

**Pilot Study: Testing the Effect of Carcass Removal on *Ceratomyxa shasta*  
Levels in Bogus Creek**

**Project Report**

**Submitted By**

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**Summary:**

A pilot study examining the effect of adult carcass removal on *Ceratomyxa shasta* myxospore release was conducted in Bogus Creek during the fall of 2008. A total of 907 fall-run Chinook salmon carcasses were removed from the lower reach of Bogus Creek (19% of the total Bogus Creek run) while water samples were collected and assayed for *C. shasta* DNA. Study cooperators included Oregon State University, Yurok Tribal Fisheries, California Department of Fish and Game, and the California Nevada Fish Health Center (FHC). Study results demonstrated that the actinospore stage of the parasite was not present in the creek during the spawning period and thus all parasite detection in water samples was the myxospore stage from the adult salmon. The incidence of *C. shasta* myxospores visually detected in intestinal scrapings of adult salmon was 30%, although molecular detection demonstrated a much higher prevalence of infection. The number of myxospores varied between 3000 and 14.7 million per gram of scraping with no trend for collection date or fish sex. However, decomposed carcasses had higher myxospore loads than fresh carcasses. Results of water sampling conducted concurrently with carcass removal supported the hypothesis that Bogus Creek contributes a significant amount of myxospores to the Klamath River mainstem. During the period of sampling parasite abundance rarely exceeded 1 myxospore/L; however, if this occurred on a sustained basis it would represent a contribution of approximately 57 million spores daily.

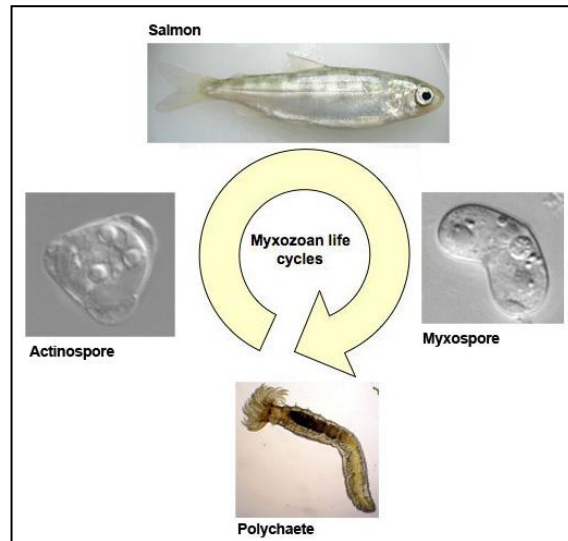
Based on this study, we feel that carcass removal from Bogus Creek is logistically feasible and that water filtration can be applied for assessing success. We also recommend a subsequent study on Bogus Creek based on what was learned in 2008, in which the following changes would be made: 1) removal of carcasses could be done weekly rather than daily, 2) water monitoring should be done year-round at the mouth of Bogus Creek, but is only necessary above and below the reach from which carcasses are removed and 3) sentinel exposures no longer are needed as we have multi-year data that the parasite life cycle is not completed in Bogus Creek (i.e. actinospores are not present).

## **Introduction:**

Severe infection, of juvenile Klamath River Chinook salmon and coho salmon, by the myxozoan parasite *Ceratomyxa shasta* may be a contributing factor in declining adult returns in the basin. The incidence of *C. shasta* infection, observed in histological sections of juvenile Chinook collected in the Klamath River above the confluence of the Trinity River between May and July, has ranged from 21 – 35% (Nichols et al. 2008). This incidence is 10 – 27% higher in samples assayed by the more sensitive quantitative polymerase chain reaction assay (QPCR). Approximately 70% of the histological positive samples demonstrated pathology due to the infection. The high prevalence and severity of infection, in native fish that should have high resistance to the disease, indicates this parasite is a key factor limiting salmon recovery in Klamath River.

*Ceratomyxa shasta* has a complex life cycle, involving an invertebrate (polychaete worm) host as well as salmon (Bartholomew et al. 1997; Fig. 1). A section of the lower Klamath River has been identified to be highly infectious to salmon (Stocking et al. 2006) and should be a focus for management actions to disrupt the parasite's life cycle.

In August 2007, a multidisciplinary panel of fish disease experts and fishery managers met to develop a research plan focused on management actions to reduce disease levels (ceratomyxosis) in natural juvenile salmon of the Klamath R.



One of the proposed management actions was removal of adult salmon carcasses with the goal of reducing numbers of myxospores released back into the system. The hypothesized effect of this action would be reduction of infection in polychaete populations and thus reduction of actinospores released to infect juvenile fish the following spring. Results from the in-field study would provide data on actual spore reductions and test the basic logistical requirements for larger scale removal if desired. Results from carcass removal in Bogus creek will be combined with concurrent laboratory studies to provide additional data for a disease model that is being developed to provide information on the extent of removal that would need to occur to result in meaningful disease reduction.

This report summarizes results of the collaborative studies by Oregon State University (OSU), the USFWS Fish Health Center (FHC) and the Yurok Tribe to address the following objectives:

1. To determine the practical feasibility of removing salmon carcasses from the tributary (Yurok Tribe).  
This effort was assessed by removal of carcasses from a control section of Bogus Creek
2. To assess whether removal of carcasses results in reduction in parasite numbers

(Yurok Tribe, OSU).

This was measured by quantification of parasites from water samples collected at 5 locations using automated water samples.

3. To determine if infectious *C. shasta* actinospores as well as myxospores are present in lower Bogus creek (FHC).

This was assessed with sentinel trout challenges is complementary to the water filtration / *C shasta* DNA assay to track myxospore concentration in the creek.

4. To determine the contribution of myxospores from individual carcasses and any correlation with state of decomposition or time during the spawning period (FHC).

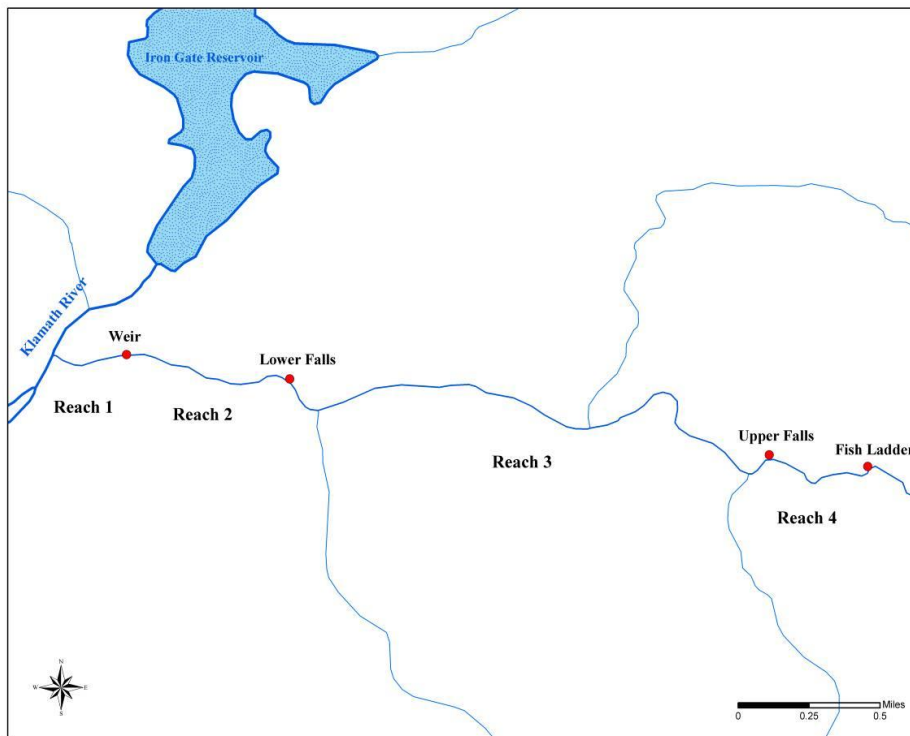
Salmon carcasses collected in Bogus creek (reach 2) were examined for myxospore presence and to provide an estimate of spore contribution.

5. To determine how long myxospores survive (OSU).

Spores extracted from fish were “aged” and examined at different times to determine survival.

### **Methods:**

***Identify study site and scope:*** A meeting between the three collaborators (Jerri Bartholomew, OSU; Scott Foott, FHC; and Josh Strange, Yurok Tribe) and members of California Dept of Fish and Game (CDFG) was held in August 2008 to identify a study site on Bogus Creek. Also discussed at this meeting were logistics for collection and



**Figure 2. Map of spawning ground survey reaches on Bogus Creek used since the 2003 field season. The weir denotes the location of the Bogus Creek Fish Counting Facility (M. Hampton, CDFG).**

removal of carcasses and any concerns that CDFG have about the study plan. Figure 2 shows the study reaches described in following sections.

**Carcass removal:** All salmon carcasses were removed from Reach 1 of Bogus Creek, from the video weir to the mouth, for approximately six weeks starting from the beginning of the spawning season. An equidistant reach upstream served as the control with no carcass removal. Complete removal of all carcasses was conducted daily over the study period during October and November of 2008.

**Water monitoring:** Reduction in parasite (myxospore) numbers was monitored using a series of four automated (solar powered) water samplers (ISCOs) that collected creek water 2-3 times weekly during the period that carcass removal occurs and for at least 2 weeks prior to and following removal. Samplers were placed at 4 locations in Bogus Creek; 3 above the study section and 1 immediately below the reach where carcass removal occurs, with an additional ISCO in the mainstem Klamath River below Bogus Creek. Samplers collected 1 liter of water every 2 hours for 24 h; this was repeated 2-3 times per week through early December 2008. The Yurok tribe was responsible for removal of the samples and their filtration. Filtered samples were shipped to OSU.

Water samples were assayed using a quantitative molecular assay (QPCR) for the parasite according to standard protocols currently used for parasite monitoring in the Klamath River (Hallett and Bartholomew 2006).

In addition to data collected from this study, we also used concurrent datasets collected as part of ongoing monitoring to interpret results.

**Sentinels:** One hundred and sixty-one Rainbow trout (Roaring River stock) received September 18, 2008 were transported to the FHC wet laboratory. Three exposures were conducted (below California Department of Fish & Game (CDFG) Klamath River Project Bogus Creek barrier weir located approximately 0.3 miles upstream from the confluence with the Klamath River) between 24 Sept. and 05 Nov (Table 1). This period coincided with the presence of adult Fall-run Chinook salmon returning to spawn in Bogus Creek. Exposures lasted for thirteen consecutive days and consisted of 45 RBT housed in a 302 L aluminum live box. The live box was checked and fish fed once per week by Iron Gate Hatchery personnel. At the end of each exposure period, fish were transported back to the FHC wet laboratory for observation and sampling.

**Table 1. Starting dates and number of fish exposed (in) and recovered for observation (out).**

Exposure Dates	No. of Fish In	No. of Fish Out	In-River Mortalities
9/24/08	45	45	0
10/8/08	45	44	1 (jumper)
10/22/08	45	44	1

Following the 24 Sept. exposure, fish were placed directly into a 750L observation tank at temperatures similar to that of Bogus Creek ( $\bar{x}$  creek= 13.2°C,  $\bar{x}$  holding tank = 15.0°C). Fish from exposures 2 & 3 were allowed 24h of acclimation at 11.0°C to reduce temperature shock before movement into the 15°C observation tank (Table 2, Figure 3). Fish from all three exposures were held for a 20 d observation period (total of 33 days post exposure (dpe)) prior to sampling. Five unexposed (control) fish were sampled at the start of each exposure period.

**Table 2. Daily water temperature data in Bogus Creek during the 3 exposures (minimum, maximum and mean  $\{\bar{x}\}$ ).**

Group ID	Min (°C)	Max (°C)	$\bar{x}$ (°C)
Exposure 1	8.9	17.0	13.2
Exposure 2	4.7	13.3	8.6
Exposure 3	5.3	11.5	8.6

**Figure 3: Daily mean water temperature in Bogus Creek for the 3 exposures.**

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***Carcass myxospore determination*** – Chinook carcasses were sampled in reach 2 of Bogus creek (0.6mi reach from weir to waterfall) on October 24, 31, November 5, and 14 (Fig. 1). Fork length, sex, carcass condition (fresh = eyes normal and gill with red or pink color or decomposed), spawn status of female (spawn = few eggs remaining in peritoneal cavity or not spawned) was recorded for each sample. The intestinal tract (junction of stomach and small intestine to rectum) was dissected, placed into individually numbered plastic bags for refrigerated transport back to the laboratory, and held in a 6°C water bath for 2 – 4d prior to processing. On 24 Oct, liver, kidney, and spleen was also collected and placed into a separate pooled-organ bag. The weight of each sample was measured prior to cutting the intestine into 8 – 12 cm pieces. An intestinal tract content sample (scraping) was obtained by grasping the end of the section with forceps and pushing the backside of a #21 scalpel blade, held at 45° angle, along the outside of the intestine. This process was repeated several times until only the serosa to stratum compactum layers remained. The scraping subsample was weighed, diluted 3x with PBS (4x final dilution), poured into tubes, vortex mixed, and allowed to settle for 1 -3 min. The supernatant in duplicate 10 µL samples was examined for the presence of *C. shasta* myxospores by 40x phase microscopy. Four hemocytometer counts on positive samples quantified the myxospore concentration per gram of sample. Two values are expressed in this report: myxospore / g scraping = derived from the hemocytometer count [(10 x dil.)/ 4 WBC squares X average spore count], and myxospore / scraping = myxospore / g scraping x grams of scraping subsample. We consider the myxospore / scraping to represent the minimum spore load for a given fish. The liver-kidney-spleen samples from myxospore positive carcasses were processed as above except the final dilution was 2x. A subsample of myxospore positive scrapings were assayed by QPCR.

At 33 dpe, all surviving exposed RBT were euthanized by an overdose of MS222, measured for fork length and weight, examined for anemia (pale gills) and internal signs of ceratomyxosis such as intestinal hemorrhaging or ascites fluid. The intestinal track and kidney was removed and sampled for PCR assay and histology. QPCR samples were collected from all fish including mortalities and processed as described (True et al. 2008; Nichols and True 2007).

Histological samples were taken from all 33 dpe sentinel fish and fresh mortalities (gills still pink). Both kidney and intestine were removed (after PCR sampling), placed into

cassettes labeled with unique identifiers, fixed for 24 hours in Davidson's fixative and then transferred to 70% ethanol for storage. Select specimens, processed for 5µm paraffin sections and stained with hematoxylin and eosin, were examined using bright field microscopy at 40x and 400x magnification.

**Myxospore longevity:** Myxospores harvested from the intestinal tract of infected RbT were divided into two temperature treatments to represent either end of the spectrum in the Klamath River: 4-6°C to mimic winter and 18-22°C for summer. At each temperature, spores were held for different lengths of time (3 subtreatments): 0 days, 7 days, and 28 days.

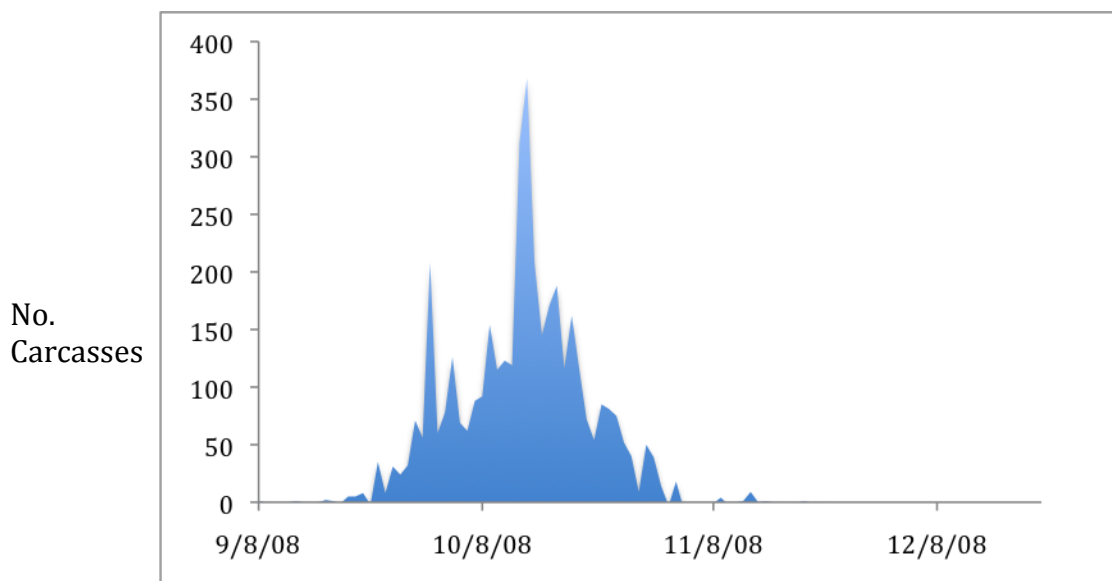
Two methods were used to assess spore viability at the different timepoints and temperatures: staining with Methylene Blue; and exposure of the invertebrate host worm. Myxospores whose cell integrity has been compromised stain dark blue with Methylene Blue and are presumed non-viable (dead) whereas unstained spores are presumed viable and infective. Definitive confirmation of viability status is confirmed by infection in the subsequent host.

Three replicates of 5000 myxospores were prepared for each subtreatment. At each time point, a subsample of spores was stained and the rest used in the worm exposure. Each worm replicate contained 50 worms (=100 spores per worm) which were housed in sediment in 10mL wells of a six-well plate at 18°C. Their water was changed twice a week and they were fed once a week. Staining also occurred at 3 months post exposure.

At 33 days post exposure, all worms from a day 0 positive control replicate were isolated and examined under a compound microscope for *C. shasta* then assessed in a PCR. One month later, another replicate was assessed. Due to low host survival, all replicates were then assessed. Day 7 and day 28 groups were all assessed with PCR at 2 months post exposure.

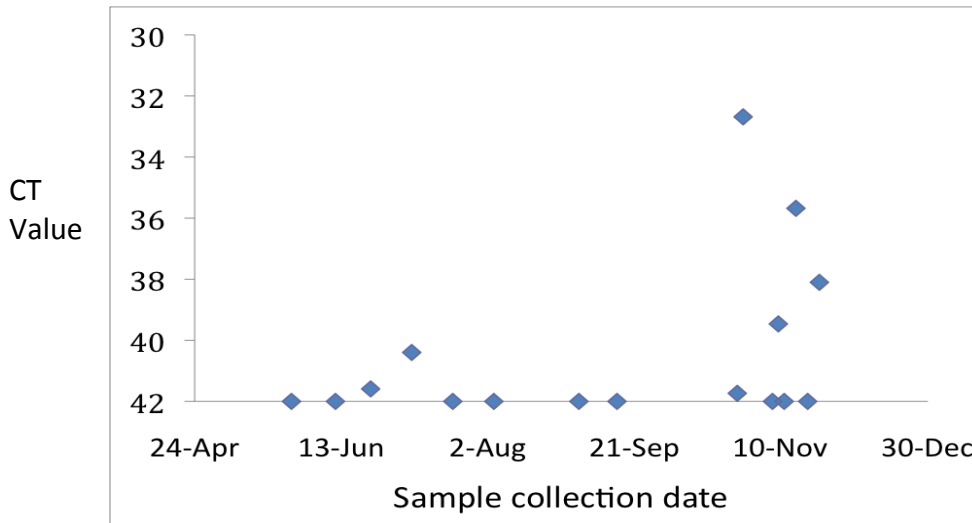
### **Results and Discussion:**

**Carcass removal:** The Yurok tribe removed 907 Chinook salmon carcasses from reach 1 during the period of 10 Oct to 24 Nov. During this period, 4,816 adult salmon were counted by CDFG (Figure 4), thus our removal of 907 fish in reach 1 constituted approximately 19% of the total run.



**Figure 4. Number of adult salmon observed at the counting facility between 8 Sept and 22 Dec 2008.**

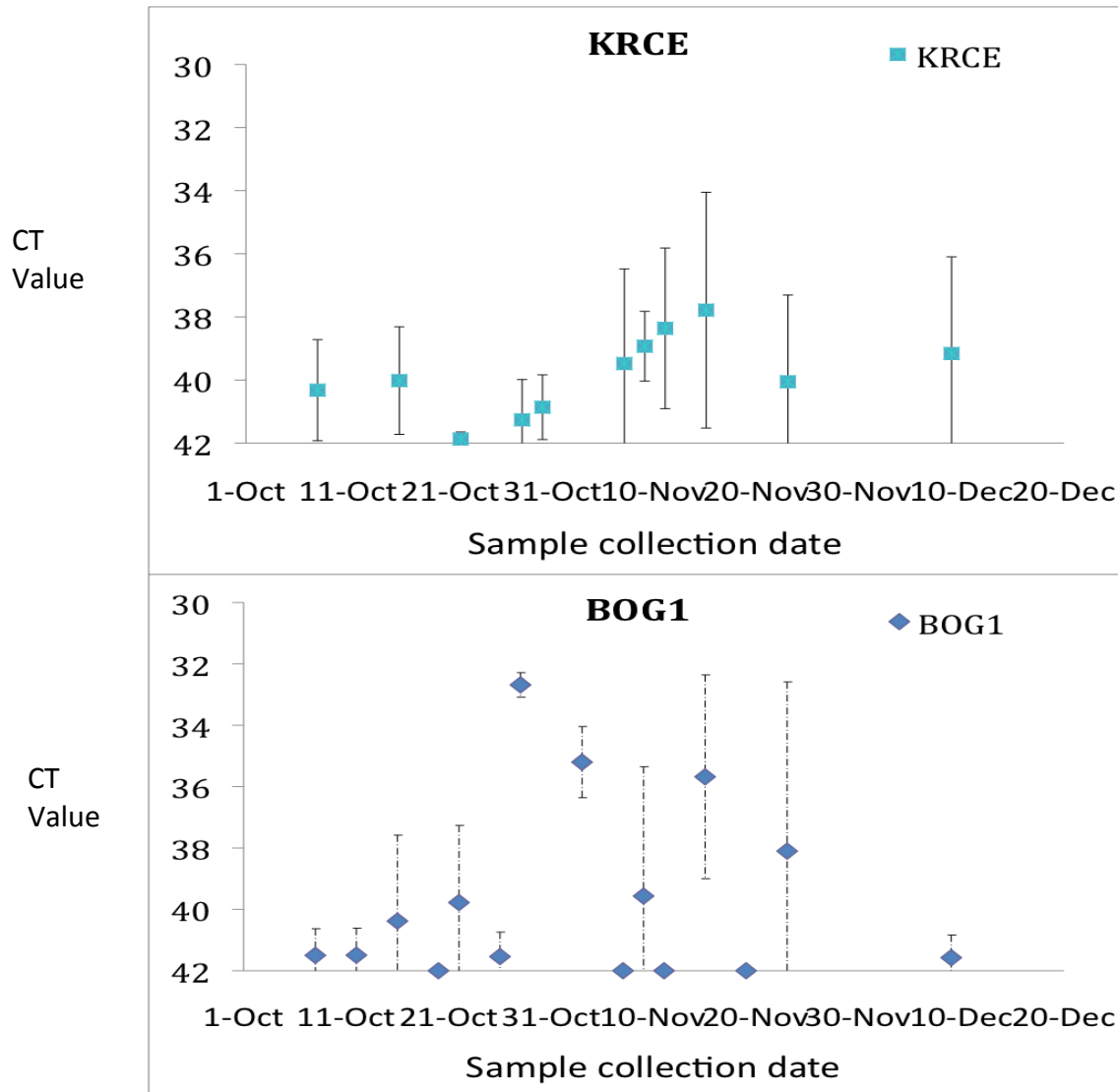
**Water monitoring:** A concurrent dataset allowed comparison of water samples collected from the mouth of Bogus Creek from May 2008 through the spawning period and ending early December 2008. This demonstrated a low and fluctuating number of parasites present in Bogus Creek beginning in late October (Figure 5). Parasite numbers remain low throughout this period, with CT values of 32-34 indicating the threshold detection of 1 myxospore/L.



**Figure 5. Quantitative PCR critical threshold (CT) values for samples collected at the mouth of Bogus Creek between May and December 2008. A CT value or 32-34 is approximately equal to 1 myxospore/L.**

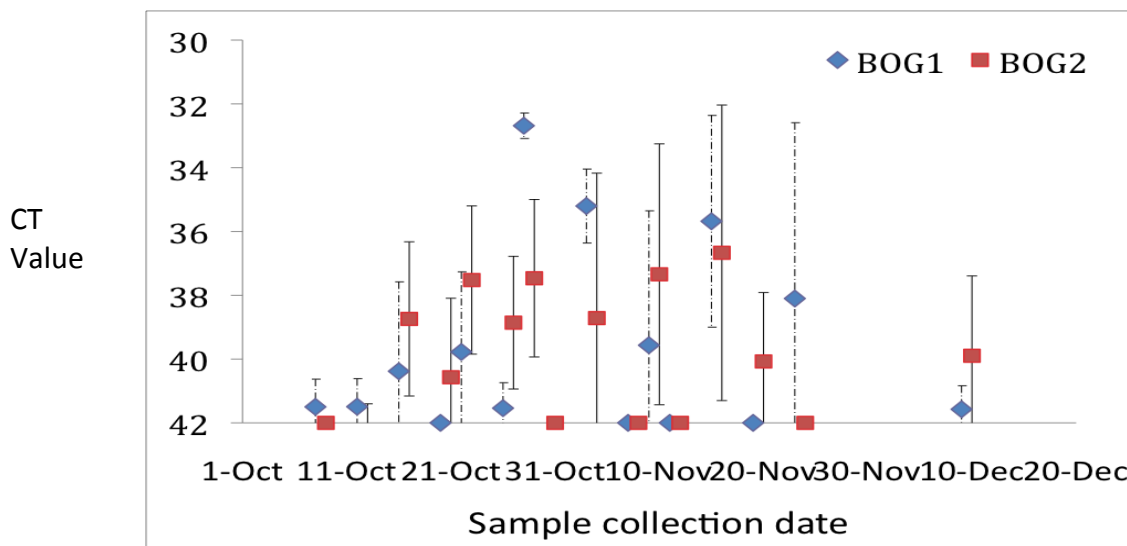
Comparison of parasite levels between Bogus Creek (BOG) and the mainstem Klamath River at Klamathon (KRCE) also showed a low parasite abundance during October-early December (Figure 6).





**Figure 6. Comparison of quantitative PCR cycle threshold (CT) values between a mainstem Klamath River site below Bogus Creek (KRCE) and the mouth of Bogus Creek (BOG 1). A CT value of 32-34 is approximately equal to 1 myxospore/L.**

Success is based on the hypothesis that if removal of carcasses from reach 1 decreased myxospore abundance, then the difference in myxospore numbers between reach 1 (BOG 1) and reach 2 (BOG 2) would not differ or would be lower at BOG 1. Because parasite levels were at or below 1 myxospore/L during this period we did not detect a decreased parasite abundance at BOG 1; however, parasite abundance did not appear to differ between the two sites (Figure 7).



**Figure 7. Comparison of quantitative PCR cycle threshold (CT) values between reach 1 in Bogus Creek, where carcass removal occurred (BOG 1) and reach 2, where carcasses were not removed (BOG 2). A CT value or 32-34 is approximately equal to 1 myxospore/L.**

To obtain a rough estimate of actual parasite numbers coming from Bogus Creek during this period, flows in Bogus Creek during mid-Nov were calculated and determined to be approximately 660 L/sec. Thus, during times when parasite abundance was 1 myxospore/L, this extrapolates to  $5.7 \times 10^7$  myxospores/d.

**Sentinel studies:** Greater than 98 % of sentinel fish survived the 13d exposure. Water temperature declined over the study from a daily mean of 13.2° to 8.6°C (Table 2 and Fig. 3). Post-exposure mortality occurred between 19 and 32 dpe in a low percentage of each exposure group (exposure 1 = 8 / 45 (18%), exposure 2 = 4/ 45 (9%), and exposure 3 = 1 / 45 (2%)). The majority of mortalities had skin lesions likely incurred during exposure. *C. shasta* was not detected by QPCR in either sentinel survivors or mortalities from all three exposures. Similarly, *C. shasta* was not observed in intestinal tract sections from 2 sentinel trout with PCR reactions showing some amplification of target DNA above the positive-negative cutoff of CT = 38.

**Carcass myxospores:** One hundred adults were sampled in reach 2 over the 4 collection dates between 24 Oct and 14 Nov (Table 3). This represents approximately 6% of the 1556 adults that passed above the DFG counting weir before 10/21/08 (M. Knechtle CDFG, pers. comm.). Intestinal scrape weight represented between 8 and 15% (collection group means) of the entire intestinal weight. Cestodes and trematodes were commonly observed in the wet mounts. The incidence of *C. shasta* myxospores detected in all adult intestinal tract scrapings was 30% (30 of 100 samples) over the entire collection period (Table 3). Prevalence of infection ranged from 13% in the last collection (14 Nov) to 40% (both 31 Oct and 05 Nov). There was wide variation in the number of myxospores / scraping within and between collection groups with no significant correlation for date of collection (Kruskal-Wallis ANOVA on ranks,  $H= 2.815$ , 3df,  $P=0.421$ ), or sex (Mann-Whitney Rank sum test  $P=0.966$ ). Samples from decomposed adults had significantly higher myxospore loads (median 182,109) than fresh carcasses (median 30,656)(Mann-Whitney Rank sum test  $P=0.036$ ).

The detection limit for wet mount examination appeared to be approximately 3,000 spores / g scraping. Large particulate matter likely reduced detection in approximately 20% of the samples. No myxospores were observed in liver, kidney, and spleen samples from 5 adults collected on 24 Oct. These fish had positive intestine scraping samples (18,750 to 174,375 spores / g scraping). The large volume of this sample type (72 – 216 g) could be a factor in the lack of detection. *C. shasta* DNA was detected in 9 of 16 (56%) scraping subsamples from the 24 Oct and 14 Nov collections (Table 4). The 45% incidence of infection from scrapings, that were negative for *C. shasta* myxospores by wet mount, suggests that either a low number of myxospores are missed or DNA from pre-sporogonic stages are present in the sample.

**Table 3. *Ceratomyxa shasta* myxospore data from reach 2 Bogus Creek Chinook carcasses: Prevalence of infection and myxospores/intestinal scraping by sex and collection date.**

Date	Prevalence	Mean (SD)	Range
24 Oct			
Male	5 / 15 (33%)	80,258 (62,304)	18,788 – 183,094
Female	<u>5 / 15 (33%)</u>	59,828 (66,097)	3,206 – 171,100
Combined	10 / 30 (33%)		
31 OCT			
Male	6 / 12 (50%)	537,848 (820,963)	2,825 – 2,159,500
Female	<u>6 / 18 (33%)</u>	3,000,808 (5,399,899)	7,125 - 13,788,600
Combined	12 / 30 (40%)		
05 NOV			
Male	4 / 8 (50%)	53,092 (49,932)	3,500 – 104,300
Female	<u>0 / 2 (0%)</u>		
Combined	4 / 10 (40%)		
14 NOV			
Male	3 / 11 (27%)	5,491 (7,997,714)	7,875 – 14,668,200
Female	<u>1 / 19 (5%)</u>		
Combined	4 / 30 (13%)		
Total	30 / 100 (30%)	1,287,327 (3,589,747)	

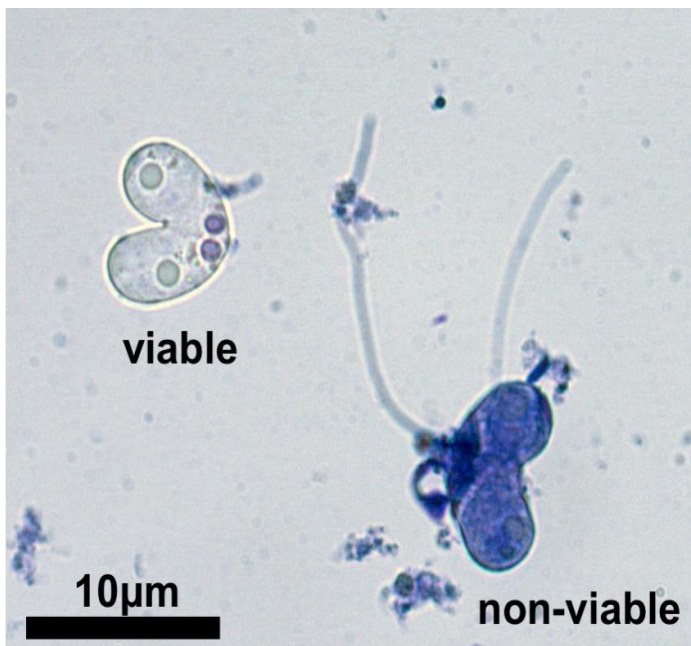
**Table 4. Prevalence of *C.shasta* DNA detection by QPCR in intestinal scrapings from 24 Oct and 14 Nov subsamples. Data includes the incidence of *C.shasta* DNA detection in myxospore positive (+) and undetected (UD) scrapings.**

Oct 24	3 / 10 (30%)
Nov 14	<u>6 / 6 (100%)</u>
Incid.	9 / 16 (56%)
Myxospore +	4 / 5 (80%)
Myxospore UD	5 / 11 (45%)

**Myxospore longevity:** Viability data was only obtained from the staining component (Figure 8) as worm survival was poor. In the day 0 group replicates 10-17 worms (out of 50) survived; however, 10 were the most that survived in any other replicate (total of 70 out of 1000).

At 33 days post exposure, 16 worms were examined microscopically for infection and developmental stages were seen in two of these (12.5%); PCR confirmed infection was by *C. shasta*. A further 15 worms examined at 2 months post exposure appeared uninfected and PCR confirmed this. None of the remaining worms were positive by PCR.

Staining indicated that spore viability was inversely proportional to both time and temperature and non-viable (dead) myxospores appear to rapidly degrade: after 1 month at 18°C, no myxospores were detected; after 3 months at 6°C, only 10% of the original number was detectable.



**Figure 8. Viable (clear) and dead (blue) myxospore stages of *Ceratomyxa shasta*.**

#### **Relationship to previous studies**

A review of Iron Gate Hatchery (IGH) Fall-run adult *C. shasta* data, collected in 2005 – 2007, indicates that incidence of *C. shasta* infection is high however the myxospore stage is primarily in carcasses and not live (spawned) fish. In 2005, histological sections from spawned adults showed an 80% incidence of infection by the pre-sporogonic stages (16 of 20 sections, CA-NV FHC unpublished data). In 2006, *C. shasta* myxospores were observed in only 1 of 60 intestinal scraping samples collected from spawned adults at IGH. *C. shasta* DNA was detected by QPCR in 12 of 20 (60%) of these intestinal scrapings and trophozoite stages were observed in one of 20 kidney sections from the same fish (CA-NV FHC unpublished data). A similar effort, by OSU and CDFG, to survey IGH Fall-run Chinook spawners by QPCR yielded 70% and 85% *C. shasta* DNA detection rates in 2005 and 2006, respectively. In 2007, *C. shasta*

myxospores were observed in the intestinal scraping of only 4% (6 of 166) of IGH spawned adults but in 34% (22 of 64) of the carcasses collected from Bogus creek, mainstem Klamath, and Shasta River (Ryan Slezak, Humboldt University, pers. comm.).

Infected juvenile salmon can also produce myxospores if they survive beyond approximately 3 weeks post-infection. In a 2008 sentinel Chinook salmon study, intestinal tracts of clinically ill fish were sampled at 20 days post-exposure, a lower intestine content smear fixed and the remaining intestine held at ambient water temperature (18°C) for 48h. No myxospores were observed in the 20d smears however the 48h samples contained 6 to 9 million myxospores / g intestine (CA-NV FHC unpublished data for 2008 Prognosis study). Bjork and Bartholomew (2008) report an average *C. shasta* myxospore load of 4.9 million from rainbow trout juveniles. Histological examination of the sentinel intestines demonstrated myxospores in necrotic muscularis layers beginning at 16 days post-exposure. The role that infected juvenile salmon play in perpetuating the lifecycle of *C. shasta* in the Klamath R. is unknown. It is unlikely that infected juvenile salmon are significant contributors of myxospores to polychaete populations in the Shasta R. to Seiad "hot zone" of the Klamath River (Stocking et al. 2006) as most clinically affected juveniles are collected in reaches below this area.

#### **Preliminary Study Conclusions:**

1. Removal of salmon carcasses from Bogus Creek was logistically feasible, at least in the lower 2 reaches.
2. Examination of salmon carcasses for myxospores indicates that spores are not present in recently spawned fish, despite heavy parasite infections. This may suggest that a maturation process is necessary or that some parasites do not successfully complete their life cycle.
3. Water monitoring appears to be a feasible method for assessing success, however, it should extend later through the winter, when myxospores are most likely being released.
4. Based on these findings, carcass removal could be done weekly, rather than daily.
5. Myxospore viability decreased more rapidly at warmer temperatures (18°C vs 6°C). However, even at colder temperatures myxospores are unlikely to survive more than one season.

#### **Acknowledgements:**

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