
	13A	**14 A**	**15 A**	**29A**	**24**	**13**	**14**	**15**	**29**
2/27/09	**1,418**	**3,565**	**9,283**	**Ns**[a]	**4,025**				
3/06/09	**8,938**	**30,870**	**13,063**	**Ns**	**650**	**Start**			
3/11/09	**925**	**10,183**	**14,013**	**Ns**	**4,333**	Ns			
3/18/09	**Ns**	**35,063**	**1,050**	**Ns**	**1,250**	Ns			
3/25/09	**1,983**	**4,565**	**528**	**Ns**	**1,660**	Ns			
3/30/09	**7,505**	**4,485**	**8,360**	**13,110**	**7,920**	Ns			
4/06/09	**9,240**	**14,835**	**1,020**	**Ns**	**3,510**	Ns	**Start**		
4/13/09	**8,613**	**5,440**	**14,750**	**390**	**8,800**	Ns	Ns		
4/20/09	**31,720**	**52,880**	**46,200**	**28,125**	**33,990**	Ns	Ns		
4/27/09	**33,040**	**43,488**	**44,673**	**23,700**	**25,885**	Ns	Ns		
5/04/09	**5,250**	**32,720**	**Ns**	**7,395**	**13,570**	Ns	Ns		
5/07/09	**19,885**	**13,200**	**6,930**	**11,558**	**6,743**	Ns	Ns		
5/11/09	**6,390**	**9,900**	**22,910**	**20,144**	**850**	Ns	Ns		
5/18/09	23,108	8,650	6,435	31,613	450	Ns	Ns		
5/21/09	Ns	Ns	7,288	Ns	Ns	Ns	Ns	**Start**	
5/25/09	12,045	0	3,760	6,228	0	Ns	Ns	Ns	
6/01/09	6,600	14,030	1,873	16,330	2,775	6,450	Ns	Ns	
6/8/09	Ns	Ns	Ns	43,125	1,236	118,869	Ns	Ns	
6/11/09	12,450	12,125	21,875	126,250	3,350	57,500	Ns	Ns	
6/18/09	5,625	625	3,125	70,000	2,500	108,750	Ns	Ns	
6/22/09	2,644	16,608	8,740	32,500	2,700	30,000	0	Ns	
6/29/09	4,156	8,025	5,400	6,250	1,760	11,875	0	Ns	
7/06/09	935	625	94	29,185	0	9,375	85	Ns	**Start**
7/13/09	0	0	0	66,250	295	335	0	Ns	Ns
7/20/09	380	0	0	56,875	2,860	10,598	135	Ns	Ns
7/27/09	775	1,331	0	61,863	640	4,148	210	0	Ns
8/03/09	4,375	109	0	18,638	2,386	3,998	0	0	Ns
8/10/09	10,046	700	0	20,781	758	9,113	0	0	Ns
8/17/09	4,883	2,625	0	26,110	9,460	1,950	0	0	Ns
8/24/09	16,650	1,000	315	77,188	0	22,916	0	0	Ns
8/31/09	3,250	3,250	156	2,638	635	32,860	0	0	Ns
9/08/09	469	0	0	4,123	0	750	0	0	Ns
9/14/09	0	0	0	16,000	0	4,095	0	0	Ns
9/21/09	0	0	0	0	0	0	0	0	0
9/28/09	[b]	[b]	[b]	[b]	[b]	0	0	0	66
10/04/09	[b]	[b]	[b]	[b]	[b]	775	0	0	0
10/12/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	0
10/19/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	0
10/27/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	188
11/02/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	0
11/09/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	80
11/16/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
11/23/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	118	0
11/30/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	200	0
12/07/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	188	0
12/14/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	[f]
12/21/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0[g]	[f]
TAM totals from pre-exposure to myxospores of *Myxobolus cerebr0alis* in Cross Creek prior to 9/30/2008									
Totals	**134,907**	**261,194**	**182,780**	**104,422**	**113,210**	**Ns**	**Ns**	**Ns**	**Ns**
TAM totals resulting from exposure to myxospores of *Myxobolus cerebralis* on or after 2/20/2009									
Totals	108,391	69,713	59,061	711,947	31,805	452,283	220	506	334

[a]: Ns indicates no sample taken on that date in that replicate.

[b]: Terminated on 9/21/09 [d]: Terminated on 11/10/09 [g] Terminated on 12/21/09

[c]: Terminated on 10/4/09 [f] Terminated on 12/07/09

Table 1F. Phase 1 Eagle River oligochaetes are a mixed population of *Tubifex tubifex* comprised of lineages I, III and V; Phase 2 Parachute Creek lineage III worms. Phase One replicates were stocked with 250 Eagle River mixed lineage *Tubifex tubifex* and exposed 12,500 myxospores of *Myxobolus cerebralis* (50 spores/worm). At intervals of 15, 45, 90 and 135 days these worms were removed from replicates 19, 20, 21 and 30 and placed into replicates 19A, 20A, 21A and 30A, respectively. For Phase Two, 250 unexposed Parachute Creek lineage III *Tubifex tubifex* oligochaetes were placed in replicates 19, 20, 21 and 30 after the Phase One worms were removed. Individual TAM counts in **BOLD** numbers are the result of pre-exposure to myxospores in Cross Creek. Individual TAM numbers not in bold numbers are most likely the result of myxospore exposure beginning on 2/20/2009 or thereafter.

Date mmddyy	**Phase One –Eagle River Is, IIIs, Vs**				**Neg. Control**	**Phase Two – Parachute Creek IIIs**			
	15 d	**45 d**	**90 d**	**135 d**		**15 d**	**45 d**	**90 d**	**135 d**
	19A	**20A**	**21A**	**30A**		**19**	**20**	**21**	**30**
2/27/09	**Start**	**Start**	**Start**	**Start**	None				
3/06/09	Ns[a]	Ns	Ns	**Ns**	None	**Start**			
4/06/09	Ns	Ns	Ns	**Ns**	None	Ns	**Start**		
4/13/09	**260**	**0**	**220**	**0**		Ns	Ns		
4/20/09	**1,200**	**0**	**0**	**0**		Ns	Ns		
4/27/09	**1,305**	**0**	**0**	**0**		Ns	Ns		
5/04/09	**823**	**0**	**0**	**186**		Ns	Ns		
5/07/09	**Ns**	**Ns**	**Ns**	**Ns**		Ns	Ns		
5/11/09	**90**	**0**	**0**	**0**		Ns	Ns		
5/18/09	0	315	0	663		Ns	Ns		
5/21/09	Ns	Ns	0	Ns		Ns	Ns	**Start**	
5/25/09	0	610	0	0		Ns	Ns	Ns	
6/01/09	0	750	0	0		8.970	Ns	Ns	
6/08/09	0	520	1,406	3,536		23,063	Ns	Ns	
6/11/09	0	2,835	385	596		411,250	Ns	Ns	
6/18/09	0	7,159	440	6,558		102,500	Ns	Ns	
6/22/09	0	0	0	1,180		205,000	6,493	Ns	
6/29/09	0	356	0	70		166,563	10,625	Ns	
7/06/09	113	0	0	4,015		52,500	0	Ns	**Start**
7/13/09	0	0	0	2,344	None	2,063	0	Ns	Ns
7/20/09	0	160	0	338	None	51,765	275	Ns	Ns
7/27/09	65	1,383	0	863	None	11,500	0	0	Ns
8/03/09	405	2,530	0	0	None	3,188	140	0	Ns
8/10/09	143	7,290	0	0	None	2.281	0	0	Ns
8/17/09	181	3,333	0	838	None	11,906	380	0	Ns
8/24/09	0	386	0	0	None	3,895	0	0	Ns
8/31/09	191	6.611	0	0	None	12,336	368	0	Ns
9/08/09	0	0	0	0	None	1,200	0	0	Ns
9/14/09	0	0	0	0	None	495	0	0	Ns
9/21/09	0	0	0	0	None	134	0	0	0
9/28/09	[b]	[b]	[b]	[b]	None	0	0	0	0
10/04/09	[b]	[b]	[b]	[b]	None	0	0	0	0
10/12/09	[b]	[b]	[b]	[b]	None	[c]	0	0	0
10/19/09	[b]	[b]	[b]	[b]	None	[c]	0	0	0
10/27/09	[b]	[b]	[b]	[b]	None	[c]	0	0	0
11/02/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	0
11/09/09	[b]	[b]	[b]	[b]	[b]	[c]	0	0	0
11/16/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
11/23/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
11/30/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
12/07/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	0
12/14/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0	[f]
12/21/09	[b]	[b]	[b]	[b]	[b]	[c]	[d]	0[g]	[f]
TAM production resulting from pre-exposure in the Eagle River prior to September 30, 2008									
Totals	**3,678**	**0**	**220**	**186**	**None**	**Ns**	**Ns**	**Ns**	**Ns**
TAM production resulting from exposure to *Myxobolus cerebralis* myxospores on 2/20/2009									
Totals	1,092	34,238	2,231	21,001	None	1,070,609	18,281	0	0

[a]**:** Ns indicates no sample taken on that date in that replicate.
[b]: Terminated on 9/21/09
[c]: Terminated on 10/4/09
[d]: Terminated on 11/10/09
[f] Terminated on 12/07/09
[g] Terminated on 12/21/09

Table 2. Myxospores aging in sand experiment. All replicates (except for 34 and 34 A) were seeded with 12,500 myxospores on February 20, 2009. Replicates 34 and 34A were seeded with 1,000,000 myxospores on that date. Replicates were each seeded with 250 lineage III *Tubifex tubifex* from Parachute Creek after the proscribed time delay.

Date	**0 Day Delay**		**15 Day Delay**		**30 Day Delay**		**60 Day Delay**	
Mmddyy	**2A**	**3A**	**16**	**16A**	**17**	**17A**	**18**	**18A**
2/27/09	**Start**	**Start**						
3/06/09	0	0	**Start**	**Start**				
3/23/09	0	0	Ns	Ns	**Start**	**Start**		
4/21/09	Ns[a]	Ns	Ns	Ns	Ns	Ns	**Start**	**Start**
4/27/09	0	0	Ns	Ns	Ns	Ns	Ns	Ns
5/04/09	0	0	0	0	Ns	Ns	Ns	Ns
5/07/09	0	0	Ns	Ns	Ns	Ns	Ns	Ns
5/11/09	0	0	0	0	Ns	Ns	Ns	Ns
5/18/09	124,110	55,825	0	0	0	0	Ns	Ns
5/21/09	Ns	191,625	Ns	Ns	Ns	Ns	Ns	Ns
5/25/09	180,960	381,550	0	0	0	0	Ns	Ns
6/01/09	394,583	1,303,999	0	63	0	0	Ns	Ns
6/08/09	500,000	291,250	135	495	0	0	Ns	Ns
6/11/09	495,000	425,000	6,150	1,380	0	0	Ns	Ns
6/18/09	420,000	80,000	41,883	4,354	100,440	78	Ns	Ns
6/22/09	457,500	572,500	295,850	93,750	25,000	149	Ns	Ns
6/29/09	100,000	90,000	437,500	81,250	171,250	4,140	2,250	0
7/06/09	30,282	16,250	32,303	91,250	96,250	2,614	0	0
7/13/09	518	21,250	142,500	21,250	11,250	215	0	0
7/20/09	13,230	21,063	45,000	47,500	17,110	6,923	1,280	9,258
7/27/09	67,620	104,558	61,875	91,250	21,315	170,325	69,491	531,975
8/03/09	27,500	179,215	51,455	50,625	35,910	65,650	87,744	314,760
8/10/09	15,625	115,000	2,290	17,631	23,993	102,265	227,500	93,750
8/17/09	66,728	312,320	26,338	111,470	60,450	40,095	1,057,500	125,000
8/24/09	12,968	132,435	30,625	60,638	61,200	58,425	992,500	510,000
8/31/09	25,755	37,500	7,220	61,880	53,078	36,435	54,175	68,530
9/08/09	486	454	20,880	31,499	6,248	43,788	174,650	17,281
9/14/09	1,473	5,591	17,200	227,700	13,438	40,058	286,650	36,691
9/21/09	1,125	0	3,080	11,588	11,040	3,445	45,063	9,010
9/28/09	[b]	[b]	2,475	2,933	2,063	7,810	87,469	38,850
10/04/09	[b]	[b]	2,625	7,475	4,360	21,138	245,473	57,500
10/12/09	[b]	[b]	1,219	34,128	900	7,695	174,400	41,200
10/19/09	[b]	[b]	258	1,525	639	2,593	218,144	50,850
10/27/09	[b]	[b]	1,188	2,209	463	2,231	44,363	6,504
11/02/09	[b]	[b]	[e]	[e]	[e]	[e]	21,850	38,163
11/09/09	[b]	[b]	[e]	[e]	[e]	[e]	9,881	4,219
11/16/09	[b]	[b]	[e]	[e]	[e]	[e]	50,400	1,688
11/23/09	[b]	[b]	[e]	[e]	[e]	[e]	31,388	7,363
11/30/09	[b]	[b]	[e]	[e]	[e]	[e]	12,218	17,063
12/07/09	[b]	[b]	[e]	[e]	[e]	[e]	20,945	15,638
12/14/09	[b]	[b]	[e]	[e]	[e]	[e]	65,455	20,813
12/21/09	[b]	[b]	[e]	[e]	[e]	[e]	5,738	35,650
12/28/09	[b]	[b]	[e]	[e]	[e]	[e]	2,800 [g]	10,725 [g]
Totals	**2,935,463**	**4,316,683**	**793,844**	**1,042,255**	**705,357**	**612,627**	**3,944,264**	**2,053,561**

[a]: Ns indicates no sample taken on that date in that replicate.
[b]: Terminated on 9/21/09. [d]: Terminated on 9/21/09. [g] Terminated on 12/28/09
[c]: Terminated on 10/13/09 [e]: Terminated on 10/27/09.

Table 2 (continued). Myxospores aging in sand experiment. All replicates (except for 34 and 34 A) were seeded with 12,500 myxospores on February 20, 2009. Replicates 34 and 34A were seeded with 1,000,000 myxospores on that date. Replicates were each seeded with 250 lineage III *Tubifex tubifex* from Parachute Creek after the proscribed time delay.

Date	**0 Days**	**0 Days**	**90 Day Delay**		**120 Day Delay**		**180 Day Delay[b]**		**180 Day Delay[c]**	
Mmddyy	**2A**	**3A**	**31**	**31A**	**32**	**32A**	**33**	**33A**	**34**	**34A**
2/27/09	**Start**	**Start**								
4/06/09	0	0								
4/13/09	Ns	Ns								
4/20/09	0	Ns[a]								
4/27/09	0	0								
5/04/09	0	0								
5/07/09	0	0								
5/11/09		0								
5/18/09	124,110	55,825								
5/21/09	Ns	191,625	**Start**	**Start**						
5/25/09	180,960	381,550	Ns	Ns						
6/01/09	394,583	1,303,999	Ns	Ns						
6/08/09	500,000	291,250	Ns	Ns						
6/11/09	495,000	425,000	Ns	Ns						
6/18/09	420,000	80,000	Ns	Ns						
6/22/09	457,500	572,500	Ns	Ns	**Start**	**Start**				
6/29/09	100,000	90,000	Ns	Ns	Ns	Ns				
7/06/09	30,282	16,250	Ns	Ns	Ns	Ns				
7/13/09	518	548	Ns	Ns	Ns	Ns				
7/20/09	13,230	21,063	Ns	Ns	Ns	Ns				
7/27/09	67,620	104,558	175	2,731	Ns	Ns				
8/03/09	27,500	179,215	7,050	0	Ns	Ns				
8/10/09	15,625	115,000	635	151	Ns	Ns				
8/17/09	66,728	312,320	281	4,450	Ns	Ns	**Start**	**Start**	**Start**	**Start**
8/24/09	12,968	132,435	4,219	613	8,284	109	Ns	Ns	Ns	Ns
8/31/09	25,755	37,500	65,783	183	1,665	458	Ns	Ns	Ns	Ns
9/08/09	485	454	20,850	788	0	731	Ns	Ns	Ns	Ns
9/14/09	1,473	5,591	96,478	345	5,340	250	Ns	Ns	Ns	Ns
9/21/09	1,125	0	3,113	0	3,360	364	Ns	Ns	Ns	Ns
9/28/09	[d]	[d]	45,688	963	163	550	Ns	Ns	Ns	Ns
10/04/09	[d]	[d]	35,175	0	220	130	Ns	Ns	Ns	Ns
10/12/09	[d]	[d]	10,220	608	600	8,918	Ns	Ns	Ns	Ns
10/19/09	[d]	[d]	29,095	108	0	4,095	Ns	Ns	Ns	Ns
10/27/09	[d]	[d]	11,869	3,975	48	675	0	0	0	0
11/02/09	[d]	[d]	19,163	1,050	176	1,269	0	0	0	0
11/09/09	[d]	[d]	2,190	308	0	176	0	0	0	0
11/16/09	[d]	[d]	4,631	639	350	155	0	0	0	0
11/23/09	[d]	[d]	9,836	285	324	975	0	0	0	95
11/30/09	[d]	[d]	3,060	0	98	0	0	0	0	10,429
12/07/09	[d]	[d]	7,226	188	0	0	0	244	3,548	56,250
12/14/09	[d]	[d]	17,523	205	998	108	0	0	15,210	172,063
12/21/09	[d]	[d]	4,945[g]	0[g]	1,398	0	0	3,990	14,700	217,838
12/28/09	[d]	[d]	[g]	[g]	0	0	0	0	49,500	205,500
Totals	**2,935,463**	**4,316,683**	**378,838**	**17,385**	**21,928**	**18,855**	**0**	**4,234**	**82,958**	**662,175**

[a]: Ns indicates no sample taken on that date in that replicate.
[b]: Each replicate of 250 worms exposed to 12,500 *Myxobolus cerebralis* myxospores.
[c]: Each replicate of 250 worms exposed to 1,000,000 *Myxobolus cerebralis* myxospores.
[d]: Terminated on 9/21/09. **[g]** Terminated on 12/21/09

Table 2 (continued). Myxospores-aging in-sand experiment. All replicates (except for 34 and 34 A) were seeded with 12,500 myxospores on February 20, 2009. Replicates 34 and 34A were seeded with 1,000,000 myxospores on that date. Replicates were each seeded with 250 lineage III *Tubifex tubifex* from Parachute Creek after the proscribed time delay.

Date	**0 Days**	**0 Days**	**90 Day Delay**		**120 Day Delay**		**180 Day Delay[b]**		**180 Day Delay[c]**	
Mmddyy	**2A**	**3A**	**31**	**31A**	**32**	**32A**	**33**	**33A**	**34**	**34A**
6/22/09	457,500	572,500	Ns	Ns	**Start**	**Start**				
6/29/09	100,000	90,000	Ns	Ns	Ns	Ns				
7/06/09	30,282	16,250	Ns	Ns	Ns	Ns				
7/13/09	518	548	Ns	Ns	Ns	Ns				
7/20/09	13,230	21,063	Ns	Ns	Ns	Ns				
7/27/09	67,620	104,558	175	2,731	Ns	Ns				
8/03/09	27,500	179,215	7,050	0	Ns	Ns				
8/10/09	15,625	115,000	635	151	Ns	Ns				
8/17/09	66,728	312,320	281	4,450	Ns	Ns	**Start**	**Start**	**Start**	**Start**
8/24/09	12,968	132,435	4,219	613	8,284	109	Ns	Ns	Ns	Ns
8/31/09	25,755	37,500	65,783	183	1,665	458	Ns	Ns	Ns	Ns
9/08/09	485	454	20,850	788	0	731	Ns	Ns	Ns	Ns
9/14/09	1,473	5,591	96,478	345	5,340	250	Ns	Ns	Ns	Ns
9/21/09	1,125	0	3,113	0	3,360	364	Ns	Ns	Ns	Ns
9/28/09	[d]	[d]	45,688	963	163	550	Ns	Ns	Ns	Ns
10/04/09	[d]	[d]	35,175	0	220	130	Ns	Ns	Ns	Ns
10/12/09	[d]	[d]	10,220	608	600	8,918	Ns	Ns	Ns	Ns
10/19/09	[d]	[d]	29,095	108	0	4,095	Ns	Ns	Ns	Ns
10/27/09	[d]	[d]	11,869	3,975	48	675	0	0	0	0
11/02/09	[d]	[d]	19,163	1,050	176	1,269	0	0	0	0
11/09/09	[d]	[d]	2,190	308	0	176	0	0	0	0
11/16/09	[d]	[d]	4,631	639	350	155	0	0	0	0
11/23/09	[d]	[d]	9,836	285	324	975	0	0	0	95
11/30/09	[d]	[d]	3,060	0	98	0	0	0	0	10,429
12/07/09	[d]	[d]	7,226	188	0	0	0	244	3,548	56,250
12/14/09	[d]	[d]	17,523	205	998	108	0	0	15,210	172,063
12/21/09	[d]	[d]	4,945	0	1,398	0	0	3,990	14,700	217,838
12/28/09	[d]	[d]	[g]	[g]	0	0	0	0	49,500	205,500
1/03/10	[d]	[d]	[g]	[g]	464	0	263	0	17,750	87,281
1/10/10	[d]	[d]	[g]	[g]	220	0	540	0	6,650	1,730,575
1/17/10	[d]	[d]	[g]	[g]	0	0	0	1,138	6,125	231,150
1/24/10	[d]	[d]	[g]	[g]	[h]	[h]	1,031	200	17,200	162,500
1/31/10	[d]	[d]	[g]	[g]	[h]	[h]	0	0	9,488	213,675
2/07/10	[d]	[d]	[g]	[g]	[h]	[h]	0	0	678	293,231
2/14/10	[d]	[d]	[g]	[g]	[h]	[h]	0	0	2,423	62,156
2/21/10	[d]	[d]	[g]	[g]	[h]	[h]	0	0	6,186	141,625
2/28/10	[d]	[d]	[g]	[g]	[h]	[h]	73	0	13,090	66,400
3/07/10	[d]	[d]	[g]	[g]	[h]	[h]	0	0	8,250	44,563
3/14/10	[d]	[d]	[g]	[g]	[h]	[h]	456	0	15,840	80,475
3/21/10	[d]	[d]	[g]	[g]	[h]	[h]	145	0	6,466	94,500
3/28/10	[d]	[d]	[g]	[g]	[h]	[h]	**0**[j]	0[j]	0[j]	13,625[j]
Totals	**2,935,463**	**4,316,683**	**378,838**	**17,385**	**22,612**	**18,855**	**1,698**	**5,572**	**192,982**	**3,883,931**

[a]: Ns indicates no sample taken on that date in that replicate.
[b]: Each replicate of 250 worms exposed to 12,500 *Myxobolus cerebralis* myxospores.
[c]: Each replicate of 250 worms exposed to 1,000,000 *Myxobolus cerebralis* myxospores.
[d]: Terminated on 9/21/09. [g]: Terminated on 12/21/09. [h:] Terminated on 1/18/10; [j:] Terminated on 3/28/2010

Table 3. All replicates were stocked with 250 *Tubifex tubifex* oligochaetes and exposed to 12,500 myxospores of *Myxobolus cerebralis* (50 spores/worm) beginning on November 22, 2009. All exposures in this experiment were terminated on July 13, 2010.

Date Mddyy	**Parachute Creek Lineage III**		**Upper Squaretop Lake Lineage VI**		**Lower Squaretop Lake Lineage III**		**Silver Dollar Lake Lineage III**		**Mt. Whitney, CA Lineage III**	
	35	**35A**	**36**	**36A**	**37**	**37A**	**38**	**38A**	**39**	**39A**
1/24/10	0	0	0	0	25,920	138	0	0	0	0
1/31/10	0	163	0	0	7,875	0	0	0	0	0
2/07/10	0	0	0	0	27,563	5,163	0	0	158	0
2/14/10	0	0	0	0	13,800	438	0	0	115	0
2/21/10	0	0	0	0	13,689	383	0	0	0	0
2/28/10	0	0	0	0	4,388	155	0	0	375	0
3/07/10	1,950	32,029	0	0	7,095	135	4,348	14,243	1,238	425
3/14/10	5,568	139,150	0	0	27,808	27,650	167,693	45,325	29,250	15,551
3/21/10	18,000	124,988	0	0	8,438	18,988	282,319	250,600	7,650	20,855
3/28/10	128,775	82,250	0	0	56,700	14,560	2,572,750	765,325	331,975	85,625
4/05/10	140,400	67,031	0	0	1,219,269	129,850	3,758,044	204,525	781,625	209,450
4/11/10	127,200	13,613	0	0	864,694	311,838	11,719,900	594,500	857,863	711,000
4/18/10	238,438	28,200	0	0	544,800	127,500	248,308	30,300	562,600	122,200
4/25/10	318,013	49,275	0	0	448,050	193,600	597,400	1,413,675	1,471,500	647,075
5/02/10	53,025	72,250	0	0	184,775	40,000	427,388	120,000	344,000	101,563
5/09/10	164,763	218,763	0	0	42,000	174,225	1,676,625	287,825	635,950	270,356
5/16/10	503,863	266,625	0	0	1,153,750	150,675	90,000	49,875	646,875	72,269
5/23/10	334,400	310,406	0	0	165,750	377,488	226,800	39,900	243,600	175,175
5/31/10	87,570	80,013	0	0	213,081	295,200	129,600	26,563	289,575	203,175
6/06/10	86,710	60,375	0	0	240,358	396,000	108,000	22,328	397,660	261,494
6/14/10	15,194	31,688	0	0	258,281	157,500	123,600	10,800	694,713	253,825
6/20/10	35,618	46,500	0	0	144,300	57,920	103,950	11,250	45,300	185,588
6/27/10	25,013	37,588	0	0	56,950	190,650	68,175	9,750	78,750	40,950
7/06/10	5,665	31,150	0	0	112,013	42,188	181,425	3,506	23,400	90,563
7/13/10	0	36,210	0	0	77,013	38,600	0	2,100	26,250	77,013
Total	**2,290,165**	**1,728,269**	**0**	**0**	**5,918,810**	**2,750,844**	**22,576,325**	**3,902,420**	**7,470,422**	**3,573,152**