FISHERY MANAGEMENT INTERVENTIONS TO ELIMINATE *MYXOBOLUS CEREBRALIS* INFECTIONS IN PLACER CREEK, COSTILLA COUNTY, IN THE SAN LUIS VALLEY, COLORADO (2003 – 2013)

A FINAL REPORT

By

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Introduction

History and Background- Whirling Disease (WD), an infection caused by the myxozoan parasite *Myxobolus cerebralis (Mc) c*an have deleterious impacts on some species of trout and salmon. Although first observed in cultured rainbow trout in Europe in the late-1800s (Plehn 1905), the complex two-host life cycle of this metazoan parasite remained an enigma for eight decades until first described by Wolf and Markiw (1984). Clinical signs of the disease can include bulging eyes, skeletal deformities, shortened gill covers, blacktail, and "whirling behavior," where severely infected trout can swim in tightly concentric circles. Myxospores, the final stage of the life cycle in trout infected by the parasite are either shed into the natural environment while alive (Nehring et al. 2002) or are released when the carcasses of dead fish decompose. Upon release into the aquatic environment these myxospores are infectious for certain strains or lineages of the aquatic oligochaete *Tubifex tubifex* (*Tt).* The final stage of the life cycle in the worm, the triactinomyxon (TAM) actinospore, is shed through the vent. TAMs are semi-buoyant and float or tumble in the water column, infecting susceptible trout or salmon upon contact. The parasite life cycle is temperature dependent and can take up to a year or more for completion in both hosts.

M. cerebralis was accidentally brought into Colorado in shipments of live trout from Idaho in the 1980s (Obmascik 1995). By the end of the 20th century, the *Mc* parasite was enzootic throughout much of Colorado, largely the result of widespread stocking of infected trout during the late 1980s and early 1990s prior to the time when population level impacts were first observed (Walker and Nehring 1995; Nehring and Walker 1996). Brown trout are highly resistant to the parasite, but can become infected upon exposure. There are no known cases of population-level impacts among brown trout in Colorado. However, cutthroat trout, brook trout, and rainbow trout, the three species of salmonids in Colorado that are most susceptible to infection (Thompson et al. 1999), can experience population-level impacts once the parasite becomes established in ponds, lakes or streams in the state (Nehring et al. 1998; Nehring and Thompson 2001; Nehring 2006).

Placer Creek – A Rio Grande cutthroat core conservation stream - Located on the east side of the San Luis Valley, Placer Creek is tributary to Sangre de Cristo Creek flowing westward from the slopes of La Veta Pass. It is fed by melting snow and rainfall cascading down mountain slopes at the northernmost part of Costilla County. Throughout most of the 1900s, Placer Creek supported a meta-population of Rio Grande native (RGN) cutthroat trout (*Oncorhynchus clarki virginalis)* and was considered a core conservation stream for the species. Even though Placer and Sangre de Cristo creeks are subject to extreme variations in flow, ranging between periodic flooding from summer thunderstorms to severe drought conditions (as occurred in the late-1970s), the RGN population was self-sustaining, protected by a gabion basket barrier from invasions by non-native salmonid competitors (Harig et al. 2000). Indeed, a population estimate conducted on Sangre de Cristo Creek approximately 1 km downstream of the confluence with Placer Creek during June and September 1978, revealed the trout population was 100% RGN cutthroat with a standing stock of 26 and 31 kg/Ha, respectively (Nehring 1979). Multiple year classes were present, even though late summer flows in both 1977 and 1978 were only 0.1 ft^3/sec. The 1977 water year was the 100-year drought of the 20th century, and 1978 was only slightly better.

In the late 1990s, a gabion barrier failed during a severe flood event and non-native brook trout (*Salvelinus fontinalis)* from Sangre de Cristo Creek soon migrated into Placer Creek. In August 2003, brook trout collected from Sangre de Cristo Creek approximately 1 km downstream of the confluence with Placer Creek were found to be infected with *M. cerebralis (s*ee Table 1). Subsequent collections and pepsin trypsin digest (PTD) testing of brook trout and cutthroat trout from numerous sites in Placer Creek during July 2005 revealed both species were heavily infected with the *Mc* parasite (see Table 1 for details). Moreover, extensive electrofishing throughout much of the drainage during summer of 2005 revealed non-native brook trout had largely replaced the RGN cutthroat trout except in the high elevation

Table 1. *Myxobolus cerebralis* myxospores in cranial tissues of brook and Rio Grande native (RGN) cutthroat trout from various locations on Sangre de Cristo Creek and Placer Creek in the San Luis Valley between 2003 and 2013.

Collection Date Mo/Da/Yr	Species	Age (Yrs)	Sample Size		Mean Myxospore Burden	Myxospores in Positive Fish	
			No.	No.+		Mean	Range
Sangre de Cristo Creek							
8/1/2003	Brook	≥1+	10	7	47,688	68,126	2,594 – 195,889
8/18/2011	Brook	≥1+	60	7	6,065	51,990	4,794 – 154,550
Placer Creek below lower barrier near Bronco Dan Gulch							
7/25/2005	Brook	≥1+	15	10	27,148	40,722	556 – 211,111
7/24/2006	Brook	≥1+	14	1	59,983	59,983	--------
6/28/2011	Brook	≥1+	60	9	3,229	21,525	3,894 – 52,811
7/10/2013	Brook	≥1+	33	0	0	0	--------
7/24/2006	RGN	≥1+	6	3	38,210	76,419	8,267 – 195,689
6/28/2011	RGN	≥1+	58	18	11,017	35,500	4,628 – 150,367
6/21/2012	RGN	≥1+	30	8	9,876	32,680	4,752 – 92,644
7/10/2013	RGN	≥1+	18	2	12,294	110,647	56,233 – 165,061
Placer Creek between the bottom barrier and the confluence with Middle Fork Placer Creek							
7/25/2006	Brook	≥1+	20	0	0	0	--------
7/25/2006	RGN	≥1+	10	4	16,900	42,250	8,528 – 95,950
6/21/2012	RGN	≥2+	30	0	0	0	--------
7/10/2013	RGN	≥2+	40	0	0	0	--------

headwater areas. In addition, lineage III *T. tubifex* worms, which are highly susceptible to infection by the *Mc* parasite (Nehring et al. 2014) were present in benthic samples collected from a senescent beaver pond on Placer Creek several kilometers upstream of the Sangre de Cristo Creek confluence (Nehring 2006). In the spring of 2006, the Malo Vega fire burned through a significant portion of the upland forest in the Placer Creek drainage basin.

Native cutthroat trout are poor competitors when occurring in sympatry with non-native salmonids, particularly brook trout (Peterson and Fausch 2003; Peterson et al. 2004). Harig et al. (2000) found that reintroductions of cutthroat trout were most often unsuccessful when reinvasions of non-native salmonids occurred due to failure of artificial barriers or incomplete removal of non-native salmonid competitors. Thompson et al. (1999) found that Colorado River, Rio Grande and greenback cutthroat

trout exposed to ambient levels of *Mc* TAMs in the Colorado during the first 2 months post-hatch suffered higher mortality than that observed in among non-native brown and rainbow trout exposed at the same or smaller sizes. Given these competitive disadvantages, the decision was made during 2006 to chemically reclaim the Placer Creek basin with rotenone, reconstruct barrier(s) to upstream migration, and reintroduce RGN cutthroat trout three years after chemical reclamation.

The reason for the 3-year delay prior to reintroduction was to see if leaving the Placer Creek ecosystem fish-free for the period of three years would result in the disappearance of the *Mc* parasite for lack of a fish host to keep the life cycle going. Results from a number of studies suggest that this might be the case. First, the viability of waterborne TAMs declines rapidly at temperatures > 15 ˚C and temperatures ≥ 20 ˚C may be lethal (El-Matbouli et al. 1999). The viability of TAMs is measured in weeks, not months or years. Second, research studies at the University of California-Davis indicated that the viability of the myxospores of the *Mc* parasite (as measured by total TAM production) was reduced by 71% when myxospores were held in aqueous suspension at 4-5˚C for 60 days prior to inoculation of lineage III *T. tubifex* (*Tt*) worms compared to a 7-day delay prior to inoculation (Hedrick et al. 2008). Third, studies at the University of Montana revealed that lineage III worms infected with the *Mc* parasite remain infected for life, and can survive (in a laboratory environment) for at least 24 months post exposure to *Mc* myxospores (Gilbert and Granath 2001).

Finally, results of a year-long experiment in Colorado revealed that the long-term viability of *Mc* myxospores is not much longer than six months, and certainly less than a year (unpublished data). In this study fixed numbers of *Mc* myxospores were incubated in aerated containers of sand and water under the same thermal regime for varying periods of time prior to the introduction of unexposed lineage III T*t* worms. The time intervals between initial inoculation of the containers with *Mc* myxospores (at day zero) and the introduction of the worms were 0, 15, 30, 60, 90, 120 and 180 days. This experiment revealed that TAM production declined at an exponential rate and at the end of the experiment, the total number of TAMs produced in the 180-day replicates was reduced by 99.94% compared to that observed among the 0-day replicates. TAM production among the 30-day replicates was reduced by 81.8% compared with that observed among the 0-day replicates. In a separate experiment, lineage III *Tt* worms were dosed with 1,000 myxospores/worm that had been refrigerated (but not frozen) in an aqueous suspension for 365 days, and then monitored for evidence of TAM production for an addition 7 months. No TAMs were ever detected during weekly water filtrations and screening during that 7-month period. All exposed worms were tested by qPCR for presence of DNA of the *Mc* parasite (Cavender et al. 2004), and all tests were negative. Taken together, the results of these studies suggest that a 3-year delay between the removal of the *Mc*-susceptible fish host and reintroduction of RGN cutthroat trout may be sufficient for elimination of the *Mc* parasite in the Placer Creek drainage basin, if barriers are installed to prevent reinvasion by infected brook trout from Sangre de Cristo Creek.

METHODS

Fish Collections **-** All fish collections and removal operations during the 9-year study were accomplished using backpack electrofishing equipment. RGN cutthroat fry or fingerlings utilized for qPCR testing for evidence of infection by the *Mc* parasite were individually preserved in 70% ETOH and tested using the protocol of Cavender et al. 2004. Fish heads saved for testing by pepsin-trypsin digest (PTD) were stored on ice after field collection then frozen, and sent to Colorado's Aquatic Animal Health Lab at Brush, Colorado for processing and testing.

Aquatic Oligochaete Rearing and Stocking – *Tubifex tubifex* worms that were transplanted into Placer Creek during 2010, 2011 and 2012 were reared from stocks of Lineage V and VI worms originally collected from the Williams Fork River and Windy Gap Reservoir in Grand County, Colorado in 2005 and 2006. The progeny from these original stocks were reared in aquaria at the Colorado Division of

Wildlife (CDOW) Area Service Center in Montrose and at the Native Aquatic Species Research Facility (NASREF) near Alamosa, Colorado. The estimated numbers of worms stocked each year was determined by doing multiple counts of 1,000 worms and weighing (wet weight) to the nearest 0.01 g. In 2010, it was determined that an averaged wet weight of 1,000 worms was 2.72 g. The oligochaete cultures were held in 3-4 gallon aquaria aerated with an air stone and fed (*ad libitum*) a ration of dehydrated spirulena discs, Tetramin pellets and Algamac 2000 ®, in a dry weight ratio of 6:3:1, ground to a fine powder in a coffee bean grinder.

Aquatic Oligochaete Sampling - Tubificid worm collections in Placer Creek were made using a hand-held benthic sampling kick net with a rectangular bag constructed with 600 µm mesh NITEX screen that allowed fine sediments to be rinsed out of the sample upon collection prior to processing for identification and enumeration of the worms in the sample. The rinsed sample was placed in a 15 L plastic pail, and small amounts of sediment and detrital matter were placed in white porcelain dissecting trays to separate the aquatic oligochaetes from the detrital matter. Oligochaetes were drawn out of the material with disposable micropipettes and placed in a small petri dish for enumeration and field classification as either non-haired or "haired" worms with pectinate chaetae (Kathmann and Brinkhurst - 1998). The worms were separated from the detrital material in the field and screened for haired and pectinate chaetae with a variable power (20X-100X) MEIJI stereozoom microscope powered by a 110 VAC generator. After screening, the worm samples were stored in 15 mL polypropylene test tubes and preserved in 70% ETOH prior to shipment to a private laboratory for determination of the lineage composition of the sample according to the protocols described in Nehring et al. 2013. QPCR testing for evidence of infection by the *Mc* parasite in the tubificid worms was conducted using the protocol of Cavender et al. 2004.

RESULTS AND DISCUSSION

The chronology of significant events over the 9-year study period is as follows:

1. Removal of approximately 5,000 brook trout by electrofishing – June & July 2005
2. Tests confirm *M. cerebralis* infection among brook & RGN trout – September 2005
3. Malo Vega fire burns through much of the Placer Creek drainage basin – June 2006
4. Migration barriers rebuilt during the summer and fall of 2006
5. First chemical reclamation project is completed – August 2007
6. Presence of a few trout detected in headwater areas – July 2008
7. Disease testing of caged sentinel fish indicates *Mc* is still present – September 2008
8. Second chemical reclamation project is completed – August 2009
9. PCR tests of sentinel fish detects no evidence of *Mc* infection – September 2009
10. 37,000 lineage V and VI *T. tubifex* worms stocked in Placer Creek – July 2010
11. 270 adult and 22,000 RGN cutthroat fry stocked in creek basin –September 2010
12. Fish and worm collections for PTD and qPCR testing – July 2011
13. 326,000 lineage V and VI *T. tubifex* worms stocked in Placer Creek – August 2011
14. 22,000 RGN cutthroat fry stocked in Placer Creek basin –October 2011
15. Off channel eutrophic pond (near site 16) is filled in and landscaped –April 2012
16. Fish and worm collections for PTD and qPCR testing – July 2012
17. 597,000 lineage V and VI *T. tubifex* worms stocked in Placer Creek – July 2012
18. Fish and worm collections for PTD and qPCR testing – July 2013

Disease Testing - The results of the PTD tests for evidence of infection by the *Mc* parasite in brook trout and RGN cutthroat trout in Placer and Sangre de Cristo creek over the study period are summarized in Tables 1 and 2. Downstream of the barrier on Placer Creek at Bronco Dan Gulch and in Sangre de Cristo Creek, *M. cerebralis* remained enzootic through the end of the study period in 2013. In 2011, qPCR testing revealed that two of 62 RGN fingerlings collected upstream of the barrier at Bronco

Dan Gulch were positive for DNA of the parasite. Both specimens were collected in the stream reach below the point where seepage from the off-channel pond flowed into Placer Creek, downstream of the confluence with the Middle Fork of Placer Creek (see Figures 1 and 2). Benthic sample site 16 is located in the backwater eddy at the point where the seepage drains into Placer Creek. This area contained fine sediment and organic black muck 20 – 30 cm in depth that provided ideal breeding and rearing habitat for tubificid worms. In 2011, all of the haired and pectinate worms collected at this site typed out as lineage III *Tt* oligochaetes (see Table 3). It is noteworthy that after this pond was back-filled, dried up and the surface area landscaped in April 2012, the number of lineage III oligocheates collected at this site declined from 155 in 2011 (Table 3), to 11 in June 2012 (Table 4) and one in July 2013 (Table 5). However, non-haired oligochaetes remained abundant at the site. It is likely that these non-haired oligochaetes were either *Limnodrilis hoffmeisteri* or *Ilyodrilus templetoni*, two species of aquatic oligochaetes that are non-susceptible hosts for the *Mc* parasite (Kerans et al. 2004).

Table 2. Estimated concentrations of *Myxobolus cerebralis* myxospores in cranial tissues of brook trout and Rio Grande native (RGN) cutthroat trout from various locations on Placer Creek in the San Luis Valley between 2006 and 2013.

Collection Date Mo/Da/Yr	Species	Age (Yrs)	Sample Size		Overall Mean Myxospore Burden	Myxospores in Positive Fish	
			No.	No.+		Mean	Range
Middle Fork of Placer Creek							
7/27/2006[a]	Brook	≥1+	30	0	0	0	--------
6/21/2012	RGN	≥2+	30	0	0	0	--------
7/10/2013	RGN	≥2+	40	0	0	0	--------
Placer Creek 5 km upstream of the confluence with the Middle Fork of Placer Creek							
7/26/2006	Brook	≥1+	10	0	0	0	--------
7/26/2006	RGN	≥1+	11	0	0	0	--------
Placer Creek 3 km above the Middle Fork Placer Creek confluence							
7/25/2005	Brook	≥1+	21	4	3,069	16,111	1,111 – 27,778
7/26/2006	Brook	≥1+	10	5	34,085	68,170	2,156 – 123,889
7/26/2006	RGN	≥1+	10	8	42,522	53,153	4,244 – 150,167
6/21/2012	RGN	≥2+	30	0	0	0	--------
7/10/2013	RGN	≥2+	40	0	0	0	--------

[a]: 10 brook trout were collected from each of three separate sites, at 0.8 km downstream of the confluence with the South Fork of Placer Creek, 0.5 km upstream of the confluence with the South Fork of Placer Creek, and 4.7 km upstream of the confluence with the mainstem of Placer Creek. Despite several days of kicknet sampling for aquatic oligochaetes at numerous sites on the Middle Fork of Placer Creek, no *Tubifex tubifex* worms were ever found on this branch of the Placer Creek.

Figure 1. Locations of RGN cutthroat trout fingerling collection sites in the Placer Creek drainage tested by PCR for DNA of *Myxobolus cerebralis* during July 2011. Twelve fingerlings were collected at each of the four locations upstream of the barriers and 14 were collected in Placer Creek between the two downstream barriers. Only 2 fingerlings of 62 tested positive for *Mc* DNA.

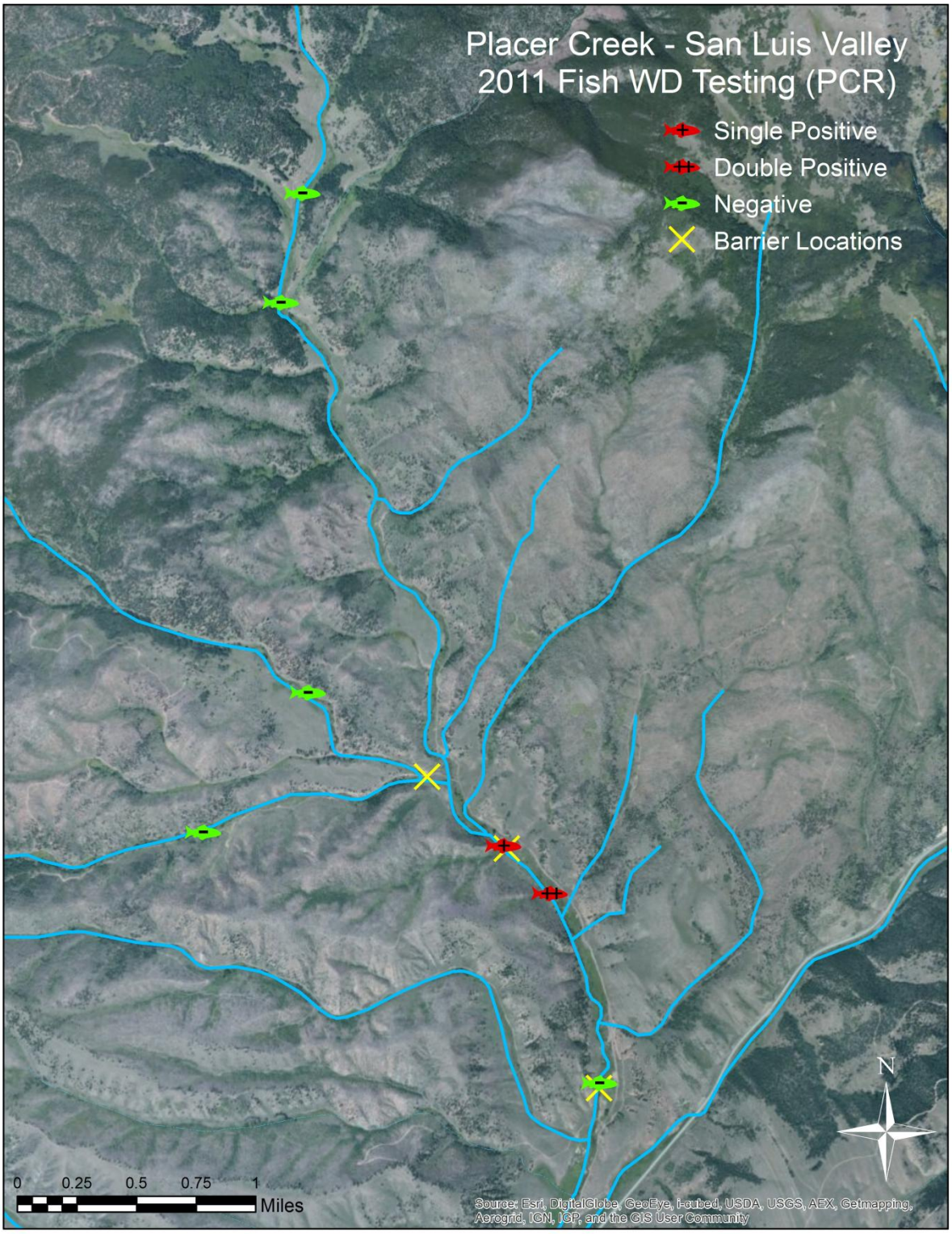
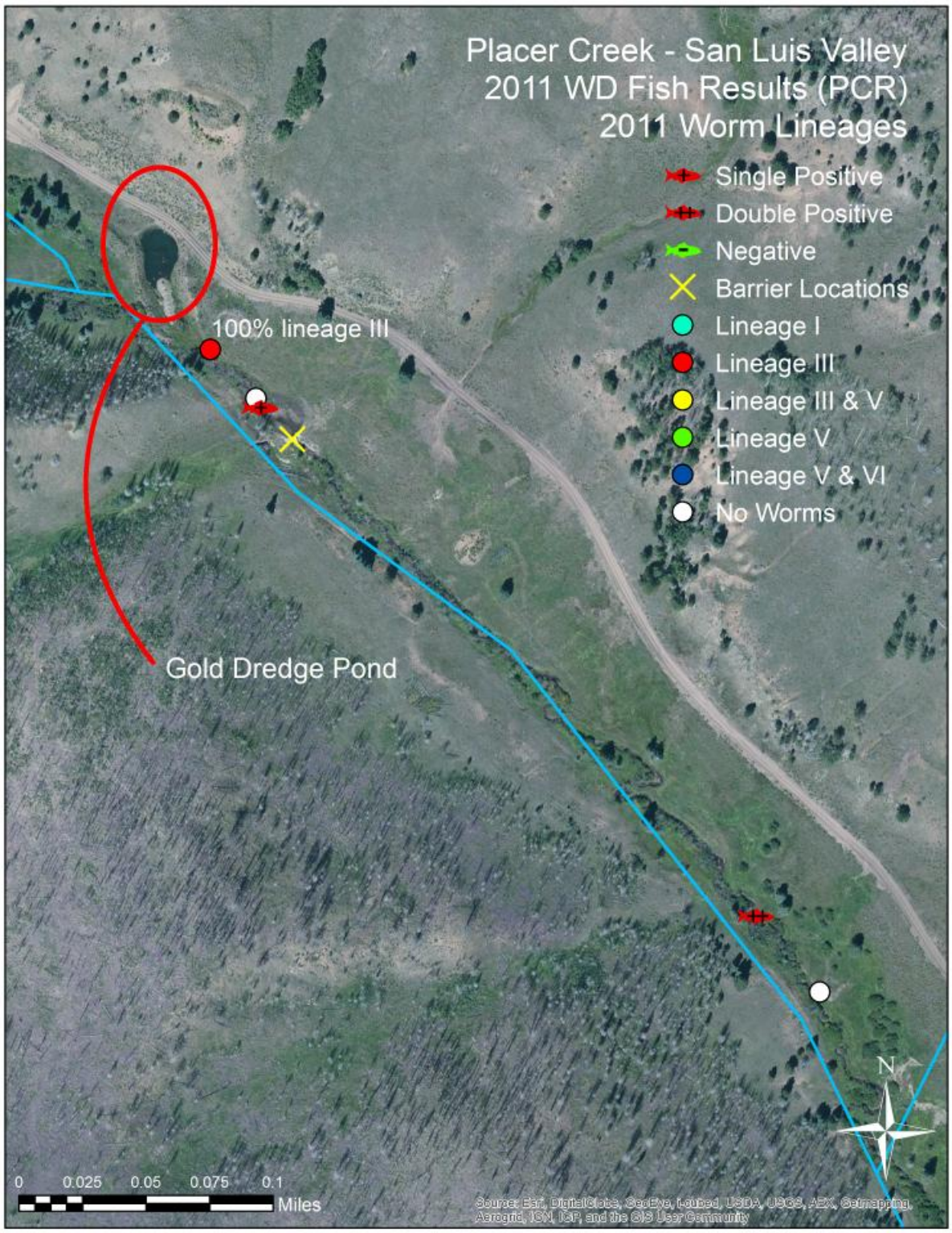


Figure 2. Satellite photo showing the location of the off-channel pond in the summer of 2011 in relation to the location of the two RGN fingerlings that tested positive (by qPCR) for DNA of the *Mc* parasite and worm collection site 16 (see Table 3), where the highest densities of lineage III *Tubifex tubifex* worms were found during benthic sample collections.



It is also noteworthy that among the 13 samples (with a total of 265 haired oligochaetes) that had DNA for lineage III *T. tubifex* (when tested by qPCR), none tested positive for DNA of the *Mc* parasite, suggesting that the prevalence of infection among *Mc*-susceptible worms in Placer Creek was extremely low. Indeed,

Table 3. Site specific locations of lineage V and VI *Tubifex tubifex* introductions into Placer Creek, tributary to Sangre de Cristo Creek, Costilla County, Colorado, on July 15-16, 2010, together with the results of qPCR testing of samples collected on April 18-20, 2011 to assess survival and test for the presence and distribution of lineage III *Tubifex tubifex* worms (that are susceptible hosts for *Myxobolus cerebralis*) as well as lineages that are resistant (I) or not susceptible(V and VI) to the *Mc* parasite.

Site No.	**Approximate Number of Worms Stocked**	**Haired Worms Collected Per site**	**qPCR Results for Lineage Testing (Expressed as a Percentage of T. tubifex DNA per Lineage)**			
			I	**III**	**V**	**VI**
1	2,460	5	0	0	100	0
2	1,230	0				
3	2,460	8	0	100	0	0
4	1,230	2	0	83	17	0
5	2,460	2	0	0	100	0
6	1,230	40	0	68	32	0
7	2,460	2*	0	0	0	0
7	2,460	1	0	0	100	0
8	1,230	0				
9	2,460	3	0	100	0	0
10	1,230	0				
11	2,460	0				
12	1,230	0				
13	2,460	0				
14	1,230	4	0	100	0	0
15	2,460	0				
16	2,460	50	0	100	0	0
16	2,460	50	0	100	0	0
16	2,460	50	0	100	0	0
16	2,460	5		100	0	0
17	1,230	0				
18	2,460	0				
19	1,230	3	0	100	0	0
20	1,230	0				

Note (*): Non-haired worm sample

there were 10 benthic samples, representing 185 worms, where the qPCR test indicated only DNA for lineage III worms was detected . This would mean that the prevalence of *Mc* infection among lineage III worms was less than 0.5%.

Among then 210 RGN cutthroat trout collected from Placer Creek and its tributaries (above the most downstream barrier at Bronco Dan Gulch) and tested by PTD for detection of *Mc* myxospores in 2012 and 2013, no evidence of infection was found (see Tables 1 and 2). Given that the last introduction of RGN cutthroat trout occurred in October 2011, all of the fish tested over that two year period had been exposed for a minimum of 9 months and a maximum of 21 months. In July 2013, 7 RGN cutthroat trout

fingerling collected from the Middle Fork of Placer Creek and 7 YOY brook trout collected on Placer Creek from below the bottom barrier at Bronco Dan Gulch were also negative when tested by qPCR for evidence of *Mc* DNA. Taken together, the PTD and qPCR test results for 2012 and 2013 support the conclusion that the life cycle of *Myxobolus cerebralis* has been broken in the Placer Creek drainage upstream of the barrier at Bronco Dan Gulch.

Table 4. Site specific locations of lineage V and VI *Tubifex tubifex* introductions into Placer Creek, tributary to Sangre de Cristo Creek, Costilla County, Colorado, on August 18, 2011, together with the results of qPCR testing of samples collected on June 14-15, 2012 to assess survival and test for the presence and distribution of lineage III *Tubifex tubifex* worms (that are susceptible hosts for *Myxobolus cerebralis*) as well as lineages that are resistant (I) or not susceptible to infection (V and VI) by the *Mc* parasite. A total of 378 grams (wet weight) of worms were stocked. Based on a ratio of 2,460 worms per 2.72 grams (determined by actual count in 2010) approximately 342,000 *T. tubifex* were stocked in 2011.

Site No.	**Approximate Number of Worms Stocked**	**Haired Worms Collected Per site**	**qPCR Results for Lineage Testing (Expressed as a Percentage of T. tubifex DNA per Lineage)**			
			I	**III**	**V**	**VI**
1	16,300	21	0	0	100	0
2	16,300	50	0	0	88	12
2	16,300	37	0	0	72	28
3	16,300	3*	0	0	0	0
3	16,300	1	100	0	0	0
4	16,300	0				
5	16,300	0				
6	16,300	2	0	0	100	0
7	16,300	2	0	0	61	39
8	16,300	0				
9	16,300	0				
10	16,300	0				
11	16,300	1	0	0	100	0
12	16,300	0				
13	16,300	0				
14	16,300	0				
15	16,300	5	0	0	69	31
16	16,300	11	0	100	0	0
17	16,300	0				
18	16,300	0				
19	16,300	0				
20	32,600	0				

Note (*): Non-haired worm sample

Lineage V and VI Worm Introductions – Almost one million lineage V and VI *T. tubifex* worms were stocked in Placer Creek between 2010 and 2012. The estimated number of worms introduced at each of the 20 sites are shown in Tables 3, 4 and 5, together with the results of the subsequent re-sampling efforts in 2011, 2012, and 2013. These data indicate that a few of the worms survived at a few locations

through at least one winter into the next summer; however, it was disappointing that the survivorship was so poor. The best survival seemed to be at sites 1 and 2 in the upper reaches of Placer Creek (see Tables 3, 4 and 5 for details). Both of these introduction sites were heavily shaded, with a substantial amount of black organic muck , an important factor for creation of a microhabitat capable of sustaining dense, thriving populations of aquatic oligochaetes (Baxa and Hedrick 2008; Nehring et al. 2013).

Table 5. Site specific locations of lineage V and VI *Tubifex tubifex* introductions into Placer Creek, tributary to Sangre de Cristo Creek, Costilla County, Colorado, on July 19, 2012, together with the results of qPCR testing of samples collected on July 10, 2013 to assess survival and test for the presence and distribution of lineage III *Tubifex tubifex* worms (that are susceptible hosts for *Myxobolus cerebralis*) as well as lineages that are resistant (I) or not susceptible to infection (V and VI) by the *Mc* parasite. A total of 660 grams of worms (wet weight) were stocked. Based on a ratio of 2,460 worms per 2.72 grams (determined by actual count in 2010) approximately 597,000 *T. tubifex* were stocked in 2012.

Site No.	**Approximate Number of Worms Stocked**	**Haired Worms Collected Per site**	**qPCR Results for Lineage Testing (Expressed as a Percentage of T. tubifex DNA per Lineage)**			
			I	**III**	**V**	**VI**
1	29,800	38	0	19	72	9
2	29,800	0				
3	29,800	2*	0	0	0	0
4	29,800	2	0	0	100	0
5	29,800	1*	0	0	0	0
6	29,800	0				
7	29,800	0				
8	29,800	0				
9	29,800	7*	0	0	0	0
10	29,800	0				
11	29,800	0				
12	29,800	1*	0	0	0	0
13	29,800	0				
14	29,800	0				
15	29,800	1	0	0	100	0
16	29,800	25*	0	0	0	0
16	29,800	42*	0	0	0	0
16	29,800	1	0	100	0	0
17	29,800	0				
18	29,800	0				
19	29,800	0				
20	29,800	0				

Note (*): Non-haired worm sample

Figures 3 through 5 display the GPS locations of the 20 sites where lineage V and VI tubificid oligochaetes were introduced from 2010 through 2012. Figures 6 through 8 provide a visual display of the occurrence and distribution of the various lineages detected during the 2011-2013 re-sampling effort. The decrease in the number of sites where lineage III *Tt* oligochaetes were found in 2012 and 2013 compared with 2011 is striking. It is also noteworthy that the number of sites where no oligochaetes of

Figure 3. Introduction sites for lineage V and VI *Tubifex tubifex* worms in July 2010.

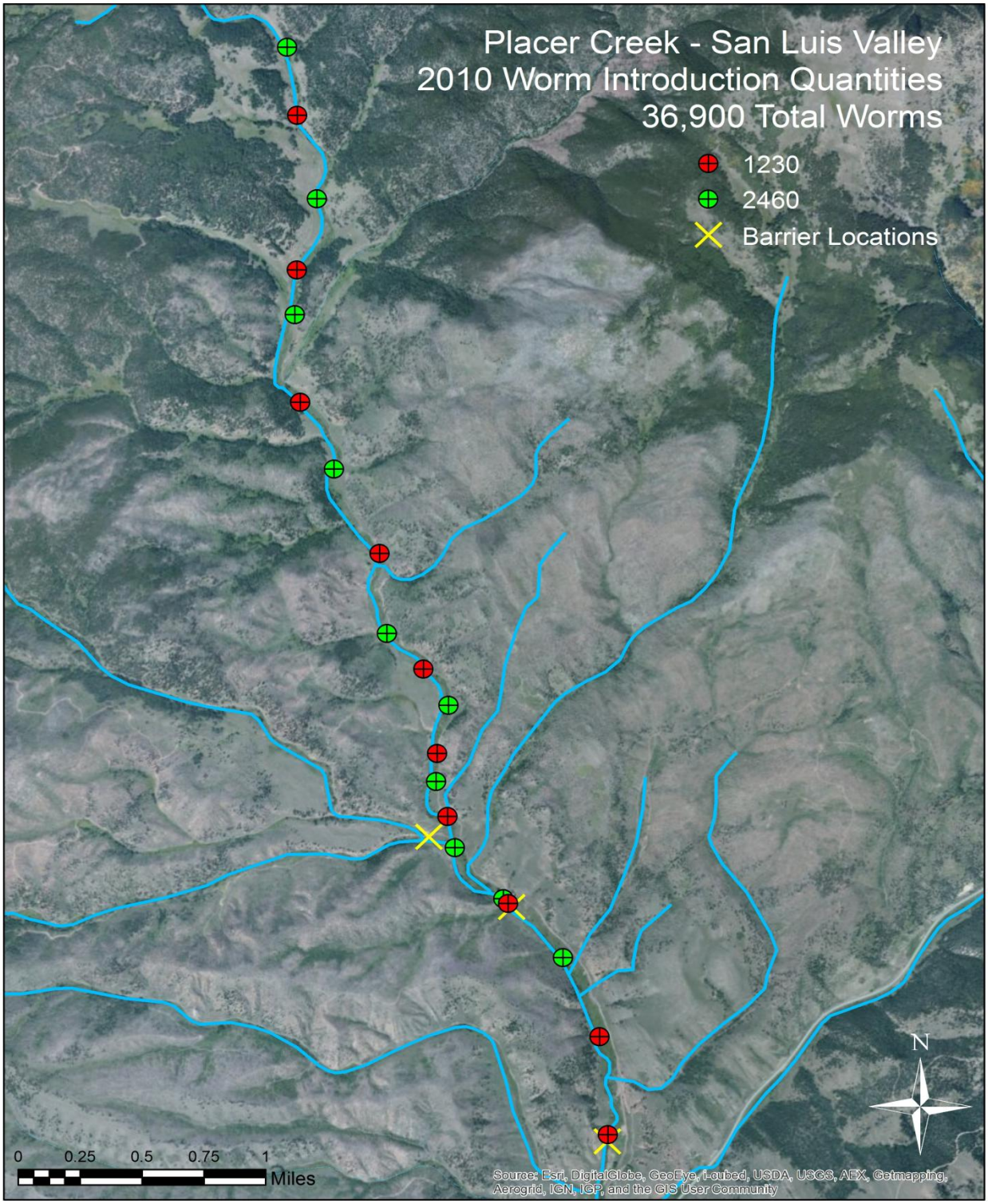


Figure 4. . Introduction sites for lineage V and VI *Tubifex tubifex* worms in July 2011.

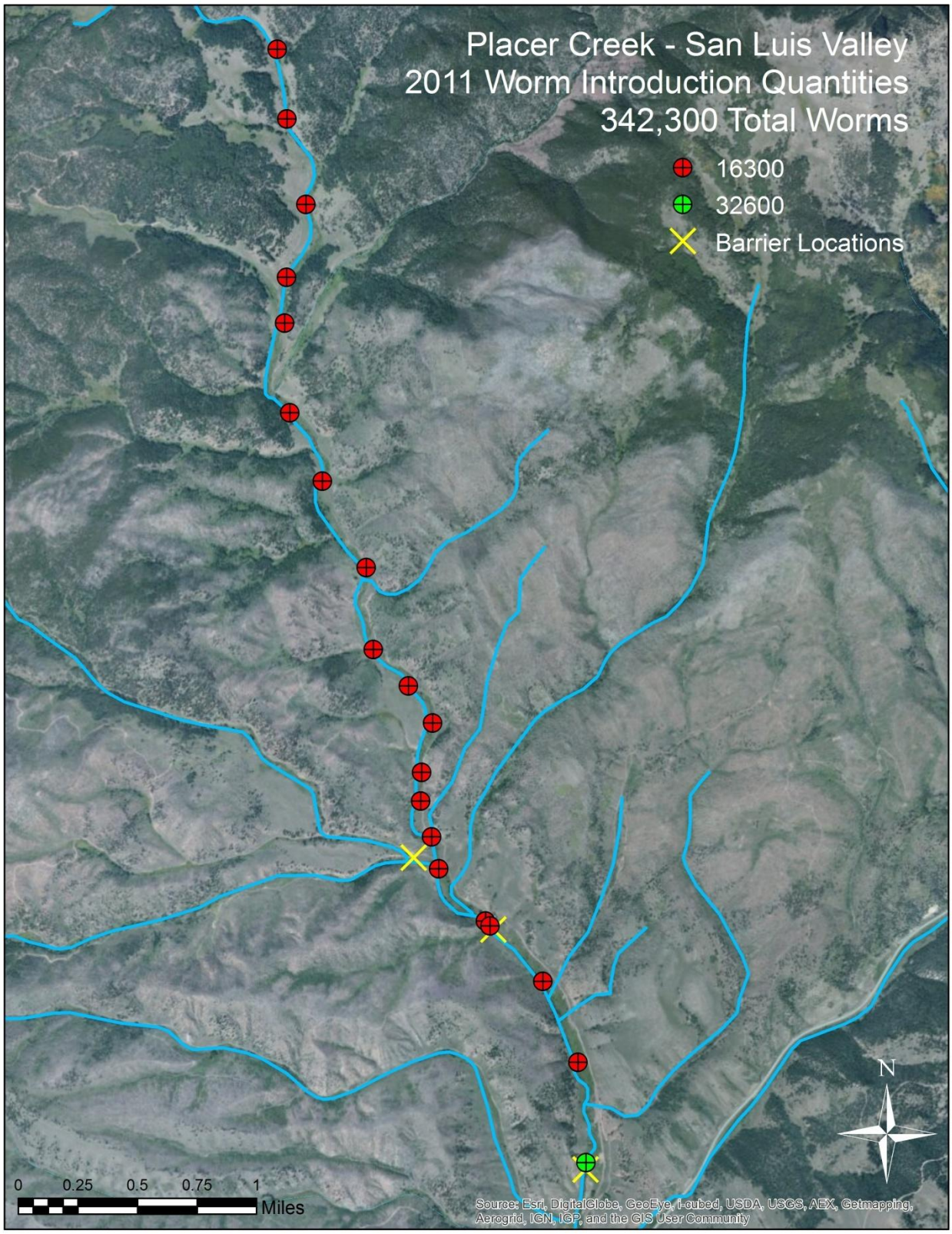


Figure 5. Introduction sites for lineage V and VI *Tubifex tubifex* worms in July 2012.

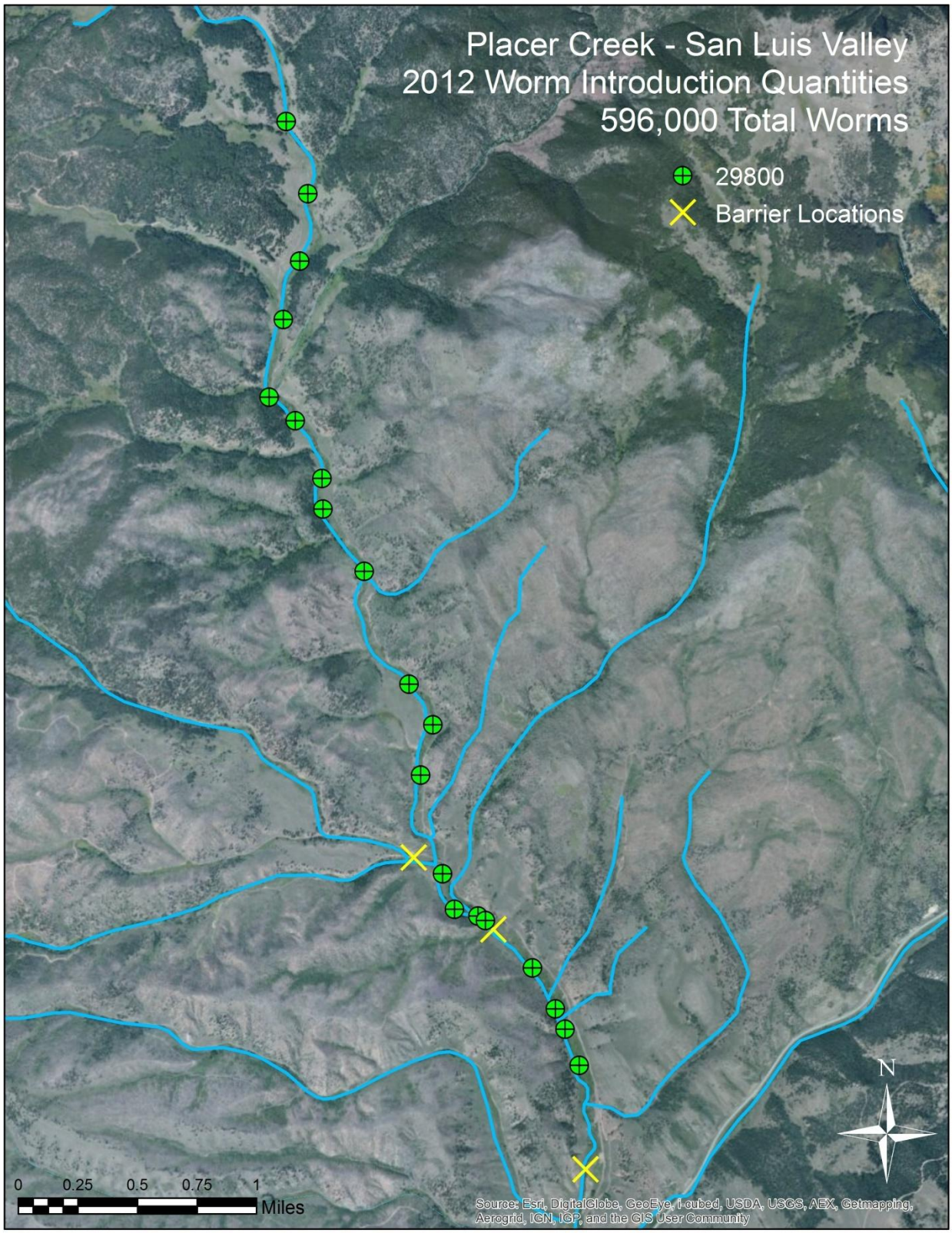


Figure 6. Results of the re-sampling of the 2010 introduction sites to assess survival and determine the lineage assemblage comprising the *Tubifex tubifex* population structure in Placer Creek in 2011.

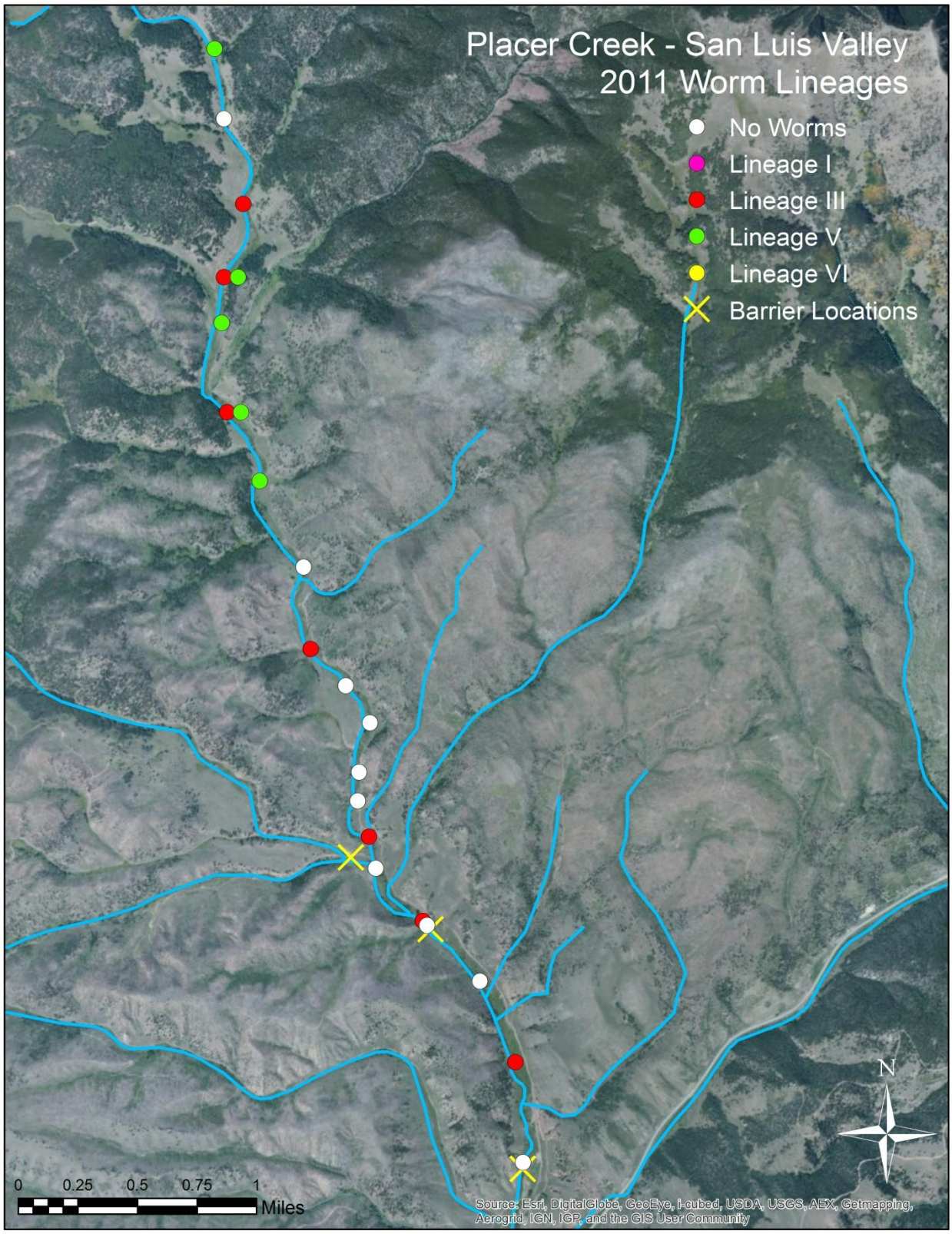


Figure 7. Results of the re-sampling of the 2011 introduction sites to assess survival and determine the lineage assemblage comprising the *Tubifex tubifex* population structure in Placer Creek in 2012.

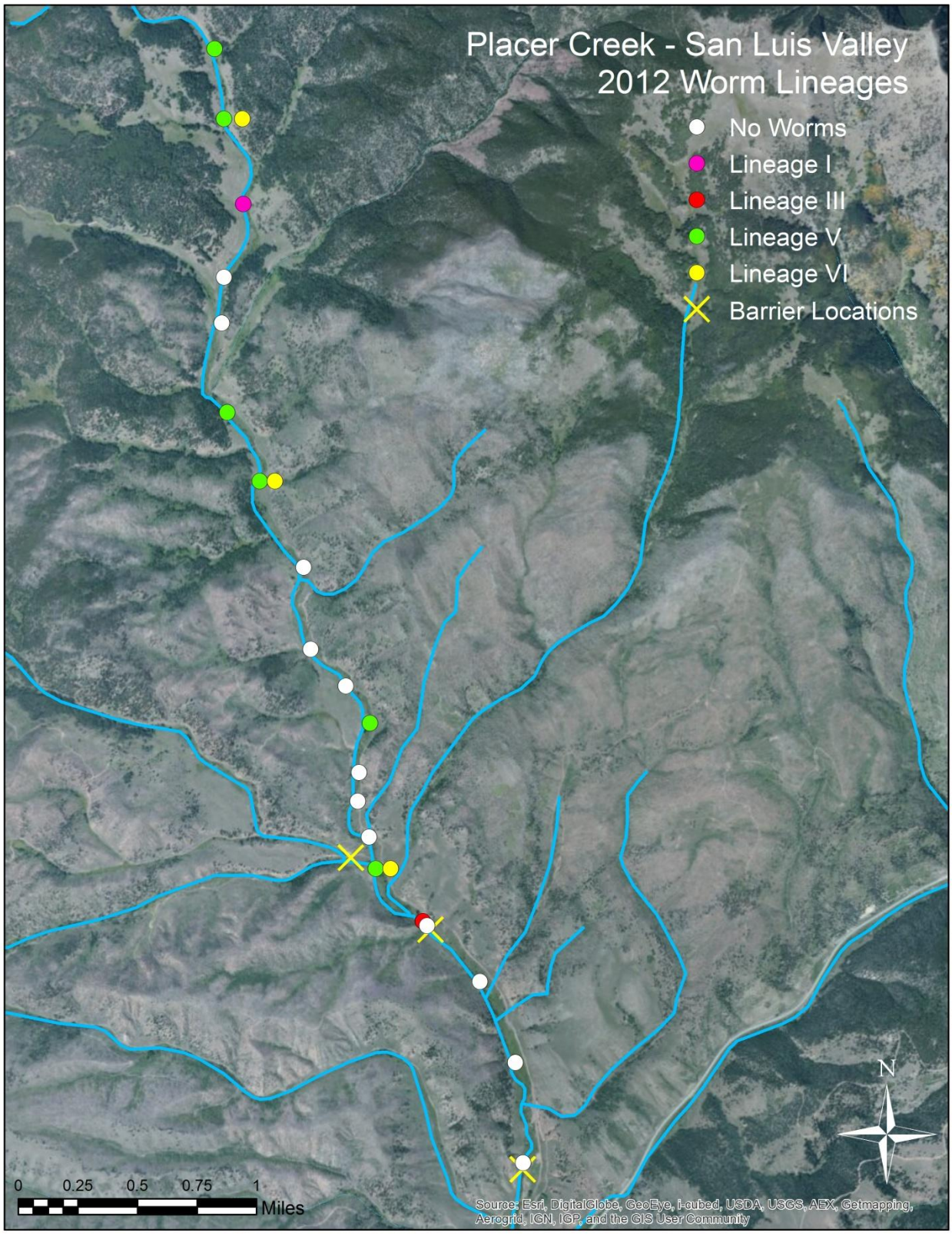
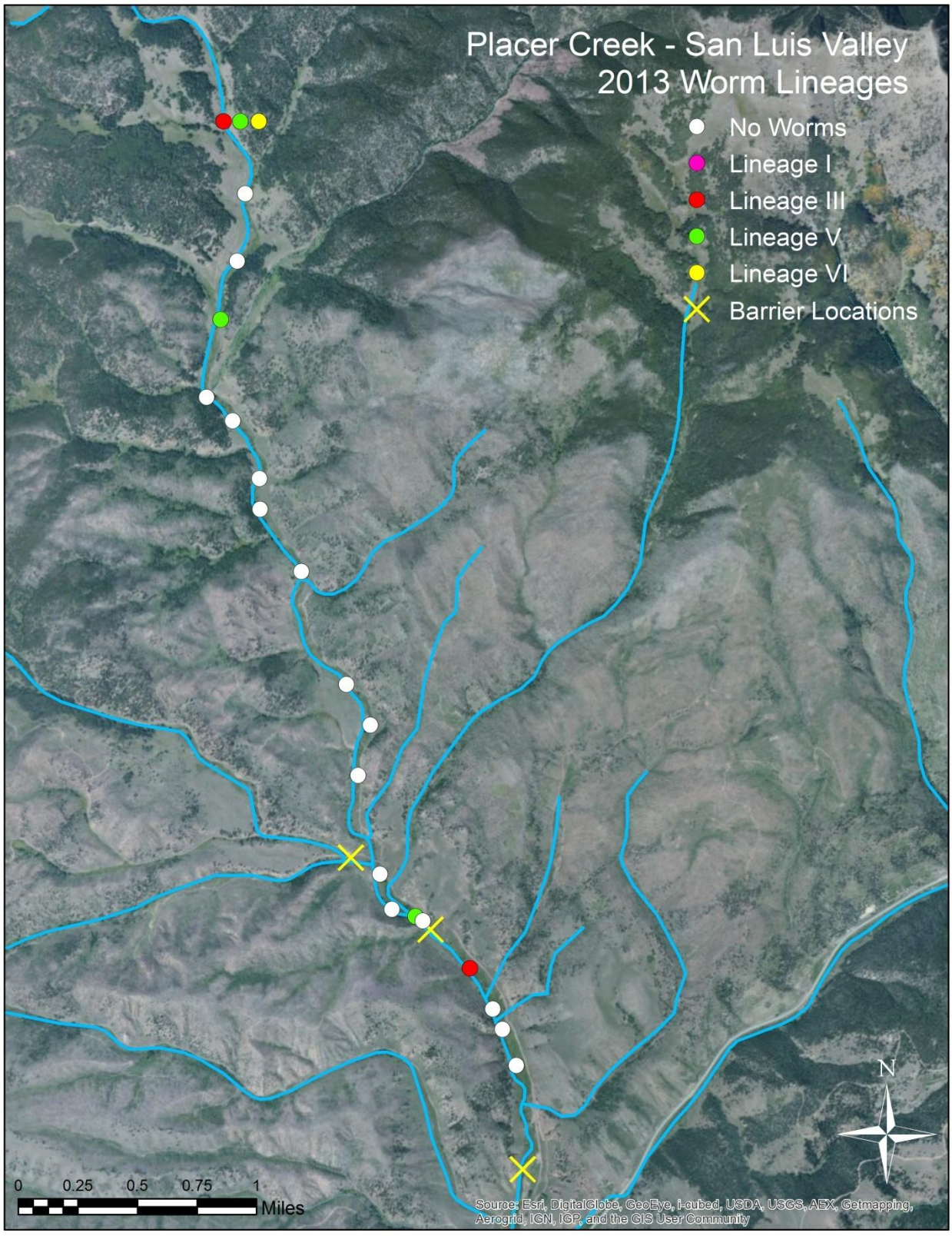


Figure 8. Results of the re-sampling of the 2012 introduction sites to assess survival and determine the lineage assemblage comprising the *Tubifex tubifex* population structure in Placer Creek in 2013.



any kind were found increased from 10 in 2011, the 12 and 16 for 2012 and 2013, respectively. This suggests that the microhabitats capable of supporting aquatic oligochaetes of any kind in Placer Creek are very limited. This is likely beneficial in that it would tend to prevent the re-establishment of *Mc* parasite in Placer Creek upstream of the barrier.

Lineage V worms were found at more locations during the 3-year resampling effort than the lineage I or VI oligochaetes, suggesting that perhaps they are the more adaptable lineage for the Placer Creek ecosystem. This is not surprising given that the previous places where lineage V worms were found to occur in relatively high densities tended to be in lake or stream environments where the primary substrate was most often comprised of fine sands without an excessive amount of putrifying back muck. The only sampling site in Placer Creek that had a large amount of anaerobic black muck was at site 16, where the organic ooze from the off-channel pond (Figure 2) was seeping into Placer Creek. This was also the site where the greatest densities of lineage III worms were found during the first resampling period in 2011. It is noteworthy that the density of lineage III worms declined rapidly in 2012 and 2013, after the off-channel pond was filled in.

RECOMMENDATIONS AND CONCLUSIONS

A collection of 60 RGN YOY fingerlings and adult trout should be made once each year for 2014 and 2015 to insure that the Placer Creek drainage basin upstream of the barrier at Bronco Dan Gulch is remaining free of *M. cerebralis.* The YOY fingerlings should be analyzed by qPCR for DNA of the parasite if funds are available for processing. However, all fish collected could be analyzed by PTD at Colorado's Aquatic Animal Health laboratory at Brush, Colorado, if monies are unavailable for qPCR testing with Pisces Molecular, LLC, at Boulder, Colorado.

A collection of adult RGN and brook trout should also be made in Placer Creek downstream of the barrier at Bronco Dan Gulch in 2014 and 2015, to determine if the prevalence and severity of *Mc* infection continues to decline.

Any time a fish removal project is undertaken utilizing gill netting, electrofishing or chemical treatment for the purpose of elimination of whirling disease and the *M. cerebralis* parasite from a lake or stream, every effort should be made to remove all of the fish carcasses in order to shorten the time period needed for the life cycle to be interrupted. Decaying fish carcasses will load the aquatic ecosystem with all of the myxospores present in the infected fish and most likely result in at least a year increase in the time required to break the life cycle of the *Mc* parasite.

The elimination of the off-channel pond in Placer Creek was likely a critical factor in breaking the life cycle of *M. cerebralis* in the stream as it removed an important source of lineage III *T. tubifex* worms in the drainage. More time will be required to determine whether or not the non-susceptible lineage V and VI oligochaetes will successfully colonize Placer Creek or not.

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