

**California Nevada Fish Health Center
FY2009 Technical Report:**

Evaluation of migration rate on ceratomyxosis in
Klamath River Chinook salmon (*Oncorhynchus
tshawytscha*) smolts.

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Summary: Juvenile Iron Gate Hatchery Chinook salmon (*Oncorhynchus tshawytscha*) were exposed in the Klamath River May 27-29, 2009. Two replicate live cages were placed in-river for 72 hours and daily for 6-8 hours at I-5 Bridge (rkm 292.7) and Community Center (rkm 258.45). Replicate live boxes were also transported in-river by raft between the two locations (distance of 34.25 rkm) at an average speed of 4.4rkm/ h, emulating migrating cohorts. During this time, 1 L composite water samples were taken every hour at Community Center and in-river throughout the migration route to ascertain actinospore load per day. Post exposure, fish were transported to the California Nevada Fish Health Center and held in parasite-free water for a 31 day observational period at temperatures similar to the Klamath River. Samples were obtained from all fish for QPCR analysis. Moribund and freshly deceased fish were sampled for histological examination and gut imprint to determine cause of death. For this study we documented prevalence of *Ceratomyxa shasta* infection, cumulative mortality, mean days to death and survival function for the different exposure groups. The data indicates that actively migrating fish had a similar risk of ceratomyxosis as caged sentinel fish.

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Introduction: Juvenile salmon in the Klamath River incur a high incidence of infection by the myxozoan parasite *Ceratomyxa shasta* (Stocking et al. 2006, Foott et al. 2004). In previous years, infection levels observed in histological sections of juvenile Chinook salmon collected in the Klamath River above the confluence with the Trinity River between May and July has ranged from 21-38% (Nichols et al. 2009). Approximately 70% of the positive samples demonstrated pathology due to severe infection. The high prevalence of infection in native fish, that should have high resistance to an endemic disease, indicated this parasite is a key factor limiting salmon recovery in the Klamath River (Foott et al. 2009). The Klamath River between the confluence with the Shasta River and Seiad Creek has been identified as highly infectious to salmon (Stocking et al. 2006). Other sentinel fish studies of in-situ cage exposures for 3 days (d) resulted in 64-77% of fish progressing to clinical disease and 10-22% for fish held 3-6 hours (Foott et al. 2007, Stone et al. 2008). This data is used to estimate the prognosis (disease mortality) of infected juvenile Chinook yet may not represent actively migrating fish.

The primary focus of this study was to compare percent cumulative mortality, mean days to death and survival function due to ceratomyxosis between juvenile salmon actively migrating through a portion of the known infectious zone with cohorts held during a similar time period in-situ above and below the exposure reach. We hypothesize that fish held in-situ at the Community Center exposure site (bottom of exposure reach) will incur the highest overall cumulative percent mortality, fewest mean days to death (rate) and have a significantly different survival function in comparison to cohorts held at I-5 (top of exposure reach) and those actively transported through the exposure reach at a rapid migration rate.

Methods:

Fish- Juvenile Chinook salmon, Klamath River stock, were obtained from the California Department of Fish and Game Iron Gate Hatchery (IGH) 40d prior to the first exposure date. Fish were brought back to the California Nevada Fish

Health Center (Ca-NV FHC) wet laboratory and held in a 352L circular tank supplied with 9.5 L min flow of single-pass, ozonated water at temperatures similar to the Klamath River. The laboratory receives Coleman National Fish Hatchery water and there is no history of *C. shasta* infection at this facility. Fish were fed (1.0mm Bio-Oregon Lifestage Diets for Fish) at 1.5% body weight per day. To reduce occurrence of columnaris disease (infection by *Flavobacterium columnare*), study fish received oxytetracycline medicated feed (10g OTC per 100lbs of fish) for 21 d prior to the first exposure. Study fish were also given a 10 minute prophylactic bath of 1 mg L⁻¹ furanase for three consecutive days post exposure. These medications do not affect infection by myxozoan parasites. Effluent from the wet lab was chlorinated.

Exposure- Three exposures were conducted May 27 – 29, 2009. Replicate live boxes containing approximately 40 fish (Appendix 1) were actively transported downriver 34.25 river kilometers (rkm) via raft from the Interstate-5 (I-5) Bridge (41°51'23.32"N 122°34'17.03E, rkm 292.7) to Community Center (41°50'23.44"N 122°50'12.57"W, rkm 258.45). Migration cages were attached to the pontoons of a 13ft Cataraft below the waterline. Average speed of travel was 4.4 river kilometers per hour based on migration rates observed in radio tagged fall Chinook juveniles released from IGH in 2008 (Foott et al 2009).

In-situ cohort groups held in live cages were exposed at the I-5 Bridge and Community Center sites during the 7-9h daily migration period (Table 1). In addition, duplicate cage groups were held for the entire 3d exposure period at both locations to simulate a typical 72h sentinel study.

Table 1. Exposure times in hours and minutes for each sentinel group (I-5 72 hr, Community Center (Com. Center) 72 hr, I-5 8 hr A&B replicates, Com. Center 8 hr A&B and Migratory cohorts 8 hr A&B) in the Klamath River over the three day study period. Data reported as: exposure day (day), group identification (Group), total exposure hours (Hours) and total number of exposure minutes (Minutes) in the Klamath River

Day	Group	Hours	Minutes
1-3	72hr I-5 Bridge	55.2	3310
1-3	72hr Com. Center	53.6	3216
1	8hr I-5	9.7	584
1	8hr Com. Center	8.1	483
1	8hr Mobile	8.9	537
2	8hr I-5	9.0	540
2	8hr Com. Center	6.8	408
2	8hr Mobile	7.5	450
3	8hr I-5	8.0	480
3	8hr Com. Center	6.1	365
3	8hr Mobile	7.2	430

Cages were constructed of 6 inch PVC pipe with 3” holes covered by quarter inch mesh for water exchange (0.34 cf. ft volume). Onset temperature probes recorded water temperature during each exposure and dissolved oxygen was measured by a Hach LDO meter. After each exposure, fish were transported back to the wet lab and reared at 18°C. Mortalities were sampled twice daily over a 31d period. All surviving fish were sampled 32 days post exposure (dpe) proportionally from each tank for histology and QPCR analysis to ascertain prevalence of *C. shasta* infection.

Water Filtration- To determine *C. shasta* actinospore concentration each day, a composite water sample was collected during the downriver transport (pooled hourly 1L grab samples) and at Community Center (500 mL every 15min for 6-9h using a 6712 portable ISCO sampler, Teledyne ISCO). Four replicate 1 L samples were removed from the daily composite water sample, transported back to the lab on ice, filtered through a 47mm 5.0µm nitrocellulose filter (Millipore Inc.) and the filters stored at -70°C until DNA extraction. Parasite DNA detection in water samples was performed in the same manner as Foott et al. (2007).

QPCR- Intestinal and kidney samples (~0.025g) were taken from all fish (mortalities or 31 day post-exposure survivors) for quantitative real-time polymerase chain reaction (QPCR) assay. Additionally, 100-fold dilutions of the water filter extracts were assayed by QPCR. The digestion, extraction, QPCR assays and analysis was performed as reported by Nichols et al. (2009). A positive C_t cutoff value of 38 was implemented in the study.

Histology- Remaining portions of the intestinal track and kidney (not used for QPCR) from moribund or freshly deceased fish were placed in Davidson's fixative for 24 h and processed for 5 μ m paraffin sections and stained with hematoxylin and eosin (Humason 1979). Slides were examined using brightfield microscopy for *C. shasta* trophozoites.

Imprint- Imprints of lower intestine content were taken from all moribund fish as well as fresh mortalities, allowed to air dried, fixed in methanol for 1 minute, stained with Leishman and Giemsa stain, and examined using brightfield microscopy at 40x magnification for *C. shasta* trophozoites to determine cause of death. The majority of mortalities were diagnosed using imprints or histology.

Statistics and data metrics – Three measures of disease were used in this study: 1) Cumulative percent mortality expresses what percent of the population died over the 31d observation period (magnitude) and is inverse to survival function which is also reported. Survival function is the probability of surviving over a given time period (time t) as a result of ceratomyxosis. More specifically, it is defined as, “the probability of an individual in the population surviving beyond time t ” (Glantz 1997). Survival curves were derived using Kaplan-Meier Survival analysis in SigmaStat 3.1. Differences in percent cumulative mortality were analyzed using a Chi-Square test. 2) Mean day to death specifies how rapidly individuals in a population die from disease and is directly related to the actinospore dosage they received during exposure periods. 3) Prevalence of

infection indicates the number of individuals in the population that were infected with *C. shasta* at the time the sample was taken.

Results:

Day 1 (May 27, 2009) - Cumulative percent mortality was significantly different between all three daily exposure groups ($P=0.008$). Fish exposed at I-5 experienced the fewest mortalities (30/80, 37.5%) and Migratory cohorts incurred the highest (19/35, 54.3%) post exposure (Figure 1). No difference in mean days to death (MDD) was observed between the three daily exposure groups ($P = 0.521$). Mean days to death for I-5A, I-5B, Migratory cohorts and Community Center ranged from 19-21d (Figure 2). A statistical difference in survival function was observed between the daily exposure groups ($P=0.001$) (Figure 3A). The probability of survival was highest at I-5 in comparison to the lower exposure sites which had a poorer survival prognosis.

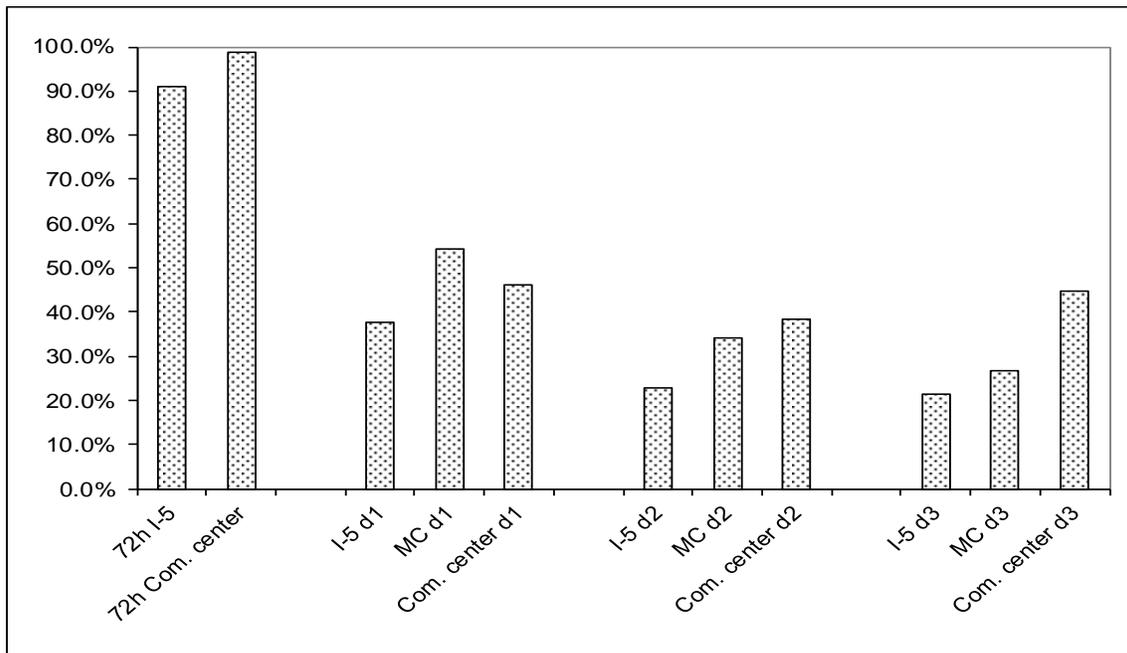


Figure 1: Percent cumulative mortality for all groups [I-5 day 1 (I-5 d1), Migratory cohorts d1 (MC d1), Community center day 1 (Com. center d1), I-5 day 2 (I-5 d2), Migratory cohorts day 2 (MC d2), Community center day 2 (Com. center d2), I-5 day 3 (I-5 d3), Migratory cohorts day 3 (MC d3), Community center day 3 (Com. center d3), 72 h I-5 and Com. Center].

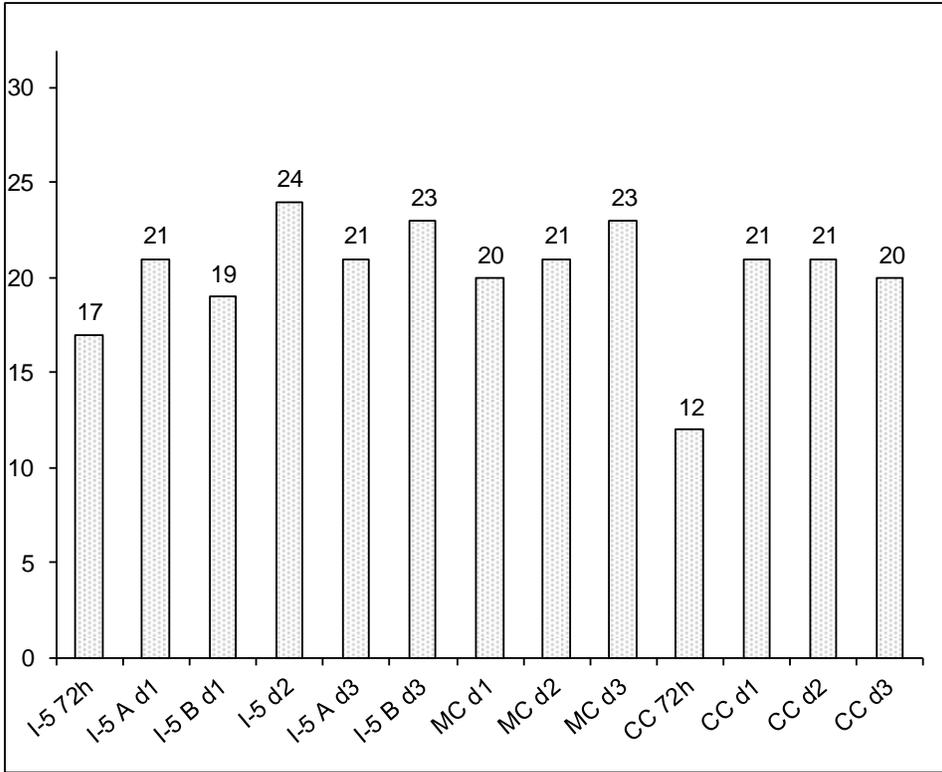


Figure 2. Mean days to death for all groups [I-5 day 1 (I-5 d1), Migratory cohorts d1 (MC d1), Community center day 1 (Com. center d1), I-5 day 2 (I-5 d2), Migratory cohorts day 2 (MC d2), Community center day 2 (Com. center d2), I-5 day 3 (I-5 d3), Migratory cohorts day 3 (MC d3), Community center day 3 (Com. center d3), 72 h I-5 and Com. Center].

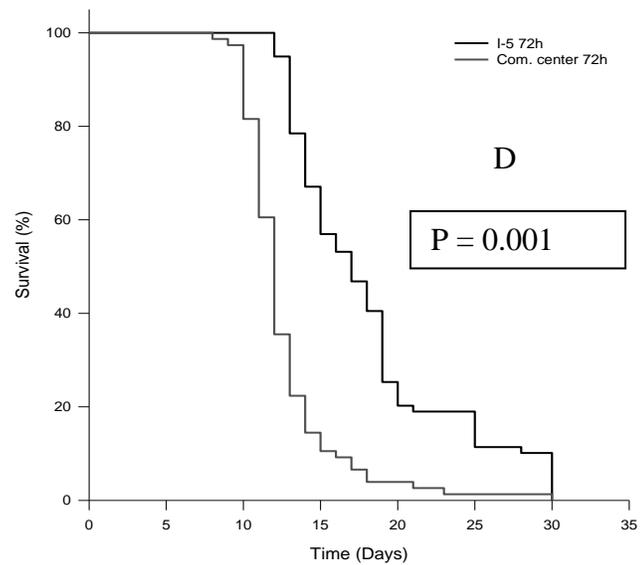
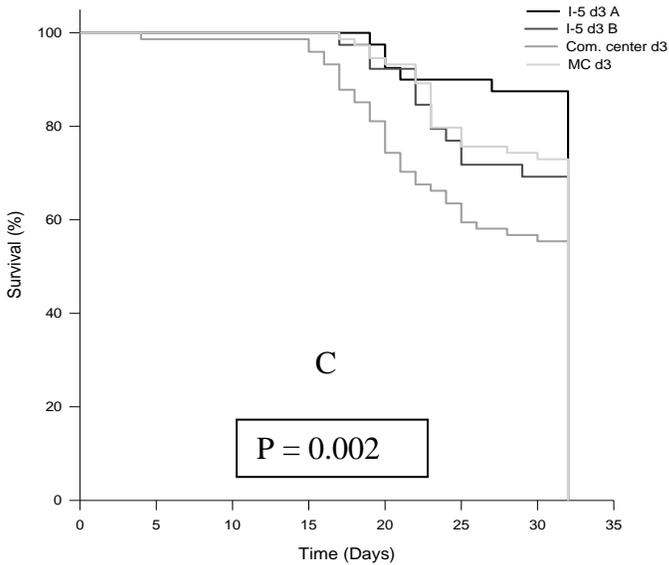
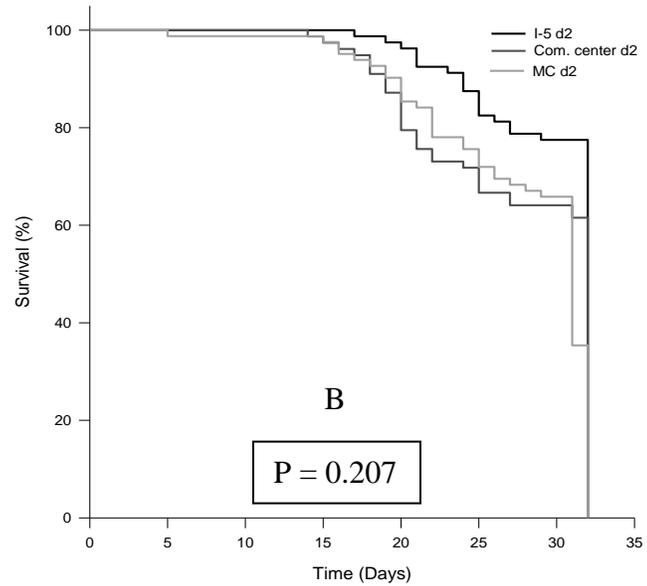
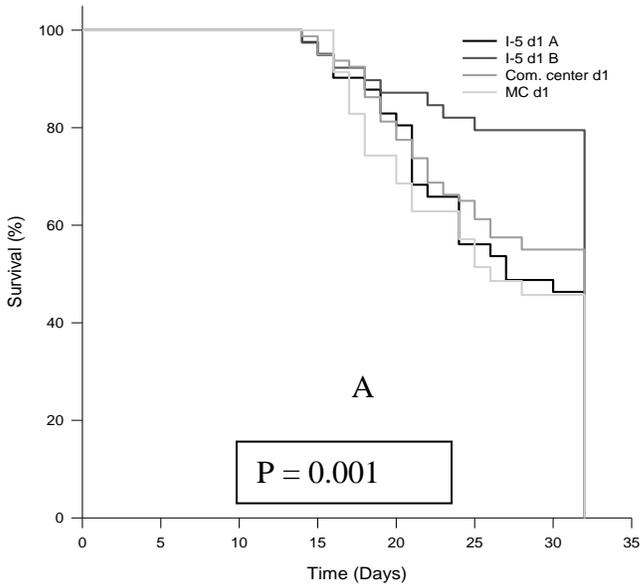


Figure 3. Survival curves by exposure day at all sites; A) I-5 day 1 (I-5 d1A, I-5 d1 B), Migratory cohorts d1 (MC d1), Community center day 1 (Com. center d1), B) I-5 day 2 (I-5 d2), Migratory cohorts day 2 (MC d2), Community center day 2 (Com. center d2), C) I-5 day 3 (I-5 d3 A, I-5 d3 B), Migratory cohorts day 3 (MC d3), Community center day 3 (Com. center d3), D) I-5 72h, Community Center 72h (Com. center 72h) Data reported as the probability of an individual in the population surviving beyond time t over the 31 day post exposure observational period as a result of ceratomyxosis.

Day 2 (May 28, 2009) - No statistical difference in cumulative percent mortality was observed amongst the daily exposure groups on 2d ($P = 0.082$). Mortality ranged from 23-39%. Fish exposed at Community Center accrued the highest cumulative mortality (30/78, 38.5%) (Figure1). There was no significant difference in mean days to death between the daily exposure groups ($P = 0.166$). Mean days to death for I-5, Migratory cohorts and Community Center ranged from 21-24d (Figure 2). The probability of survival was similar for all three daily exposure groups. No statistical difference in survival function was observed ($P=0.207$) (Figure 3A).

Day 3 (May 29, 2009) - Cumulative percent mortality was different between all three daily exposure groups ($P = 0.03$). Fish at I-5 had the lowest cumulative mortality (17 / 79, 21.5%) while the Community center exposure group accrued the highest (33 / 74, 44.6%) (Figure1). No significant difference was observed in mean days to death between any of the daily exposure groups ($P = 0.097$). Mean days to death for I-5A, I-5B, Migratory cohorts and Community Center ranged from 20-23d (Figure 2). Survival function was significantly different ($P = 0.002$) for all three daily exposure groups (Figure 3C). The probability for survival was highest for fish exposed at I-5 while survival prognosis was poor for fish exposed at Community Center and the Migratory cohorts.

Three day sentinels - The in-situ groups held at I-5 for 55.2h and Community center for 53.6h accrued high cumulative percent mortality 91.1%, (72/79) and 98.7%, (75/76) respectively (Figure 1). Survival function was different between the two 72h groups ($P = 0.001$) (Figure 3D). The probability of survival was much higher for fish held at the I-5 exposure site. Fish exposed at Community Center died at a significantly fast rate.

Water samples – Mean C_t of the 100x dilution samples was 35.12 (range 34.6 – 36.0). If the 3.2 C_t per log dilution is used to calculate the raw sample value, the

average C_t of the river water was 28.7 (35.12 – 6.4 = 28.7). This data indicates the actinospore concentration of the river was greater than 10 spores / L. Hallett and Bartholomew (2006) report a $C_{t\text{of}}$ 34.6 for 1 actinospore / L and 30.6 for 10 actinospores / L. The authors describe how Klamath R. Chinook have an infectious threshold of ≥ 10 spores/L. Water samples collected from this reach in May and June 2009, and analyzed by Oregon State University contained >100 actinospores / L (S. Hallott, pers. Communications). It appears that the river was highly infectious for the sentinels. Dissolved oxygen levels ranged from 8.20 - 10.80 mg / L (mean 10.14 mg/L) at Community Center and from the raft during in-river transport. River temperatures ranged from 16.44° C at I-5 Bridge to 20.53° C at Community Center (mean 18.45° C).

Survivors - All fish surviving 31dpe were sampled and 7 to 10 fish from each exposure group (30-100%) were assayed for *C. shasta* DNA using QPCR. Prevalence of infection was 70% (124 / 177). All control fish were QPCR negative for *C. shasta* DNA.

Discussion:

In May 2009, fish held in-situ in the Klamath River between I-5 Bridge and Community Center, for as little as 53.6h, incurred nearly 100% mortality with a mean day to death of 12.6d. Fish exposed in-situ within the same reach for as little as 6.1h had cumulative mortalities as high as 44.6% with a mean day to death of 26.7d (Appendix 1).

Between May 27 and May 29, 2009, Migratory cohorts and daily in-situ groups held at Community Center had a similar survival function over the three day exposure period ($P = 0.770$). The similarity in survival indicates that fish actively migrating through the exposure reach can still incur high level mortality in a short period of time. These results aid in validating the assumption that in-situ groups held in the Klamath River can represent feral fish.

Other than day 1, where a high number of Migratory fish were lost (55 total) due to in river transport trauma, Community Center in-situ groups had the highest cumulative mortalities. Migratory cohort survival improved on days 2 and 3 after transport cage and orientation were changed. As seen in the cumulative mortality data, actinospore concentration at I-5 Bridge was high enough to kill just as many fish as Community Center and Migratory cohorts. Actinospore exposure (dosage) for the Migratory cohorts and Community Center fish was even greater than I-5 as observed in the faster rate of death (mean day to death).

When mortality response is standardized by exposure time, Migratory cohorts had 4 – 6% mortality per hour. In June 2009, estimated ceratomyxosis mortality for juvenile Chinook migrating through the 74.6 rkm infectious zone at 4.4 rkm per hour range from 63 – 77% (17h to cover the 74.6 rkm x 4 - 6%). This estimate is in line with mortality data from sentinels held for 72h at community center (99%) and migratory cohorts exposed for 6-8 h (27 – 54% mortality). Juvenile salmon rapidly migrating through infectious zone of the lower Klamath River in 2009 will incur a high level mortality due to ceratomyxosis.

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Appendix 1. Sentinel composition and mortality data for all exposure groups (I-5 72 hr, Community Center (Com. Center) 72 hr, I-5 8 hr A&B replicates, Migratory cohorts 8 hr A&B and Com. Center 8 hr A&B). Data reported as day fish were exposed to the Klamath River (day), Group identification (Group ID), number of fish per group that were exposed in live cages (No. of Fish Exp.), total number of mortalities occurring over 31 day post exposure observational period (Total Mort.) and percent mortality per group over the entire study period (% Mort.).

Day	Group ID	No. of Fish Exp.	Total Mort.	% Mort.
1-3	I-5 72 hr	43	38	88 %
1-3	I-5 72 hr	36	34	94 %
1-3	Com. Center 72 hr	38	38	100 %
1-3	Com. Center 72 hr	38	37	97 %
1	I-5 A 8 hr	41	22	54 %
1	I-5 B 8hr	39	8	21 %
1	Migratory cohorts 8 hr A	15	8	53 %
1	Migratory cohorts 8 hr B	20	11	55 %
1	Com. Center 8 hr A	40	15	38 %
1	Com. Center 8 hr B	40	22	55 %
2	I-5 A 8 hr	40	10	25 %
2	I-5 B 8hr	39	8	21 %
2	Migratory cohorts 8 hr A	42	13	31 %
2	Migratory cohorts 8 hr B	40	15	38 %
2	Com. Center 8 hr A	39	11	28 %
2	Com. Center 8 hr B	39	19	49 %
3	I-5 A 8 hr	40	5	13 %
3	I-5 B 8hr	19	12	31 %
3	Migratory cohorts 8 hr A	37	13	35 %
3	Migratory cohorts 8 hr B	38	7	18 %
3	Com. Center 8 hr A	14	12	35 %
3	Com. Center 8 hr B	40	21	53 %
	Controls	27	0	0 %