U.S. Fish & Wildlife Service

California Nevada Fish Health Center
FY2006 Investigational Report:

Relationship between *Ceratomyxa shasta* and *Parvicapsula minibicornis* actinospore exposure in the Klamath River and infection in juvenile Chinook salmon.

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February 2007

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Summary: The infectious potential of Klamath River water (rkm 262) in June 2006 was demonstrated by the concurrent detection of low numbers of *Parvicapsula minibicornis* and *Ceratomyxa shasta* actinospores (DNA) in water samples as well as parasitic infection in salmon exposed to the same water for 3 and 6h. DNA was detected in 1 L water samples collected throughout the 6h exposure and assayed by a filtration and Quantitative Polymerase Chain Reaction (QPCR) method. Estimated *C. shasta* actinospore numbers peaked (~10 spores /L) in the afternoon while *P. minibicornis* actinospore numbers tended to decline. There was considerable variation in the *C. shasta* DNA values obtained from water samples collected from replicate livecages at the same time. While the estimated *C.shasta* actinospore exposure was 3.6x higher for salmon exposed for 6h (358,657 spores) than for 3h (99,181 spores), both infection and disease severity tended to follow the same doubling function as exposure duration. *Parvicapsula* appears to be more efficient at infecting fish than *C.shasta*. Exposure to 2 – 3x fewer actinospores than *C.shasta* resulted in 100% *P. minibicornis* infection by 17 days post-exposure compared to 34% *C.shasta* infection. The 6h exposure (estimated 358,657 *C.shasta* and 125,000 *P.minibicornis* actinospores) could be considered a biologically significant threshold for juvenile Chinook salmon as there was a 37% incidence of ceratomyxosis (with dual *Parvicapsula* infection) and 22% mortality in salmon held past 10 days post-exposure. Given the cumulative effect of even low-actinospore concentrations on fish infection, the current water filtration / QPCR method appears to be sensitive enough to predict myxozoan parasite infection in juvenile salmon rearing in the Klamath River.

Notice
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Introduction:
Juvenile Chinook salmon (*Oncorhynchus tshawytscha*) in the Klamath River incur a high incidence of infection from 2 myxozoan parasites, *Ceratomyxa shasta* and *Parvicapsula minibicornis* (Stocking et al. 2006, Foott et al. 2004). These parasites occur in a number of Pacific Northwest watersheds and both parasite life cycles include the polychaete, *Manayunkia speciosa,* as an alternate host (Hoffmaster et al. 1988, Bartholomew et al. 1997, Jones et al. 2004, Bartholomew et al. 2006). The actinospore, a stage that is infectious to salmon, is released from infected polychaetes into the water column and infections by *C. shasta* can occur from spring through fall at water temperatures $\geq$ 7°C (Ching & Munday 1984, Hendrickson et al. 1989). Myxospores develop within infected salmonids (particularly migratory adults infected during declining water temperature periods) and it is this stage that once shed from fish can infect polychaetes to complete the life cycle (Bartholomew et al. 1997). Thus it is possible to have both myxospores and actinospores present in the river at the same time. Seasonal infectivity data for *P. minibicornis* has not been reported but appears to be similar to *C. shasta* in the Klamath River. Foott et al. (in press) reported that actinospores of both myxozoans have the potential to infect salmonids for at least 7 days after release from the alternate polychaete host. A method to estimate parasite concentration in river water was developed for *C. shasta* (Hallett and Bartholomew 2006) and has also been adapted for *P. minibicornis* (Foott et al. in press) utilizing a Quantitative Polymerase Chain Reaction (K. True, publication in preparation). Our primary focus in this study was to relate parasite DNA concentration in filtered water samples to the probability of parasite infection in juvenile salmon. Specifically, we address 2 questions: 1) Does the relative concentration of parasite DNA in the exposure water and the duration of such exposure correspond to infection by both *C. shasta* and *P. minibicornis* in juvenile Chinook salmon, and 2) What histological changes are observed in juvenile Chinook tissues from limited parasite exposures?

Methods:

Salmon - Juvenile Chinook salmon (*Oncorhynchus tshawytscha*), Trinity River stock, were obtained from the California Department of Fish and Game Trinity River Hatchery (TRH) 7 d prior to the June 20, 2006 exposure and held at the California Nevada Fish Health Center wet laboratory on an ozone-treated water supply. Lewiston reservoir is the water supply for TRH and is considered to be non- infectious for *C.shasta* as there is no history of ceratomyxosis at the hatchery and there have been no observations of the parasite in 6 years of histological survey of juvenile TRH salmon prior to release. Similarly, *P. minibicornis* has not been detected in juvenile TRH salmon prior to release by histological examination of kidney sections, however QPCR analysis has not been conducted to date. *Parvicapsula* is observed in both juvenile and adult salmon collected in the Trinity River.

Exposure – Four cages, each containing 25 salmon, were placed into the Klamath River at 10:30 on June 20, 2006 approximately 1 mile above Beaver

Creek (rkm 262, UTM 10 516058E 4634926N). The 1.27 ft long cages were constructed of 12 inch PVC pipe (chamber radius = 0.49 ft) with ¼ inch mesh screens at both ends (cross-sectional area = 0.75 sq. ft., total volume = 0.96 cu. ft.). Each cage contained a 7/16 inch hose with ¼ inch mesh cap that was suspended in the middle of the cage and attached to a hand bilge pump. The mesh end of the cages was orientated into the current flowing along the bank (see title page photo) and the cages were attached to clips spaced 15 ft apart on an anchor line held at 2.5 ft depth. Two cages were removed to the transport tank after 3 h of river exposure followed 3h later by the other two cages. These replicate exposure groups are referred as 3h and 6h. The transport tank contained 17°C water from the wet lab and was continuously aerated. Mean river temperature during the exposure was 19.8°C (18.6 – 21.2°C) and water velocity through the cages averaged 0.3 feet / s (Std Dev. 0.08). Flow was measured with a Global Water Instrumentation flowmeter (Fair Oaks, California) directly behind the rear mesh of each cage at 11:00, 12:30, and 14:30. A group of 25 salmon were held at the wetlab as non-exposed controls. In order to reduce the occurrence of columnaris disease (infection by *Flavobacterium columnare*), each group received a 10 min prophylactic bath of 1 mg L^{-1} furanase one day following the exposure period. Salmon were fed a commercial salmon diet and held in separate 10.6 gal aquaria supplied with 5 gpm flow of aerated water (mean temperature 19.9°C, range = 17.5 – 22.7°C). Coleman National Fish Hatchery uses this water supply and has no history of either *C.shasta* or *P minibicornis* infection. Effluent from the wet lab was chlorinated.

At 10 days post – exposure (dpe), ten fish from each tank were sampled for histological examination as well as fork length, weight, and hematocrit measurements. At 17 – 24 dpe, a total of 15 moribund salmon were removed from the exposed groups and sampled for histology. All survivors at 31 dpe were sampled for histology and measured for weight and fork length. Intestinal tract (including the caeca) and kidney was placed in Davidson's fixative, processed for 5 μm paraffin sections and stained with hematoxylin and eosin (Humason 1979). Previous work has shown these post-exposure periods to produce clinical infections dominated by trophozoite stages (Foott et al. 2004). All tissues for a given fish were placed on a single slide and identified by a unique code number. Each slide was examined at both low (40X) and high magnification (400X) without knowledge of the sample group. Parasite infection was arbitrarily scored as moderate (#1) or diseased (#2) when associated inflammation was observed in less than or greater than 30% of the affected tissue, respectively. If no parasites were detected in the section the fish received a zero score.

Spore DNA filtration – A 1 L sample was pumped from each cage at 30 min intervals during river exposure. Each sampled was divided in half and each 500 mL was filtered through a 47mm 5.0μm nitrocellulose filter (Millipore Corp., Bedford MA). The filter apparatus was washed with distilled water and wiped dry between each 1L water sample. The 2 filters were rolled, placed together into a 2 mL snap-cap tube, and frozen on dry ice (Hallett and Bartholomew, 2006). Filters were stored at -70° C until DNA extraction was performed. Two aliquots of filtrate water were also frozen to test for inhibition. Each filter was cut into

small pieces with scissors (wiped with DNAaway™ solution and distilled water between samples), the 2 filters were digested together in 1 mL of an 8x dilution of Proteinase K (20µg/mL in Applied Biosystems digest buffer) for 1 h at 55°C, centrifuged at 10,000xg x 10min, and the supernatant used for DNA extraction. DNA was extracted with an Applied Biosystems Model 6100 Nucleic Acid Prep Station using reagents and procedures provided by Applied Biosystems (http://docs.appliedbiosystems.com.; ABI PRISM™ 6100 Nucleic Acid PrepStation: User Guide: Rev B 4326242B).

Filter DNA extracts were tested for both *P. minibicornis* and *C. shasta* 18S ribosomal DNA by Quantitative Polymerase Chain Reaction (QPCR). The *C. shasta* assay was conducted by the method of Hallett and Bartholomew (2006). The *P. minibicornis* assay was conducted by the method of True (unpublished data) using a Taqman Minor Binding Grove probe. Primers for *P. minibicornis* were designed using ABI Primer Express software targeting the 18S ribosomal DNA sequence. Both a river filtrate and molecular-grade water sample (5µL) were spiked with a high concentration *C. shasta* control extract and compared to determine whether inhibitors were present in the water samples. A similar spiked control for *P. minibicornis* was inadvertently not run with the river samples but there are plans to assay this control in the spring of 2007 during QPCR analysis of smolt samples. The 30µL QPCR reaction mixture consisted of Taqman universal master mix (Applied Biosystems, Foster City, CA), 800nM of each primer, 200nm Taqman® Probe, and molecular grade water containing 250 ng / µL Bovine Serum albumin (to reduce inhibition effects). A 5µl volume of total DNA template was added to each reaction mixture. Tests were run in an Applied Biosystems 7500 Sequence Detection System (http://docs.appliedbiosystems.com. "Data Analysis on the ABI PRISM® 7700 Sequence Detection System: Setting Baselines and Thresholds: Guide: Rev A 4370923A").

Detection thresholds for each assay were set using known *C. shasta* positive fish tissue, and *P. minibicornis* DNA plasmid controls. Quantity of parasite DNA is reported as Cycle threshold (CT); the number of amplification cycles required for the fluorescent signal to surpass normalized background levels and produce an exponential fluorescent signal curve indicative of target DNA amplification. The negative-positive threshold for both assays occurred at CT values of 38-39. All low level test results near the threshold were confirmed by visual inspection of amplification curves and verification of a significant change in normalized fluorescent signal (Δ Rn of at least 100,000 fluorescent units).

Results and discussion:

Fish size - Mean (std. dev.) fork length, weight, and condition factor (KFL) at 10 dpe was 76 mm (6), 5.2 g (1.3), and 1.165 (0.112), respectively. Hematocrits were normal (mean 40% (std. dev. 3%)) at this time point. Salmon in all groups grew an average of 0.33 mm / d and had mean (St. dev.) fork length, weight, and KFL at 31 dpe of 83 mm (5), 6.2 g (1.3), and 1.101 (0.134), respectively. Hematocrits were not taken at 31 dpe as only 1 fish with pale gill was observed in the sample groups.

Parasite infection - The prevalence of infection for *C.shasta* ranged between 30% in the 3h salmon and 40 – 70% in the 6h salmon sampled at 10 dpe (Table 1 and Fig. 1). The few trophozoites observed in the sections tended to be exterior of the intestinal tract and were associated with mild focal inflammation of the intestinal serosa or visceral adipose tissue. Low numbers of *P. minibicornis* were seen in glomeruli but not kidney tubules in 50 – 100% of the salmon (Table 1). At this time point, no glomerulonephritis was associated with the infection.

Moribund condition was observed in 4 of 49 (8%) 3h and 11 of 50 (22%) 6h salmon between 17 and 24 dpe (Table 1 and Fig. 1). These fish had pale gills and swollen hemorrhagic intestines indicative of ceratomyxosis. This diagnosis was confirmed by the intestinal sections. All moribund salmon were also infected with *P. minibicornis* and glomerulonephritis was observed in the kidney sections from 2 of 11 (18%) moribund 6h salmon (Fig. 2 and 3).

The prevalence of infection for *C.shasta* in salmon surviving to 31 dpe was 8 and 15% in the 3h salmon replicates while the 6h salmon survivors had 10 and 11% prevalence (Table 1). One salmon in the 3h-B replicate demonstrated signs of clinical ceratomyxosis (ascites, intestinal hemorrhages with focal lesion seen in the histological section). The observation of focal granulomas on the serosal surface of the intestine, containing low numbers of myxospores but no pre-sporogonic forms, in the *C. shasta* positive 6h salmon and one 3h salmon indicate that 31 dpe coincided with the resolution of infection . Given that ceratomyxosis did not occur prior to 17dpe, severity of infection could only be ascertained from samples collected after 10dpe. If the 10dpe sample total is removed from the population totals, the incidence of ceratomyxosis (Cs2 score) was 14 % (4 of 29 fish) in the 3h and 37% (11 of 30 fish) in the 6h groups. Granulomatous tissue without associated parasites was seen in the visceral adipose tissue and serosal surface of 5 fish (10%) from the 31 dpe collection (Fig. 4). All exposed salmon sampled at 31 dpe were infected with *P. minibicornis,* however 42% of the 6h salmon were rated as diseased (#2 score) compared to 8% in the 3h group (Fig. 5). Neither parasite was observed in the kidney and intestinal sections of the 25 non-exposure controls.

Table 1. Prevalence of infection (POI = number positive / total (percent)) and severity of parasite infection (1 or 2) in replicate (A and B) salmon groups exposed for 3 or 6 h to the Klamath River and sampled at 10 and 31 days post-exposure (dpe). Severity ratings indicate the presence of *C.shasta* (Cs) or *P. minibicornis* (Pm) with (#2 diseased) or without significant inflammation (#1 moderate). Infection data for moribund salmon sampled between 17 – 24 dpe from each exposure group is also reported.

	3h –A	3h- B	6h – A	6h - B
10dpe				
Cs POI	3 / 10 (30)	3 / 10 (30)	4 / 10 (40)	7 / 10 (70)
Cs1	3	3	4	6
Cs2	0	0	0	1
Pm POI	5 / 10 (50)	7 / 10 (70)	10 / 10 (100)	9 / 10 (90)
Pm1	5	7	10	9
Pm2	0	0	0	0
31dpe				
Cs POI	1 / 12 (8)	2 / 13 (15)	1 / 9 (11)	1 / 10 (10)
Cs1	1	1 *	1 *	1 *
Cs2	0	1	0	0
Pm POI	12 / 12 (100)	13 / 13(100)	9 / 9 (100)	10 / 10 (100)
Pm1	11	12	3	8
Pm2	1	1	6	2
Moribund				
Total no.	3	1	6	5
Cs2	3	1	6	5
Pm1	3	1	5	4
Pm2	0	0	1	1

* Focal granuloma containing only myxospores

Figure 1. Percentage of moribund (morb),10, and 31 day post- exposure (d) samples of the 3h and 6h exposure salmon with *Ceratomyxa shasta* infection rated as moderate (Cs1) or associated with >30% intestinal lesion (Cs2).

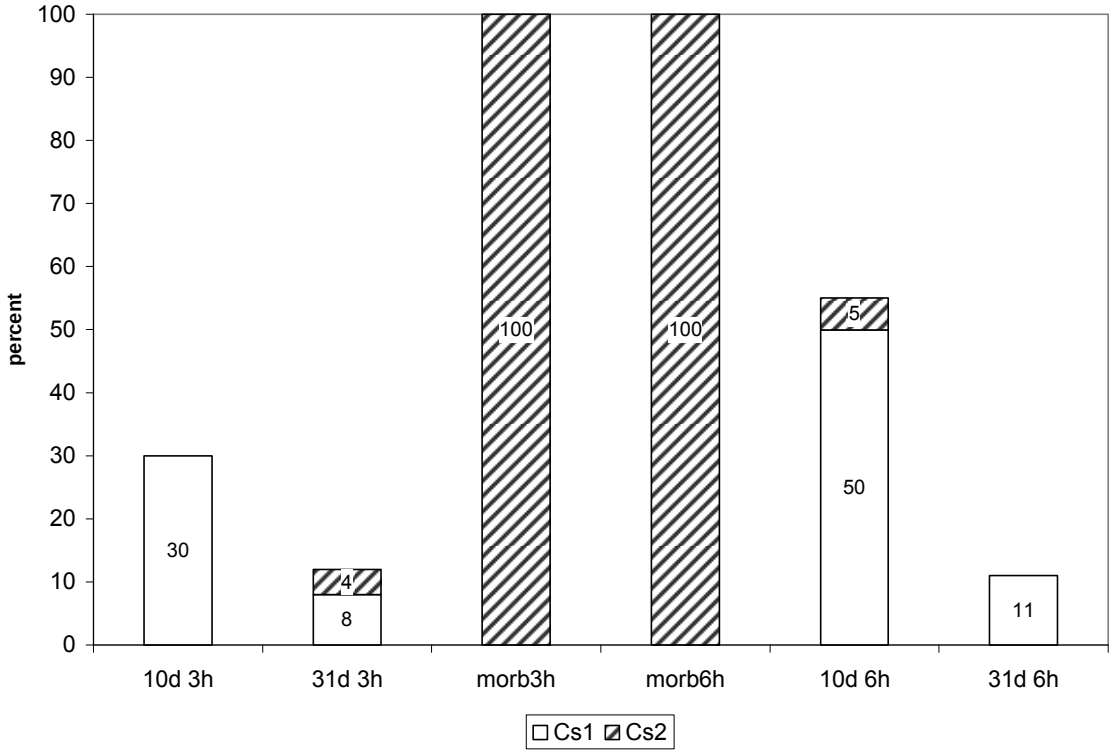
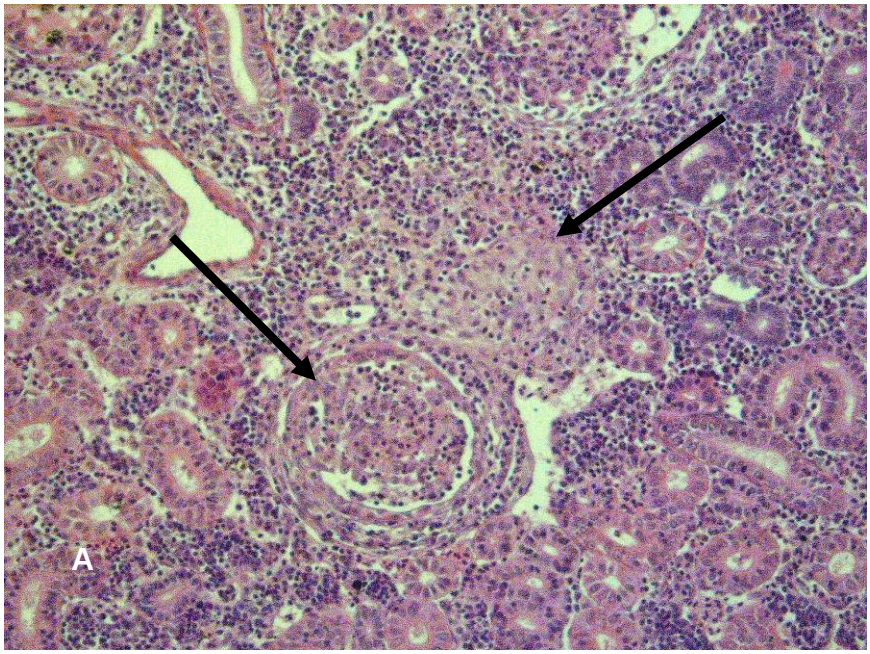


Figure 2.
(A) Low magnification photo of glomerulonephritis (black arrows) in kidney section from a 6h exposure salmon infected with *Parvicapsula minibicornis.* Granulomatous tissue has filled in the vasculature and Bowman's capsule (H&E stain).

(B) High magnification photo of normal salmon glomerulus showing space (white arrow) within the Bowman's capsule (H&E stain).



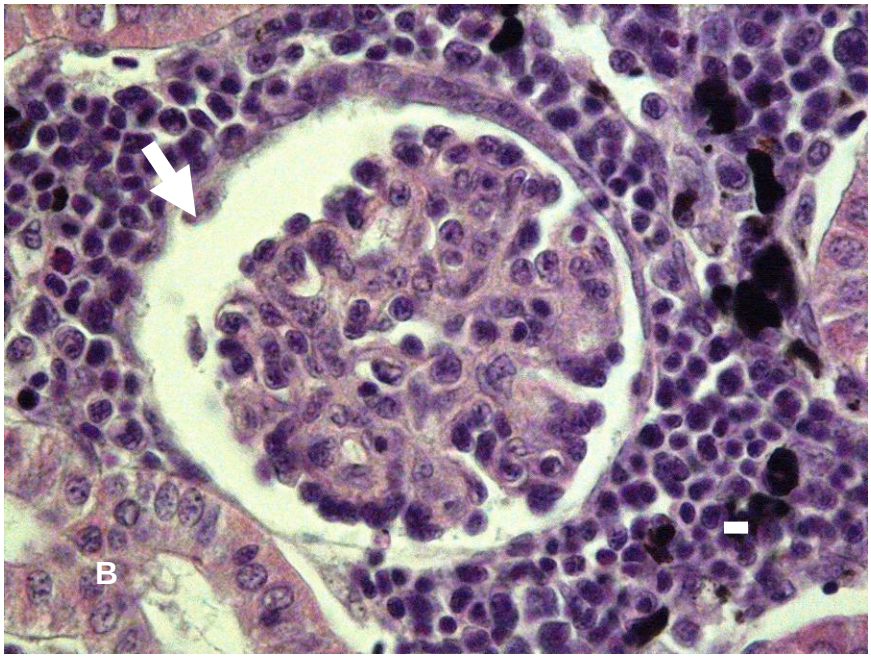


Figure 3. *Parvicapsula minibicornis* within vasculature spaces of glomerulus and tubule lumen (arrows).

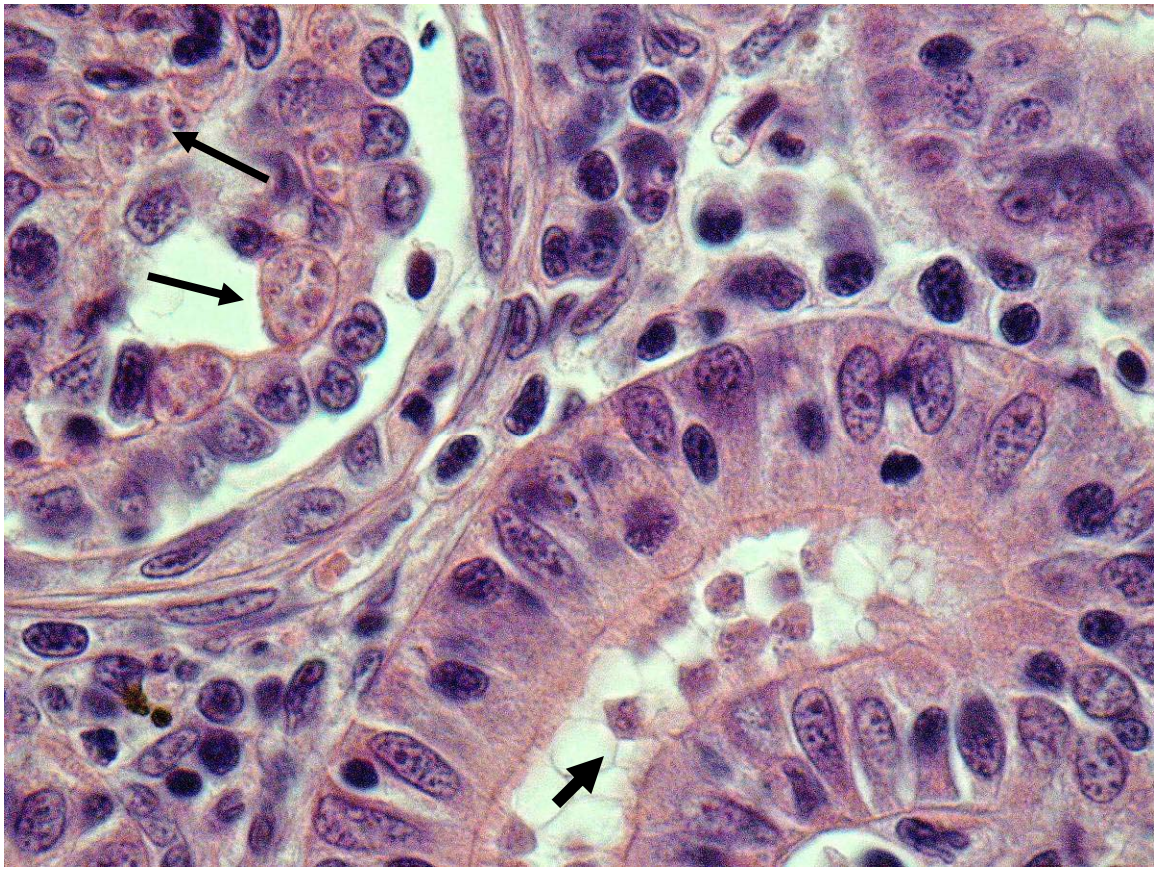
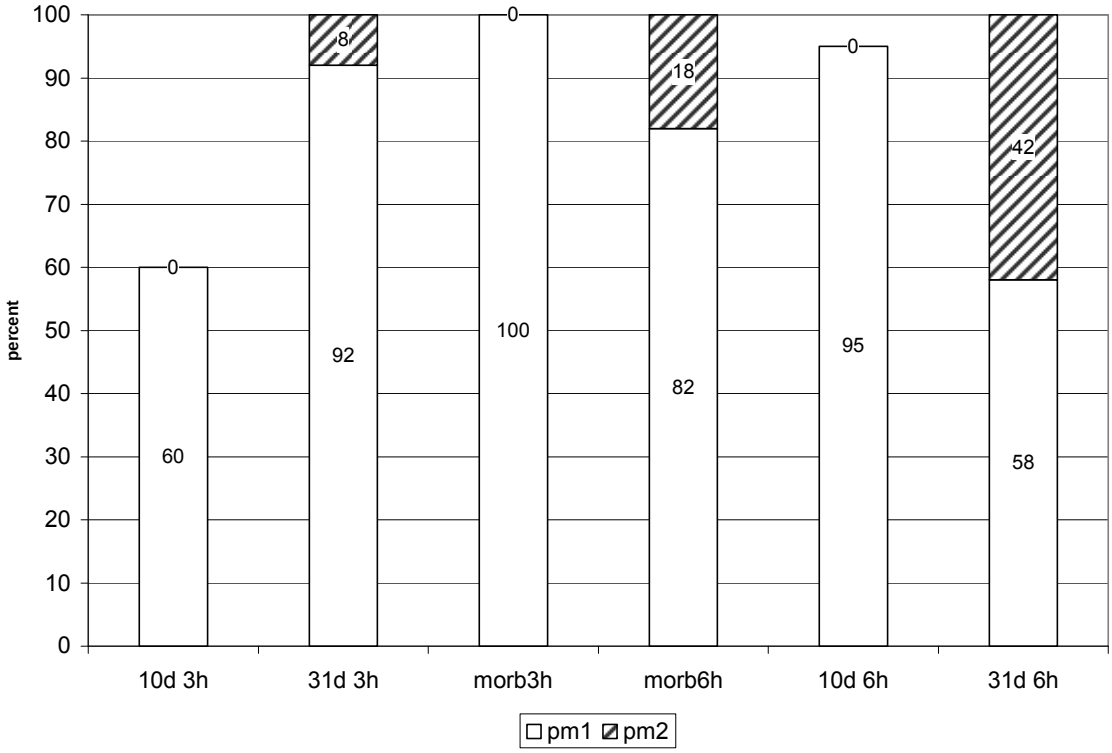


Figure 4. Photomicrograph of focal inflammation, without *C.shasta* trophozoites, associated with the serosa (tella subserosa) of small intestine from a 31dpe salmon.

Figure 5. Percentage of moribund (morb),10, and 31 day post- exposure (d) samples of the 3h and 6h exposure salmon with *Parvicapsula minibicornis* infection rated as moderate (Pm1) or associated with >30% glomerulonephritis (Pm2).



Spore DNA concentrations - Ceratomyxa shasta DNA was detected in all 36 water samples collected over the 6 h exposure period and CT values ranged from 29.8 – 38.0. The average CT value of 34.5 (std. dev. 1.7) was close to the 1 actinospore / L value (CT = 34.6) reported by Hallett and Bartholomew (2006). No obvious inhibition was detected as spiked river filtrate water varied only 0.2 CT from the *C.shasta* spiked molecular-grade water. The estimated spore / L value for each water sample was derived from the equation: (CT – 34.736) / -4.0166 = log (spore /L) (Hallett and Bartholomew, 2006).

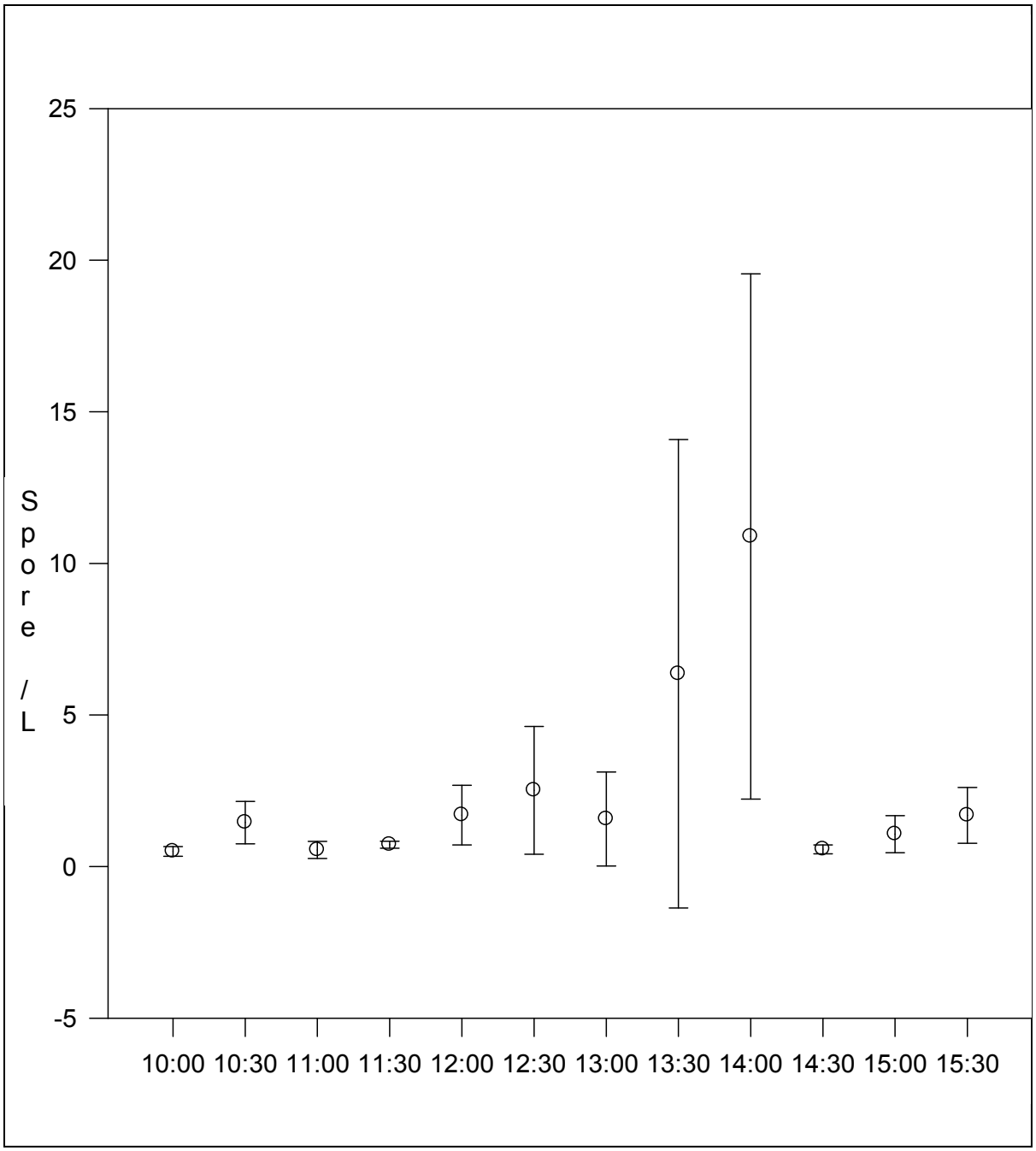
The average hourly spore / L estimates ranged from 0.50 to 10.89 with a marked increase occurring in the 13:30 and 14:00 samples (Fig. 6). There was considerable variation in the CT (spore / L) values obtained from the replicate live cages for any 30 min interval with coefficient of variation ranging from 15 – 121%. The estimates of less than one spore / L is not surprising given that there are multiple copies of the 18S rRNA gene in each spore and the filter/QPCR assay reportedly can detect 1/1000th of a spore (Hallett and Bartholomew 2006). When the average velocity measurement of 0.3 ft/s is multiplied by the cross-sectional area of the livecage (0.75 sq. ft) it yields a flow rate of 0.225 cfs or 6.37 L/s. The estimated total actinospore exposure for each group was calculated as follows:

1. (6.37 L/s x 60s/min x 30min) = 11,466 L passing through the cage in a 30 min period
2. Summation of [11,466L x spore/L value** for each 30 min. period] = estimated total actinospore exposure

** the value of 1 was arbitrarily assigned to any 30 min spore/L estimate < 1.

The 3h cages were estimated to be exposed to 3.6x fewer *C. shasta* actinospores (99,181 spores) than the 6h groups (358,657 spores) due to marked increases in CT values at 13:30 and 14:00. The increased actinospore exposure was demonstrated in both the overall incidence of infection and the percentage of 6h salmon showing disease (Fig. 1). Despite the 3.6x higher estimated spore exposure, infectivity roughly followed the doubling in exposure time between the 3 and 6h groups. The 6h group incurred about a 1.8x higher incidence of infection (46% vs 26%) and 2.4x higher incidence of disease (22% vs 10% clinical ceratomyxosis) than the 3h salmon. Given the lower sensitivity of histological examination as compared to QPCR for detection of asymptomatic *C. shasta* infection, the incidence of actual infection was likely higher in both groups. It appears that many fish were able to destroy limited numbers of invading parasites as there was a marked decrease in prevalence of infection between the 10 (POI = 50 – 100%) and 31dpe (POI = 8 – 15%) samples. This opinion is further supported by the observation of chronic inflammation within visceral adipose tissue or along the intestinal serosa in 18% (8 of 44) of the salmon sampled at 31 dpe. Granulomatous tissue was associated both with limited numbers of *C. shasta* myxospores or without observed parasites in 31 dpe salmon. The 6h exposure (approximately 358,657 *C.shasta* and 125,400 *P.minibicornis* actinospores) could be considered a biological significant

Figure 6. Estimated mean *C.shasta* spore / L values with standard error bars for water samples collected at each 30 min interval.



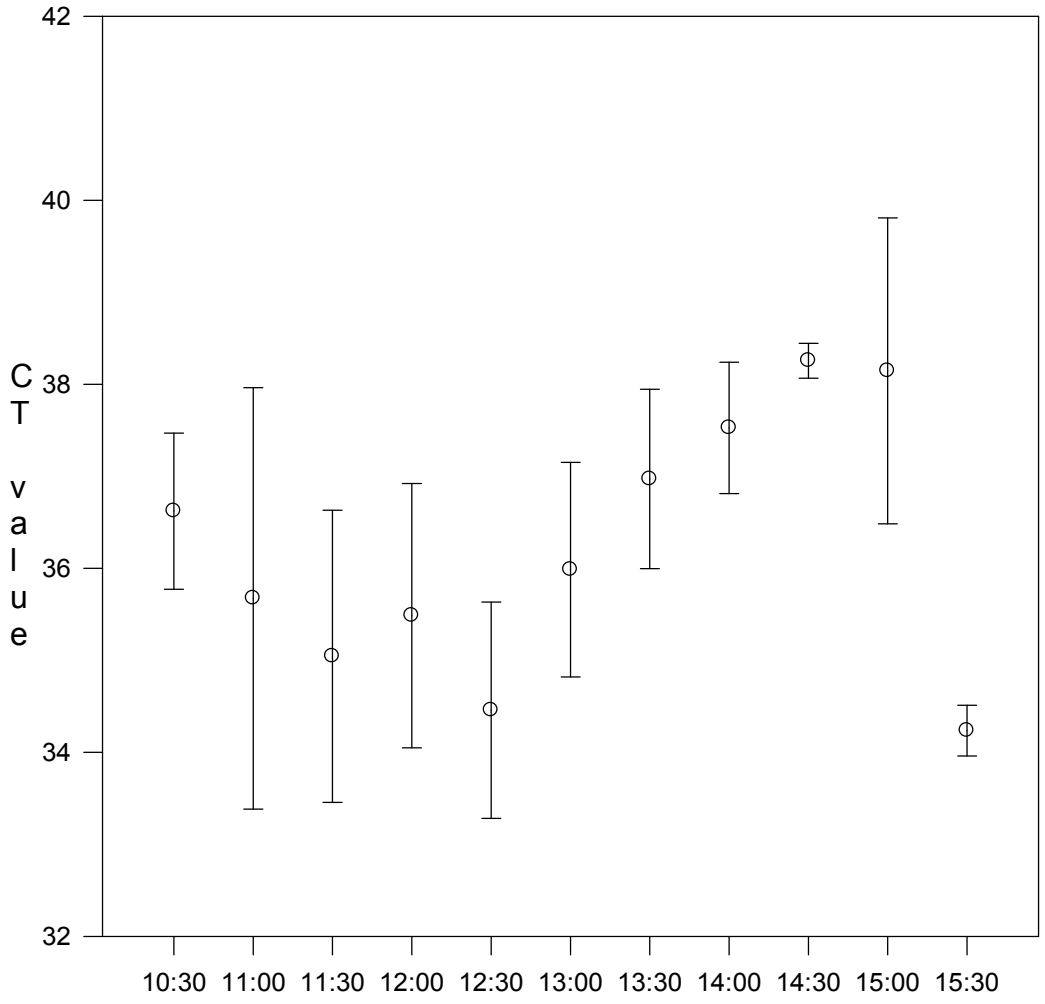
threshold as there was a 37% incidence of ceratomyxosis (with dual *Parvicapsula* infection) observed in salmon held past 10dpe.

We suggest actinospores, and not myxospores, are the source of parasite DNA in the collected water samples as it is quite rare to observe myxospores in juvenile Klamath R. salmonids during the spring and summer. In addition, the detection of parasites in the sentinel salmon indicates that actinospores were present in concentrations high enough to produce infection. Myxospores have been detected in adult salmon sampled at Iron Gate Hatchery during the late fall (2005 unpubl. data, CA-NV FHC) and thus we believe this is the time when these spores are likely to be found in abundance in the water column. This hypothesis could be altered through an examination of water samples collected during these periods. The contribution of other salmonid species and life stages to the total myxospore input to the river is an area for future study.

Parvicapsula was detected in 29 of 35 water samples with the CT range of positive samples ranging from 33.4 – 39.3. A 10:00 sample was removed from the dataset as it had a CT of 12.0 (demonstrating higher DNA content than the highest level Pm plasmid DNA standard of 1.25×10^{7} molecules). This sample was likely cross-contaminated with a Pm plasmid spiked fish tissue sample extracted in an adjacent well. The coefficient of variation for positive CT values (<40) among replicate water samples collected during the same 30 min period ranged from 0.8 – 6.4%. Presently, we do not have data to convert the CT score to actinospore / L but estimate that a CT of approximately 30 reflects 1 actinospore (S. Hallett, personal comm.). Similarly, work in our laboratory found a single, un-extracted *P. minibicornis* actinospore collected from *M. speciosa* worms to produce a CT value of 30.4. This CT value is also equivalent to the 1250 molecules of Pm template plasmid positive control value.

Unlike the *C. shasta* actinospore profile, there appeared to be a slight decrease in *P. minibicornis* DNA (higher CT values indicates lower DNA content of sample) during the afternoon and no detections occurred in 5 of 7 water samples collected at the beginning (10:00) and 30 min later (Fig 7). If we assume that the positive CT values (and therefore remove the first 30 min of exposure from each group) reflect approximately 1 *P. minibicornis* actinospore / L, then the estimated number of actinospores that passed through a cage of fish over 3h was 57,000 (22,800L /h x 2.5h) and 125,400 (22,800L/h x 5.5h) through the 6h cages. Comparing the incidence of infection for both parasites in 3h salmon (84% for *P. minibicornis* and 27% for *C. shasta*) to the estimated actinospore exposure (57,000 for *P. minibicornis* and 99,181 spores for *C. shasta*), suggests that *Parvicapsula* actinospores may be more efficient at invading the fish host than *C.shasta*. Similar results of salmon incurring a higher incidence of infection from *P. minibicornis* than *C. shasta* when exposed to small volumes of Klamath River that had been aged for up to 7 days were reported by Foott et al. (in press).

Figure 7. Mean CT values for *Parvicapsula* DNA content in positive water samples collected at 30 min intervals (standard error bars).



It appears that the limited *C. shasta* infections were successfully contained in over half of the infected salmon in both exposure groups. Given the cumulative effect of even low actinospore concentrations in flowing systems on fish infection, the current water filtration / QPCR method appears to be sensitive enough to predict myxozoan parasite infection in juvenile salmon rearing in the Klamath River. Future research with the water filtration / QPCR method could examine the efficiency of actinospore infection at given flow rates. Another area of study is to develop methods to reduce the effect of actinospore variability per volume of water on actinospore enumeration.

Acknowledgements:
Partial funding for this work came from the Klamath River Basin Conservation Area Restoration Program (81230-1311-1006-8K02). We thank Trinity River Hatchery for Chinook salmon, and Paul Zedonis and Jerri Bartholomew for their comments on the manuscript.

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Appendix 1 **Reviewer 1**

pg4 methods –"Do you know what the temp range was at that site?"

Temp range during exposure given

pg4 "Reiterating what I say below – flow confuses me but doesn't it have to be cubic ft?"

flow changed to velocity

pg5 "Do you have any reason for this arbitrary scoring, a ref from previous paper perhaps? Did you score uninfected fish 0?"

Score criteria was arbitrary, added 0 score criteria

Methods "I get a little confused if you are keeping the 2 samples separate or combining them. If the latter, you could make it more clear by saying the apparatus was cleaned between 1L samples"

Language inserted

Table 1 "This might be easier to interpret if you listed POI first, as in the legend, then had a separate headings for inf severity e.g.:
Cs POI
Cs Inf. Sev."

Done

Results – "An additional reason for actinospores being the source of DNA in the water samples would be the fact that the fish became infected, which is evidence of presence of that stage. As you say above (see Paul's comment) that myxospores were present, do you still think this is rare or is it that they are in low numbers?" Or by examination of the contribution by other species?

Language inserted

Although in the graph it looks like 24% had fairly severe lesions?

Even the 6h group had a majority of fish that did not develop ceratomyxosis. Over a third of the group held past 10dpe did get sick and we consider this exposure level significant to the population.

Actinospore graphs - Is this the mean for all 4 cages? It actually looks pretty tight except for that afternoon sample set. Would it be worth showing data from all 4 cages? Also, this graph and the one below need a Y-axis title.

Looks like the variability is higher here, but I guess that is a reflection of reporting by Ct value rather than estimated spores. Need Y-axis title

Y-axis title inserted

Reviewer #2

Histology scoring and Cs scores in table – " Not clearly defined according to your methodology. " **additional language on criteria added**

Results linking Pm standard CT values – "Confusing with statement below about the standard of 1 spore representing a CT value of 30.4"

Language dropped