LONG-TERM FISH DISEASE MONITORING PROGRAM IN THE LOWER KLAMATH RIVER

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Funding: Bureau of Reclamation

FIGURE 1.1. Map of sites for exposures of sentinel fish (except Tully Creek) and collection of water samples in 2009 with site abbreviations and river kilometers (Rkm).

Objective 1:

Task 1.1 SELECTION OF INDEX SITES
Young’s Bar was substituted for Tully Creek in 2008. Fish were not held at Tully Creek in 2009 but access was possible for water sampling; remaining index sites consistent with previous year. The Williamson River site is located at the Nature Conservancy and the Klamathon site is at the Klamath River Country Estates near Klamathon Bridge.
Task 1.2 SENTINEL FISH EXPOSURES
Sentinel exposures in 2009 were conducted following the same protocol used in previous years to determine:

1. How infection levels this year compare with levels in previous years.
2. If the distribution of the parasite has changed.
3. The relative susceptibility of Klamath River Chinook and coho salmon.
4. The effects of post-exposure water temperature on disease progress in Chinook and coho salmon.
5. The relationship between parasite numbers measured in water samples and biological effects in the different fish species.

Methods
In 2009, exposures were conducted for 72 hr each during the months of April, May, June, September and October. The April 17-20, September 11-14 and October 27-30 exposures occurred in the Klamath River near Beaver Creek only; this is the first year exposures were conducted in April and October. Exposures during May 12-15 and June 16-19 occurred at 6 locations in the upper and lower river (Figure 1.1).

A known C. shasta susceptible rainbow trout stock from Roaring River Hatchery (Oregon Department of Fish and Wildlife) and Klamath River fall Chinook and coho salmon from Iron Gate Hatchery (California Department of Fish and Game) were held at all sites with the exception that coho salmon were not exposed at Keno Eddy in the upper Klamath River. Generally, 40 fish of each stock were held in live cages at each site. After exposure, each group of fish was brought to the Salmon Disease Laboratory (SDL), Corvallis, Oregon, held in well water at water temperatures similar to river temperatures occurring during the 72 hr exposure and observed for loss and disease signs for 90 or more days. The fish were given preventative treatments for external parasites and columnaris disease. In April, the sentinel fish were approximately 0.5-1.5 g size, May 1.5-2.5 g, June 2-9 g, September 8-35 g, and October 10-58 g. The post-exposure rearing temperature used in April was 13°C, May was 16°C, and June, September and October were 18°C. In October, post-exposure rearing temperature was higher than the exposure river temperature of 12°C. All fish that died were evaluated for C. shasta infection by microscopic examination of a sample from the lower intestine for the presence of myxospores. A subsample of dying fish from each group was necropsied for other parasite and bacterial infections. PCR testing for C. shasta was done on those fish that were negative by microscopic examination.

To study the effect of water temperature on C. shasta infections in the Chinook and coho juveniles, during the May, June, September and October exposures in the Klamath River near Beaver Creek, 80 fish of each stock were exposed and then each divided into 2 tanks upon return to the SDL, one receiving water at 13°C and the other at 16°C (May) or 18°C for the June, September, and October exposures. These fish were reared for 90 days and mortality examined as described above.
Results & Discussion

Average water temperatures during the 72 hr exposures are shown in Table 1.1. For June, the water temperature near Beaver Creek was about 1-2 °C warmer in 2009 than in June 2008.

TABLE 1.1. Average Klamath River water temperatures (°C) at sentinel sites during the 72-hour fish exposures in 2009.

<table>
<thead>
<tr>
<th>Site</th>
<th>April 17-20</th>
<th>May 12-15</th>
<th>June 16-19</th>
<th>September 11-14</th>
<th>October 27-30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower Williamson Rv</td>
<td>16</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Keno Eddy</td>
<td>14</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Klamathon</td>
<td>14</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Near Beaver Creek</td>
<td>12</td>
<td>15</td>
<td>21</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>Seiad Valley</td>
<td>14</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orleans</td>
<td>12</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results of the sentinel exposures in April, May, June, September and October are listed in Figures 1.2-8. The values of percent loss represent fish that had died and were found to have spores of C. shasta or were PCR positive for this parasite.

Susceptible rainbow trout exposures—The temporal occurrence of C. shasta infections in the known susceptible rainbow trout stock from Oregon after exposure in the Klamath River above Beaver Creek confluence is shown in Figure 1.2. This location is part of the “infectious zone” for C. shasta based on previous year’s studies. In 2009, an exposure as early as April 17-20 resulted in a 97% fatal infection of C. shasta and 100% loss with C. shasta for May, June and September. However for the October 27-30th exposure, only 7% of the rainbow trout died from C. shasta, indicating the abundance of this parasite in the river had decreased dramatically in the fall.

FIGURE 1.2. Temporal occurrence of C. shasta infection in known susceptible juvenile rainbow trout exposed in the Klamath River near Beaver Creek for 72 hr each month.
The May and June sentinel exposures of the susceptible rainbow trout were conducted at all 6 sentinel sites. The results (Figure 1.3) demonstrate the wide occurrence of *C. shasta* infection with high severity at most locations. Similar results were observed in previous years, with high loss of the rainbow trout exposed in the lower Williamson River and in the Klamath River from near Beaver Creek and downriver to Orleans. The lowest loss occurred at Keno Eddy, located above JC Boyle Dam, where only 30% died with *C. shasta* in May, increasing to 75% in June. Previously at this site mortality had been even lower: 7% in May and 0% in June 2008; 30% in May and 21% in June 2007. Below Iron Gate Dam near Klamathon Bridge the rainbow trout losses ranged from 60-88%.

**FIGURE 1.3.** Percent loss of susceptible rainbow trout with *C. shasta* infections exposed at six sentinel sites in the Klamath River basin for 72 hours each in May and June 2009. See Figure 1.1 for site abbreviations.
April 2009 exposures—The April 17-20 exposure conducted near Beaver Creek resulted in high loss of the susceptible rainbow trout and 16.7% loss of the Iron Gate Hatchery (IGH) fall Chinook juveniles (Figure 1.4). No loss due to C. shasta was observed in the IGH coho. This exposure demonstrated that by mid-April there was already a lethal level of C. shasta affecting the fall Chinook juveniles.

**FIGURE 1.4.** Percent loss with *C. shasta* infection of rainbow trout, IGH fall Chinook and coho salmon exposed April 17-20 (72 hr) in the Klamath River near Beaver Creek and held for 90+ days after exposure.

May 2009 exposure—**Figure 1.5** shows the *C. shasta* percent mortality for juvenile rainbow trout, IGH fall Chinook and coho salmon after exposure at 6 sites on May 12-15, 2009. Results for the rainbow trout have already been shown in Figures 1.2 and 1.3. No infections of *C. shasta* were detected in IGH fall Chinook exposed in the lower Williamson River, at Keno Eddy, or near Klamathon Bridge below Iron Gate Dam. High loss from *C. shasta* occurred near Beaver Creek (78%), Seiad Valley (44%) and low loss at Orleans (5%). So by mid-May, the IGH fall Chinook exposed for 72 hr were dying at a high rate from *C. shasta*. Juvenile coho did not die from *C. shasta* in the lower Williamson River and were not tested at Keno Eddy. Near Klamathon Bridge, 10% of the coho died from *C. shasta*, 27% at Beaver Creek, 13% at Seiad Valley and 2% at Orleans. The loss was lower at Orleans in both species, probably a result of dilution effects from the tributaries entering below Beaver Creek.
FIGURE 1.5. Percent loss with *C. shasta* infection of rainbow trout, IGH fall Chinook and coho salmon exposed May 12-15, 2009 at six sentinel sites in the Klamath River basin and held for 90+ days post-exposure. See Figure 1.1 for site abbreviations.

June 2009 exposure-The June 16-19 sentinel exposure results (Figure 1.6) show similar results to May but with some increase in mortality due to *C. shasta* at most sites. In the upper river, no IGH Chinook died after exposure in the lower Williamson River or Keno Eddy. One coho salmon juvenile died with a *C. shasta* infection after exposure in the lower Williamson River. Above Klamathon Bridge at the Klamath River Country Estates site only 4% of the Chinook died and 2.5% of the coho salmon. High loss of IGH Chinook occurred near Beaver Creek (87%), Seiad Valley (75%) but much lower at Orleans (13.5%). Loss of the coho also was higher than May at Beaver Creek (58%), Seiad Valley (41%) and lower at Orleans (8%). So, 2009 was similar to 2008 in that high losses occurred with *C. shasta* infections in the “infectious zone” around Beaver Creek and Seiad Valley.
FIGURE 1.6. Percent loss with *C. shasta* infection of rainbow trout, IGH fall Chinook and coho salmon exposed June 16-19, 2009 at six sentinel sites in the Klamath River basin and held for 90+ days post-exposure. See Figure 1.1 for site abbreviations.

September and October 2009 exposures-The percent *C. shasta* mortality for the September and October exposures near Beaver Creek is shown in Figure 1.7. No IGH fall Chinook died after exposure in either September or October. For the coho juveniles, 7% died with *C. shasta* infections in September but no infections were detected in October. The infectivity of *C. shasta* near Beaver Creek had decreased dramatically in the fall.

FIGURE 1.7. Percent loss with *C. shasta* infection of rainbow trout, IGH fall Chinook and coho salmon exposed September 11-14 or October 27-30, 2009 in the Klamath River near Beaver Creek and held for 90+ days after exposure.
Effect of post-exposure rearing water temperature on infections of C. shasta in Klamath River fish stocks - Results of the comparison of percent loss of Chinook and coho with C. shasta infection when exposed near Beaver Creek in May, June, September and October and held at the laboratory at 2 different water temperatures are shown in Figure 1.8. The known susceptible rainbow trout stock consistently suffered high loss even at the lower temperature of 13°C for all months except October. For the Chinook salmon, the loss was high at both temperatures in May and June. For the coho salmon, loss was similar in May but showed a large difference in June with 5% loss at 13°C compared with 58% at 18°C. In 2009, higher water temperature post-exposure had a greater effect on infection in the juvenile coho.

FIGURE 1.8. Comparison of post-exposure water temperature on percent C. shasta loss for groups of rainbow trout, IGH fall Chinook and coho salmon exposed for 72 hr near Beaver Creek in May, June, September or October 2009 and then divided in half and one portion held at 13°C and the other at 16 or 18°C for 90 days.

Comparison of sentinel results for the IGH Chinook and coho salmon exposed near Beaver Creek in 2007, 2008, and 2009 - From 2007 to 2009 there appears to be shift toward more severe effects of C. shasta on the Chinook when compared to coho (Figure 1.9). In 2007 the loss of juvenile coho was very high while the Chinook loss was lower. In 2008 both species suffered high loss in May and June. In 2009, the greatest loss occurred in May and June in the fall Chinook. In general however, losses for both species due to C. shasta has been high in May and June for all 3 years.
FIGURE 1.9. Comparison of percent loss with C. shasta infection of juvenile IGH fall Chinook and coho salmon exposed in the Klamath River near Beaver Creek in 2007, 2008 and 2009 sentinel studies.

Ceratomyxa shasta eye infections in sentinel juvenile coho salmon- C. shasta infections of the eye (Figure 1.10) were observed in some of the juvenile coho salmon, with loss similar to what has occurred in previous years. Giemsa stains of histological sections of the affected eye revealed presence of many myxospores (Figure 1.11). These infections were evident in fish held for greater than 45 days post-exposure. In all cases, where PCR tests were done for C. shasta on gut tissue from fish with eye infections, the gut samples were negative for C. shasta. Infections of the eye were observed in coho from the May, June and September exposures at Beaver Creek, Seiad Valley and Orleans. Figure 1.12 shows the percent of coho that had C. shasta infections of the intestine and eye for June. Greater than 20% of the coho exposed near Beaver Creek or Seiad Valley in June had C. shasta infections of the eye.
FIGURE 1.10. Juvenile coho with *C. shasta* infection of the eye.

FIGURE 1.11. Giemsa stained histological section of a *C. shasta*-infected coho eye showing the presence of many myxospores.
Summary of the sentinel results-

- *Ceratomyxa shasta* infections were detected in all months tested in 2009, i.e. April, May, June, September and October. Near Beaver Creek, as early as mid April, infections of the fall Chinook were detected (~17%). Similar to 2008, high loss occurred in Chinook exposed near Beaver Creek in May (78%) and June (87%). Coho exposed near Beaver Creek suffered 27% loss in May and 58% loss in June. No Chinook died in the fall exposures and only 7% of the coho in September. By late October the susceptible rainbow trout suffered only a 7% loss from *C. shasta*.

- *C. shasta* infection of juvenile Chinook and coho salmon occurred below Iron Gate Dam and was most severe in the “infectious zone” near Beaver Creek and Seiad Valley, similar to sentinel results in previous years.

- The lower Williamson River in the upper Klamath Basin was tested for *C. shasta* infection in May and June. The rainbow trout died at nearly 100%, but no infections were detected in fall Chinook salmon. No coho died of infection in May and only 1 fish was found infected with *C. shasta* after the June exposure.

- When comparing sentinel study results for 2007, 2008 and 2009 for fish exposed near Beaver Creek, in 2007 coho loss in May and June was much higher than for the Chinook. In 2008, both species suffered very high losses after exposure. In 2009, the loss of Chinook was higher than coho from *C. shasta*.

- In the comparison of effect of the post-exposure water temperature on loss from *C. shasta*, coho salmon suffer higher loss when held 18°C versus 13°C.

- Similar to previous years sentinel studies, some of the coho juveniles developed eye infections of *C. shasta* without also having detectable intestinal infections.
Task 1.3 WATER SAMPLE COLLECTION AND PARASITE DENSITY DETERMINATION

Water samples were taken to determine:

1. The spatial and temporal distribution of *Ceratomyxa shasta* in the Klamath River.
2. How abundance this year compares with levels in previous years.
3. If the distribution of the parasite has changed.
4. The relationship between parasite numbers measured in water samples and biological effects in the different fish species.

**Methods**

Water samples were collected from 5 mainstem sites and 3 tributaries at regular intervals from March through September in 2009, as follows.

**TABLE 1.2**

<table>
<thead>
<tr>
<th>SITE</th>
<th>FREQUENCY OF COLLECTION</th>
<th>METHOD OF COLLECTION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mainstem</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Klamathon (KKB)</td>
<td>once a week</td>
<td>ISCO (automatic sampler)</td>
</tr>
<tr>
<td>Beaver Creek (KBC)</td>
<td>twice a week</td>
<td>ISCO</td>
</tr>
<tr>
<td>Seiad Valley (KSV)</td>
<td>twice a week</td>
<td>ISCO</td>
</tr>
<tr>
<td>Orleans (KOR)</td>
<td>once a week</td>
<td>ISCO</td>
</tr>
<tr>
<td>Tully Creek (KTC)</td>
<td>once a week</td>
<td>ISCO</td>
</tr>
<tr>
<td><strong>Tributaries</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bogus Creek (BOG)</td>
<td>once every two weeks</td>
<td>ISCO</td>
</tr>
<tr>
<td>Shasta River (SHR)</td>
<td>once every two weeks</td>
<td>grab sample (manual collection)</td>
</tr>
<tr>
<td>Scott River (SCT)</td>
<td>once every two weeks</td>
<td>grab sample</td>
</tr>
</tbody>
</table>

Water was sampled either manually (grab sample) or using an automatic sampler (ISCO). For grab samples, 4 x 1 L was collected from the river at 1 time point. The ISCOs were set for 24 h sampling beginning 8 am Sunday and Wednesday; 1 L was collected from the river every 2 hr for 24 hr, then the total sample mixed manually and 4 x 1 L samples taken and filtered. The samples were chilled until filtering, within 24 hr of collection.

Water samples were also taken at the 6 sentinel fish sites during the exposures in May, June; only at Beaver Creek in April, September and October. 4 x 1 L samples were taken at the start and end of exposure.

In 2009, the DNA extraction procedure was modified; the filter paper was dissolved in a series of acetone/ethanol washes prior to kit digestion. DNA was then extracted from 3 of the 4 frozen 1 L samples collected at each site. An inhibition test was performed on 1 sample from each site then all 3 samples were assessed for presence of *C. shasta*. Each sample was run in duplicate and sample pairs with values having a standard deviation greater than 1 were rerun. Positive and negative controls were included in each QPCR run. Samples that were undetected were assigned a Cq value of 42 and
included in the average. Each data point on a graph represents the average of the 3 water samplings at that time point; error bars display the standard deviation of those 3 samples. Following the guidelines of Bustin et al. 2009, we now use the term ‘quantification cycle’ (Cq) instead of ‘cycle threshold’ (Ct).

Results & Discussion
The modified sample processing protocol was more sensitive and generated Cq values 1.6 less than the method used in 2008; it also reduced inhibition. The temporal and spatial distribution of the parasite differed this year. Abundance peaked earlier, with levels above 10 spores/L at 4 of 5 mainstem sites in early May (Figure 1.13). At the end of June, abundance dropped dramatically in the mainstem to 1 spore/L or less at all sites. Spore levels increase again in July but are now predominantly below 10 spores/L. Traditionally, Beaver Creek experiences the highest levels of C. shasta, however, this year 22% (average) of samples collected from Seiad Valley surpassed these levels.

FIGURE 1.13. *Ceratomyxa shasta* abundance at five mainstem sites in 2009. Each data point is the average Cq of 3 x 1L water samples. A lower Cq value indicates more parasite is present. Site abbreviations are explained in Table 1 above.
Only very low amounts of \textit{C. shasta} DNA (<1 spore/L) were detected in the tributaries (Bogus Creek, Scott and Shasta Rivers); the most DNA detected was Cq 36.7 in the Scott River in July (Figure 1.14).

**FIGURE 1.14.** \textit{Ceratomyxa shasta} abundance at three tributary sites in 2009. Each data point is the average Cq of 3 x 1L water samples. A lower Cq value indicates more parasite is present. Site abbreviations are explained in Table 1.2 above.

Higher levels of \textit{C. shasta} were detected in May than June and yet more fish (of all 3 species assessed) died in June than in May; the difference is most pronounced at Orleans (Figures 1.16, 1.17). Ambient river temperature was higher in June (up to 8 C at Orleans; Table 1.1) and fish were held at 18 versus 16 C in the lab, however, based on the temperature comparison study, this difference seems insufficient to fully explain the differences observed in mortality. Flow also varied between exposures (Figure 1.19); discharge from Iron Gate Dam was ~ 100 cfs greater in June than May and thus the actual exposure at Klamathon and Beaver Creek was likely to be higher; however, flow at Seiad Valley and Orleans was ~1000 – 5000 cfs lower in June than May. Interpretation of the correlation between sentinel fish mortality and parasite abundance in water samples may be hampered by differences in methodology; water samples (3) are obtained at only 2 time points (beginning and end of the exposure) to represent the levels of parasite present during the 3 day exposure, whereas the fish remain in the water for the 3 days.
Parasite levels do not correlate clearly with mortality this year and disease thresholds are not as obvious (Figures 1.15-1.18). Parasite levels less than 1 spore/L again this year proved lethal for susceptible non-native rainbow trout, including Keno Eddy this year and more than 10 spores/L caused 90-100% mortality. But for all species, the level of parasite measured in water samples was not consistently proportional to percent mortality attributable to *C. shasta*. Consistent with previous years, at Beaver Creek more than 10 spores/L were detected in April, May and June and mortalities occurred in Chinook, and less than 1 spore/L was detected in September and there was no mortality (Figures 1.15 – 1.18). In contrast, in June, less than 1 spore/L was detected at Orleans and yet Chinook suffered ceratomyxosis and there were 10-fold fewer waterborne parasites at Seiad Valley than May, yet mortality was higher (Figure 1.17). These disparities may be attributable to the notable increase in river water temperature between subsequent exposure periods (Table 1.1). At Klamathon Bridge, less than 1 spore/L induced low level mortality (<10%) in both Chinook and coho, however there is considerable variability in spore abundance at this site (Figures 1.16, 1.17). At Orleans, flow was considerably less this year (~3500 cfs versus 6000 cfs in 2008) which likely improved transmission of the parasite (lower velocity provides increased opportunity for spores to attach to their fish host) and may explain increased mortality despite decreased concentration (Figure 1.20). Multiple strains of *C. shasta* have been identified in the Klamath River. Inconsistencies between fish and water data warrant further study to determine if the strain composition explains the incongruent datasets observed this year.

**FIGURE 1.15.** April sentinel water data with corresponding sentinel fish data (% mortality due to *Ceratomyxa shasta*). The water data point is the average of 3 x 1 L water samples taken at the beginning of the 72 hour exposure. Rbt = rainbow trout, Chf = fall Chinook salmon. A lower Cq value indicates more parasite is present. Site abbreviations are explained in Table 1 above; KED, Keno Eddy, WMR NC, Williamson River Nature Conservatory.
FIGURE 1.16. May sentinel water data with corresponding sentinel fish data (% mortality due to Ceratomyxa shasta). Each water data point is the average of 3 x 1 L water samples taken either at the beginning or end of the 72 hour exposure. Rbt = rainbow trout, Chf = fall Chinook salmon. A lower Cq value indicates more parasite is present. Site abbreviations are explained in Table 1 above; KED, Keno Eddy, WMR NC, Williamson River Nature Conservatory.

FIGURE 1.17. June sentinel water data with corresponding sentinel fish data (% mortality due to Ceratomyxa shasta). Each water data point is the average of 3 x 1 L water samples taken at the beginning or end of the 72 hour exposure. Rbt = rainbow trout, Chf = fall Chinook salmon. A lower Cq value indicates more parasite is present. Site abbreviations are explained in Table 1 above; KED, Keno Eddy, WMR NC, Williamson River Nature Conservatory.
FIGURE 1.18. September and October sentinel water data with corresponding sentinel fish data (% mortality due to *Ceratomyxa shasta*) for Beaver Creek. Each water data point is the average of 3 x 1 L water samples taken at the beginning or end of the 72 hour exposure. Rbt = rainbow trout, Chf = fall Chinook salmon. A lower Cq value indicates more parasite is present.

FIGURE 1.19. Daily mean discharge from Iron Gate Dam, Seiad Valley and Orleans from March through September, 2009.
Summary of water sample data:

- The temporal and spatial distribution of the parasite differed this year.
- Abundance was above 1 spore/L at all 5 mainstem sites at the beginning of sampling in April but only 2 of the 5 sites had levels above 1 spore/L at the end of the sampling period in September.
- Abundance peaked earlier in 2009, with levels above 10 spores/L at 4 of 5 mainstem sites in early May.
- In previous years, the highest levels of *C. shasta* are detected at Beaver Creek, however, this year 22% (average) of samples collected from Seiad Valley surpassed these levels.
- Only very low amounts of *C. shasta* DNA (<1 spore/L) were detected in the tributaries (Bogus Creek, Scott and Shasta Rivers).
- Parasite levels do not correlate as clearly with mortality this year and disease thresholds are not as obvious. However, a threshold of 10 actinospores/liter still appears to be an appropriate correlative for high mortality in Klamath River salmon.
Task 1.4 POLYCHAETE ABUNDANCE AND INFECTION PREVALENCE
Polychaete samples were collected at three sites: Up river from the I-5 Bridge ~.5 Rkm, at the Tree of Heaven campground, and down river from the Seiad Creek confluence (~5 Rkm). At each site in May and June, three separate samples were collected using a Hess sampler and preserved in alcohol. In the lab, 3 random subsamples from each sample were examined and polychaetes separated from their habitat. Estimates of mean field density are shown in Figure 1.21. Prevalence of infection is yet to be determined.

FIGURE 1.21. Mean field density of polychaetes at two Klamath River sites in May (orange bar) and three sites in June (blue bars), 2009. Three samples were collected at each site.

Task 1.5 PROJECT COORDINATION
In November, 2009, a meeting was held in Corvallis, OR with members of the OSU, USFWS and HSU laboratories and Yurok tribe; a Karuk tribe representative joined us via conference call. Attendees presented a summary of research conducted in 2009 and that planned for 2010. There was discussion of protocols, outstanding research questions and data gaps and the 2007 management report was revisited. A follow-up meeting was held at HSU with representatives from each research group in March, 2010, immediately preceding the Klamath River Fish Health Workshop.

Objective 2. DEVELOPMENT OF A MODEL TO ADDRESS CRITICAL UNCERTAINTIES IN PARASITE TRANSMISSION IN RELATION TO ENVIRONMENTAL PARAMETERS SUCH AS WATER TEMPERATURE AND FLOW.

Task 2.1 IDENTIFICATION OF CRITICAL UNCERTAINTIES IN OUR UNDERSTANDING OF PARASITE TRANSMISSION
Key biological questions were identified through discussions at a number of meetings and many of these are being addressed either as part of this study, or by other funded research in our laboratory and in other laboratories. For example, infection prevalence and average myxospore load in adult fish was investigated by the USFWS in collaboration with HSU, see http://www.fws.gov/canvfhc/showReport.aspx?id=102; and the infectious dose for juvenile fish and the resulting myxospore load was determined at OSU, see below.
Task 2.2. EFFECTS OF WATER TEMPERATURE
Data for comparison of the effects of temperature (18°C versus 13°C) on coho and Chinook salmon is presented under the sentinel studies portion of this report. When held at the higher temperature following exposure the coho salmon suffered higher loss, while mortality was similar for Chinook salmon held at either temperature. This has been observed in previous years and suggests that coho are more sensitive to the effects of temperature.

In 2008 a study was conducted to assess the effects of four different temperatures (13, 15, 18 and 21 °C) on survival of Chinook and coho salmon and stocks of steelhead from Iron Gate and Trinity River Hatcheries. Because of the very high parasite exposure that occurred in June 2008, mortality was too high to see a clear difference as a result of temperature. We had planned to repeat that study this year, but continued analysis of the data did show a clear trend of increasing mortality with temperature and given that parasite abundance was equally high in 2009 we did not feel we would be able to see any differences in these data by repeating the study.

Task 2.3 MODELING DISEASE TRANSMISSION IN THE KLAMATH RIVER
Adam Ray, under the supervision of Dr. Phillipe Rossignol, has developed a mathematical model of disease transmission to examine the basic host-parasite interactions necessary for *C. shasta* to complete its life cycle (Figure 2.1). In 2008, field experiments were conducted to quantify the parameters related to the infection of and mortality from *C. shasta* in the salmonid host, as previously reported. Further analysis of these data led to the identification of a threshold for *C. shasta*-induced mortality for Iron Gate Hatchery Chinook salmon. This threshold provides a target for management that does not require the complete eradication of the parasite. Also, water samples were collected from aquaria that contained infected juvenile Chinook to determine if and when myxospores are released from infected individuals. During this past field season, additional experiments were conducted to directly quantify the transmission rate of actinospores to the salmonid host. The transmission parameter had been measured previously, indirectly from the field exposures conducted in 2008, but was deemed too inaccurate to justify using as part of the model. Further transmission experiments are scheduled for the 2010 field season, to provide a wider range of values for this parameter and to further explore environmental interactions.
FIGURE 2.1. Epidemiological model of ceratomyxosis developed for the Klamath River. The grayed-in areas are parameters that have valid estimations.

Threshold identification
From field exposures conducted in the summer of 2008, we quantified a range of values for a portion of the model representing the actinospore dose (A), the resulting proportion of infected salmon (S) and the parasite induced mortality (δ). IGH Chinook salmon were held in live cages in the Klamath River for different durations to represent various doses of actinospores in both June and September. The mortality rate ranged from 98 – 0 % with a corresponding range of actinospore doses of $6.12 - 4.4 \times 10^6$. Further analysis of these data led to the identification of a mortality threshold of $5.5 - 9.9 \times 10^5$ actinospores per fish (Figure 2.2). The development of the model and quantification of this threshold has been submitted for publication in *Diseases of Aquatic Organisms*. 
Juvenile myxospore production

Chinook salmon exposed for 72 hours in the Klamath River were returned to the Salmon Disease Lab (SDL) at Oregon State University and observed for morbidity and mortality from *C. shasta*. Medicated feed and other chemical treatments were administered for the first 7 days post exposure (DPE) to reduce the effect of any other environmental pathogen that may have been contracted during the exposure period. After the 7 days of treatment, water samples were collected from the out-flow of each of the 4 aquaria every other day until all the fish succumbed to the infection (~26 days DPE). These samples were then analyzed by qPCR assay to quantify the amount of *C. shasta* DNA, if any, was present. Very high levels (~20 Cq) of the parasite were detected in the first sampling period, which is surprising as we did not realize the parasite could progress so quickly through the salmonid host (Figure 2.3). As more fish succumbed to the infection, the amount of waterborne parasite DNA decreased. While these data are very interesting, they have yet to be incorporated into the model as the current theory is that returning adult salmon are the main contributors of myxospores into the Klamath that perpetuate the life cycle.
FIGURE 2.3. Timing and relative quantity of myxospores released from juvenile IGH Chinook salmon exposed in the Klamath River for 72 hours in June 2008. DPE = days post exposure.

Transmission study

Methods
In summer 2009, field exposures of Chinook salmon were conducted in the Klamath River to determine the transmission rate of actinospores to the salmonid host. This experiment was developed to provide data which can be inserted into the epidemiological model for ceratomyxosis. In June, IGH Fall Chinook were exposed in the main stem Klamath River, up river of the confluence of Beaver Creek. Fish were held in 4 live cages for 3 and 6 hour durations. The water velocity was recorded for each of the 8 live cages, every 30 minutes to estimate the total volume of water experienced by the fish. Water samples were collected every half hour and pooled into hourly samples for the duration of the experiment. These samples were analyzed by qPCR to quantify the actinospore dose encountered by the Chinook. After each exposure period, 10 fish were euthanized and their gills were removed. Half of the gill was placed into EtOH for qPCR analysis and the other half was placed in Davidson’s fixative to be examined by histology. After the exposure period, the remaining fish were transferred to the SDL and monitored for signs of infection, for up to 90 days post exposure. Sick and moribund fish were visually examined for the myxospore stage. If visually negative, a piece of intestinal tissue was collected and assayed by PCR. The qPCR measurements of the gills will be compared to those recorded from the water samples to estimate the transmission rate of the actinospore to the salmon host.
Results
A rough approximation of the actinospores measured from the water and gill samples and their corresponding transmission rates are presented in Table 2.1. With just a doubling of exposure from 3 to 6 hours we observed a 10-fold increase in parasite DNA on the gills. These results need to be further validated and standardized, as this difference is most likely due to rapid proliferation of the parasite within the host tissue. The gill tissue saved for histology has not yet been analyzed and may provide some insight on the rate of parasite proliferation.

Table 2.1. Estimate of the transmission rate of the actinospore to the salmonid host from field exposures conducted in June 2009.

<table>
<thead>
<tr>
<th></th>
<th>3 Hour Exposure</th>
<th>6 Hour Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-river actinospore dose</td>
<td>1.59 X 10^6</td>
<td>5.31 X 10^6</td>
</tr>
<tr>
<td>C. shasta on the whole gill</td>
<td>2.33 X 10^3</td>
<td>2.09 X 10^4</td>
</tr>
<tr>
<td>Transmission rate (η₁)</td>
<td>1.45 X 10^3</td>
<td>4.05 X 10^-3</td>
</tr>
</tbody>
</table>

Objective 3. DEVELOPMENT OF A QPCR ASSAY TO DIFFERENTIATE BETWEEN THE DIFFERENT ITS1 STRAINS OF CERATOMYXA SHASTA.

Task 3.1. DEVELOPMENT OF A STRAIN-SPECIFIC QPCR ASSAY FOR C. SHASTA.
This task is still underway as it required exploring more technologies than we anticipated due to the complicated nature of our samples and the information we wish to obtain from each. We strove for a method that would identify and quantify (at least ratio, preferably absolute amount) all genotypes present in a sample (fish, polychaete or water) and allow notification of novel genotypes. Sanger sequencing currently achieves this for us, but the procedure is labor intensive, expensive and time consuming; we wished to reduce all these.

The first technology we pursued was ‘HRM’ which combined qPCR with high resolution melting curve analysis, and all occurred in one machine. Following manufacturer’s guidelines, we designed pairs of C. shasta-consensus primers that flanked the ITS1 variable region and assessed samples with different combinations of genotypes (0-III) present in different ratios (0-100%). In preliminary tests, the main genotypes could be distinguished with this approach, being represented by a characteristic melting curve (Figure 3.1), but this was not consistent across all samples. Furthermore, several genotypes contain multiple sub-genotypes and these were not as readily identifiable. Additionally, it was difficult to characterize samples containing different proportions of genotypes.
FIGURE 3.1. *Ceratomyxa shasta* ITS1 genotype I (left, blue curve), which preferentially infects Chinook salmon, can be readily distinguished in an HRM analysis from genotype II (right, yellow), which infects coho, by its lower melting temperature. The green curve is a 50:50 mix of the two genotypes.

Having deemed the HRM approach unsuitable for our sample types, we pursued probe-qPCR that focused on the ITS1 region (unlike the regular qPCR that targets the 18S rRNA and quantifies all *C. shasta* present). However, this method only permits limited multiplexing (only up to 2 genotypes can be identified at a time) and does not provide information on any novel genotypes that may be present in a sample.

We are currently exploring a method called pyrosequencing and a second gene, which codes for the heat shock protein 70. Pyrosequencing will amplify and quantify known genotypes and alert the user of unknown genotypes present in a sample. The method is also faster and cheaper than Sanger sequencing. We attempted to use pyrosequencing on the ITS gene but QIAGEN (molecular company) representatives informed us that there is too much variability in this gene for this approach. Thus, we are now focusing on the hsp gene and are in the process of sequencing sufficient representatives (n=10) of each ITS1 genotype to proceed with primer design and pyrosequencing. We hope to identify a gene with less DNA sequence variation than the ITS1 gene but with more than the 18S rRNA gene and have sample sequences group consistent with their ITS1 genotypes.

**Objective 4. PROVIDE FINER RESOLUTION OF THE INFECTIOUS ZONE IN THE LOWER KLAMATH RIVER**

**Task 4.1-4.2. COLLECTION AND ASSESSMENT OF WATER SAMPLES**

Developed water sampling and molecular methods were used to more accurately define a zone of the lower mainstem Klamath River previously identified as being highly infectious through sentinel fish studies, water sampling and polychaete studies.

**Methods**

During spring/early summer 2009, water samples were collected in a longitudinal river transect, at 16 approximately equidistant sites, from Seiad Valley to east of the I5 bridge (Figure 4.1). Sampling occurred on three days, 3 weeks apart: May 13 (A), June 3 (B) and June 24 (C). Each day, 3 × 1L water samples were collected from each site (exception: only 1 sample was collected at each time point at
Rkm 231 and 234 on June 24), 3 times at 3 hour intervals. Every other site was sampled simultaneously: either at 9am, 12 noon and 3pm or at 10am, 1pm and 4pm. Samples (total of 144 per day) were chilled during transportation to the Salmon Disease Laboratory, OSU, where they were filtered within 24 hours. On June 24, an additional 6 downstream sites were included in the study. These were sampled (3 x 1L) once in the morning and once in the afternoon.

Figure 4.1. Overview of water sampling sites on the lower Klamath River. The 16 sites from Rkm 208-287 (yellow pins) were sampled on all three dates, May 13, June 3 and June 24, whereas the 6 from Rkm 132-195 (blue pins) were included only on the third sampling day. The Klamath River flows westward into the Pacific Ocean.

In the laboratory, each water sample was filtered through a nitrocellulose membrane using a vacuum pump, the filter paper folded into a microfuge tube and frozen. The filter paper was dissolved in a series of acetone/ethanol washes, removed, and the remaining DNA extracted using a kit (QIAGEN DNeasy Blood and Tissue). Any *C. shasta* DNA present was quantified by a TaqMan PCR assay on a 7300 platform (Applied Biosystems) (Hallett and Bartholomew 2006).
Reference samples with known numbers of *C. shasta* spores were processed in the same manner as the field samples. Positive (tissue) and negative (water) controls were included on each plate. All samples were run in duplicate (i.e. 2 wells each). Data generated by the SDS software (Applied Biosystems) was exported into Excel where averages and standard deviations were calculated. Any sample whose 2 wells differed by greater than 1 standard deviation was rerun.

**Results & Discussion**

Parasite abundance on May 13, June 3 and June 24 is displayed in **Figures 4.2-4**. All water samples contained *C. shasta* DNA. Levels varied from <1 - >100 spores/L and overall site averages were all >10 spores/L (**Figure 4.5, 6**). Daily abundance was consistent at some sites (<1 Cq) whereas it fluctuated over 10-fold (>4 Cq) at other sites.

Parasite abundance was unusually high in May and decreased at the beginning of June, notably at KBC. Discharge rates from hydrographs don’t explain the data, but there was a 9 C increase in temperature between sample days. At the end of June the overall pattern had changed and spore abundances were higher at some sites but lower at others.

There was no consistent pattern in abundance between days. Levels increase for about 20 Rkm downstream of the Shasta River confluence to peak east of KBC, thus providing some basis for defining an upstream limit of the infectious zone. On the first 2 collection days, levels were clearly lower downstream of the confluence of the Scott River, suggesting this as a downstream limit; however, on the third day levels increased markedly both above and below these locations. These levels are more variable between dates than expected, and likely reflect changes in water flow and temperature which affect the polychaete host, release of the parasite from the polycathaete and subsequent survival of the waterborne stage infective to fish. For example, flows from the Scott River (data from Seiad Valley gauge, **Figure 4.7**) decreased approximately 1000 cfs over the course of the study and thus contributed less to parasite dilution during the latter sample period.
Figure 4.2. Abundance of Ceratomyxa shasta in water samples collected from 16 lower Klamath River sites. Each data point is the average of 3 x 1L water samples. Samples were taken at 3 time points at each site, at 9 or 10 am, 12 or 1pm and 3 or 4pm on May 13, 2009 (IZA). The lower the Cq value the more parasite DNA is present. Approx. 3.3 Cq equates to a 10-fold difference in spore numbers.
Figure 4.3. Abundance of *Ceratomyxa shasta* in water samples collected from 16 lower Klamath River sites. Each data point is the average of 3 x 1L water samples. Samples were taken at 3 time points at each site, at 9 or 10 am, 12 or 1pm and 3 or 4pm on June 3, 2009 (IZB). The lower the Cq value the more parasite DNA is present. Approx. 3.3 Cq equates to a 10-fold difference in spore numbers.

Figure 4.4. Abundance of *Ceratomyxa shasta* in water samples collected from 16 lower Klamath River sites. Each data point is the average of 3 x 1L water samples. Samples were taken at 3 time points at each site, at 9 or 10 am, 12 or 1pm and 3 or 4pm on June 24, 2009 (IZC). The lower the Cq value the more parasite DNA is present. Approx. 3.3 Cq equates to a 10-fold difference in spore numbers.
Figure 4.5. Comparison of abundance of *Ceratomyxa shasta* in water samples collected from 22 lower Klamath River sites on three dates, three weeks apart: May 24 (IZA), June 3 (IZB) and June 24, 2009 (IZC). Each data point is the average of 6-9 x 1L water samples. Samples were taken at 2 time points at the lower 6 sites and at 3 time points at the upper 16 sites. The lower the Cq value the more parasite DNA is present. Approx. 3.3 Cq equates to a 10-fold difference in spore numbers.

Figure 4.6. Overall abundance of *Ceratomyxa shasta* in water samples collected from 22 lower Klamath River sites on three dates, three weeks apart: May 24 (IZA), June 3 (IZB) and June 24, 2009 (IZC). Each data point is the average of 6-27 x 1L water samples. The lower the Cq value the more parasite DNA is present. Approx. 3.3 Cq equates to a 10-fold difference in spore numbers. KSV=Seiad Valley index site, KBC = Beaver Creek index site.
This study characterizes spore abundance in the water column, it does not identify the source of the spores i.e. where the infected polychaetes are located. Decreases in spore abundance, e.g. as progress downstream towards Scott River, may be caused by fewer spores being released in this region, or spores being contributed upstream are degrading as they disperse downstream due to high water temperature.

Also, note that water flows have decreased over the past four years (Figure 4.7). This may have allowed downstream accumulation of materials suitable for polychaete habitat and conducive to polychaete and parasite propagation.

Figure 4.7. Hydrographs of daily mean discharges from Iron Gate Dam from 2005 – 2009 (upper), Iron Gate Dam May-June 2009 (lower left) and Seiad Valley May-June 2009 (lower right).

We thank: California Department of Fish and Game, especially Kim Rushton and crew at the Iron Gate Hatchery for providing Klamath River fall Chinook and coho salmon juveniles for our sentinel studies; Roaring River Hatchery, Oregon Department of Fish and Wildlife, Scio, OR for susceptible rainbow trout; Scott Vanderkooi and crew USGS, Klamath Falls, OR who assisted with sentinel cage studies at Keno Eddy, Keno, OR.

We also thank land owners who allow access to conduct sentinel studies and water sampling: The Nature Conservancy, Klamath Falls, OR for access at the lower Williamson River; The Sportsman’s Park Club near Keno, OR; Klamath River Country Estates near Klamathon, CA; Fisher’s RV Park at Klamath River, CA; Wally Johnson, Seiad Valley; Sandy Bar Resort, Orleans, CA.