

## U.S. Fish & Wildlife Service

California Nevada Fish Health Center

FY2015 Technical Report:

### **Prevalence of *Ichthyophthirius multifiliis* in both resident and sentinel Speckled dace (*Rhinichthys osculus*) in the Lower Klamath River (August 5- September 9, 2015).**



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**Summary:** A low prevalence of single *Ichthyophthirius multifiliis* (Ich) trophont infection (3% by histology and 8% by wet mount microscopy) was observed in resident speckled dace (*Rhinichthys osculus*) in the Lower Klamath River between 05 August – 09 September 2015. Gill hyperplasia (disease) was not associated with the observed trophonts. Other parasites were more common, particularly trematode metacercaria ( $\geq 68\%$  of each sample group). Similarly, sentinel dace held for 1 week in the river, at the highway 101 bridge and below Blue Creek, showed a low prevalence of Ich infection over this same period. Ich was detected in 2% and 9% of the highway 101, and 0% and 23% of the Blue Creek sentinel gills by microscopy and quantitative polymerase chain reaction (QPCR) respectively. Water samples taken at these sample sites showed a consistent low level ( $< 1$  parasite/L) of Ich DNA concentration, with greater concentrations found at Highway 101 than Blue Creek. There was no trend observed between sentinel infection and water DNA. The higher sensitivity of QPCR shows potential for monitoring efforts. High resident fish density with low prevalence of Ich infection could be the source of infectious Ich theronts for returning salmon in the Lower river.

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## Background:

Severe infection, by the ciliate parasite *Ichthyophthirius multifiliis* (Ich), has been observed on gills of adult Chinook salmon (*Oncorhynchus tshawytscha*) migrating through the Lower Klamath River in 2002 and 2014. This parasite, along with external bacterial infections (*Flavobacterium columnare*), were associated with the 2002 fish mortality event. Despite multiple observations of salmon with heavy Ich trophont infection of the gill, no large scale mortality was observed in 2014 (Belchik 2015). The question of reservoir hosts and distribution of infective stage (theront) of Ich in the Lower Klamath River is the basis for this cooperative study. Our objectives are to determine the prevalence and severity of Ich infection in native resident fish (primarily speckled dace) in the upper estuary (near Highway 101 bridge) and directly below the mouth of Blue Creek (river mile (RM) 16). Additionally, we collected water samples for later Ich DNA assay (this aspect will be done by the Oregon State University) to compare it to sentinel dace held for 1 week at the above sites. The use of QPCR to assay both water and gill tissue was a pilot effort.

## Methods:

*Sentinels* - Approximately 300 speckled dace (*Rhinichthys osculus*) were captured by California Department of Fish and Wildlife in the upper Shasta River (Nature Conservancy property), transported to the California Nevada Fish Health Center (CA-NV FHC) wet lab, and treated with 3 consecutive 20 minute, 100 mg/L formalin baths to remove ectoparasites. After this treatment and prior to each sentinel group transport, five fish were sampled for skin and gill tissue to examine by phase microscopy for external parasites. Dace were fed daily with salmon diet and reared at ambient (16 - 22°C) temperature within a 3,800 L tank. Twenty dace were also examined for external parasites at the conclusion of the study.

Between 05 August and 09 September 2015, a weekly sentinel cage exposure of 20 dace at 2 locations was conducted in the Lower Klamath River. Cages were constructed of 153mm diameter PVC pipe with 76mm holes covered by 1mm mesh for water exchange (0.01 cu. m volume). Dace were transported in oxygen filled plastic bags containing 1.5 L of water. Two cages of ten sentinel dace each were placed at the following locations: 1) left bank at the highway 101 bridge (101) and 2) approximately one eighth of a mile downriver of Blue Creek (BC) mouth (Figure 1). These locations are approximately 10 RM apart and represent exposure sites for migrating salmon moving up from the estuary (101) and holding habitat in the thermal refugium at the mouth of Blue Creek. Blue Creek is also an adult salmon Ich sample site for Yurok Tribal Fishery biologists. Dissolved oxygen was measured at each site with a Hach HQ40d meter (Hach Company, Loveland, Colorado) and temperature recorded hourly with an HOBO Water Temp Pro v2 Model Number U22 001 (Onset Computer Corporation, Bourne, Massachusetts). After 7 days sentinels were removed from the cages, rapidly euthanized in Tricaine Methanesulfonate, and measured for fork length. Two to three gill filaments were then dissected for wet mount phase microscopy (left side). Gill tissue (right side) from 10 dace was collected for a QPCR sample, held in 600µL microcentrifuge tubes on ice, and stored at -20°C. The heads from sentinel dace, not selected for QPCR, were preserved in Davidson's fixative as an archive sample. Skin scrape preparations were also examined by phase microscopy for the

first exposure groups (02 August). This practice was discontinued as no parasites were observed in either the sentinel or resident skin scraping preparations and due to time constraints in the field.

*Resident dace* - Resident wild speckled dace (target of 20 / site) were captured by beach seine near the 2 sentinel sites and sampled weekly as described above. Gill filaments were examined by both phase microscopy (on site) and histology. Due to their small size, all fixed filaments on one side (ventral aspect) were initially de-calcified (Rapid Bone Decalcifier DCR0257, American Master Tech, Lodi California), prepared for paraffin blocks, sectioned at 5µm, and stained with hematoxylin and eosin. Slides were examined at 400 – 2,000x total magnification for parasites and abnormalities.

*Ich DNA in river water* – Four one liter water samples were collected at each sentinel site at the beginning and end of each exposure, and refrigerated on ice until filtered 8 to 9 hours post-collection. One 1-L water sample was filtered through each 47mm 5.0µm nitrocellulose filter (Millipore Inc.) and the filters stored at -70°C until shipped to Dr. Hallett at Oregon State University for *Ichthyophthirius multifiliis* DNA analysis by QPCR. The vacuum funnel was washed with distilled water between samples from a given site. Between sites the funnel was wiped with DNAaway (Thermos Scientific) and rinsed with distilled water. Filters were processed as per Hallett and Bartholomew (2006). Briefly, filter membranes were dried, dissolved in acetone, and the dried pellet removed for total DNA extraction by QIAGEN DNeasy kit. Sample DNA was diluted 1 to 4 in water, assayed on a StepOnePlus (Applied Biosystems) in duplicate on a single plate with a 10-fold dilution series of purified *Ich* trophont DNA positive controls using a SYTO9 qPCR assay with the primers of Jousson et al. (2005). The samples were run again on a second plate in a standard IPC inhibition assay (Applied Biosystems TaqMan Exogenous Internal Positive Control; Hallett et al 2012). Deviation occurred at concentrations corresponding to a quantification cycle ( $C_q$ ) threshold of approximately 33 which was selected as a preliminary detection threshold. Positive control dilutions were linear over at least 6 orders of magnitude (data not shown).

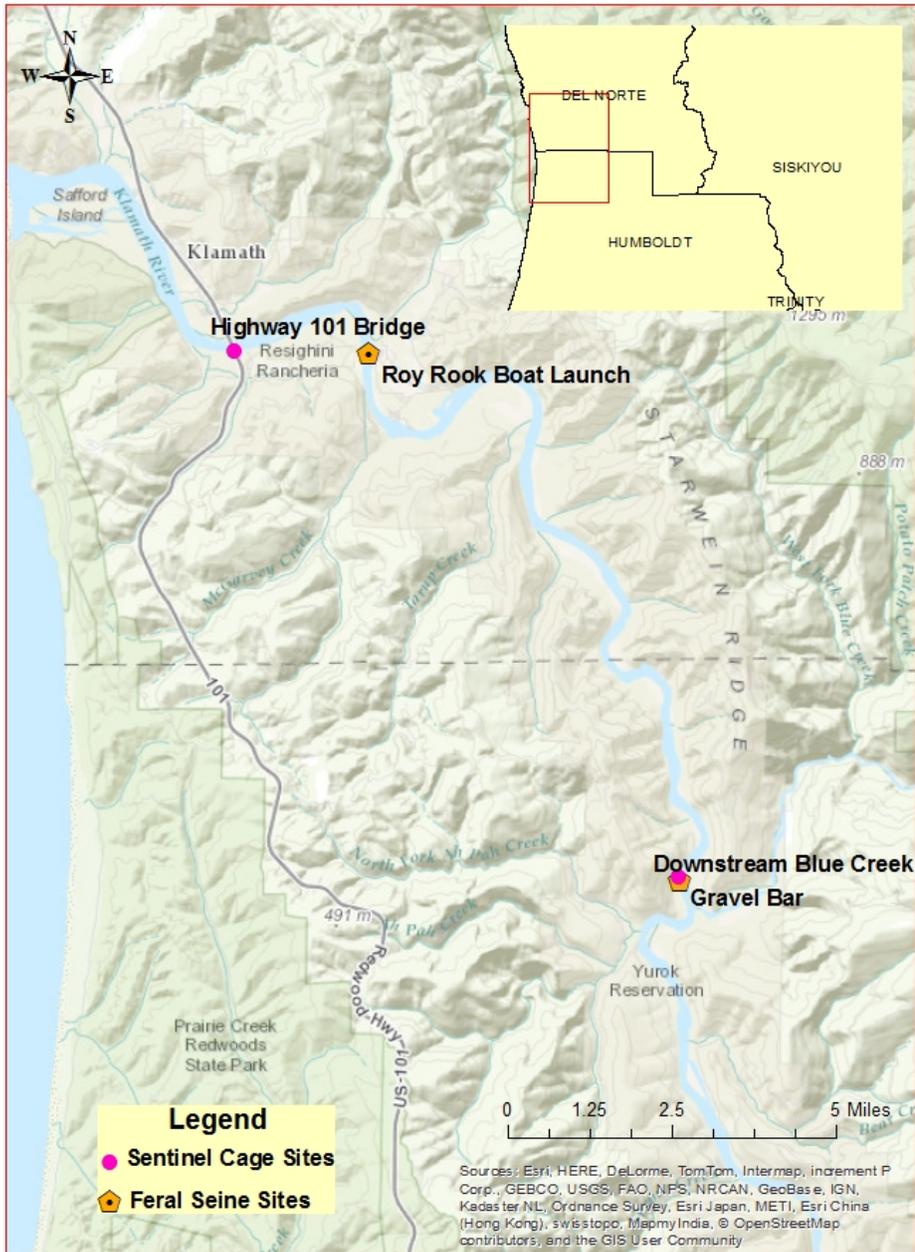
*Ich Gill Tissue Assay* – A Taqman MGB probe assay, and optimized primers, were designed in Primer Express software (Applied Biosystems) to target specific *I. multifiliis* rDNA in a previously published SYBR QPCR (Jousson et al., 2005). The MGB assay uses a probe (IKT10) TG CAA ACC AAA CTC starting at bp 674, and forward primer TCT TAG GCT AAA ATG GGC ATA CG (bp 650) and reverse primer CCC CAA GTC GAA CCA GTG AA (bp712). Assay standards were developed from adult Chinook salmon gill tissue, with confirmed *Ich* infection levels by microscopy and histology. Adult gill tissue was stored in Z-fix until extracted with Qiagen DNeasy kit and assayed in 10-fold serial dilutions to assay endpoint with an ABI 7300 Real Time PCR System. Reaction volumes of 50µL, containing 5µL DNA template and environmental master mix (Invitrogen) under the following amplification conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. The reliable endpoint was determined by examining the standard deviation of  $C_q$  values of 8 replicates for each standard dilution in the series. Three appropriate standard concentrations, within the dynamic range of the 7300 SDS instrument, were assayed a second time in 8 replicates to ensure assay repeatability and determine the mean  $C_q$  values for a relative standard curve for

the QPCR assay. Standard curve slope was -3.27, y-intercept was 25.0 and  $R_2$  value was 0.999. Amplification efficiency was >102%. Positive control aliquots were stored at -20°C until use.

BLAST results included other *I. multifiliis* 18S rDNA sequences (n=3) and some similarity to *Tetrahymena thermophila*, a free-living ciliate protozoa common in pond water. Specificity testing of extracted *T. thermophila* genomic DNA (strain WH6, ATCC# 30007D) was performed under the above assay conditions. Low-level amplification ( $C_q$  37.8) was observed for the highest concentration (4pg/10uL reaction volume) and no amplification in further dilutions of 1:2 through 0.5 pg final dilution. Therefore for this pilot study, assay threshold for a positive fish test result was set conservatively ( $C_q$  36) to preclude false-positives due to potential *T. thermophila* parasite DNA cross-reactivity.

Gill tissues were digested in 100µL MagMAX Proteinase K Buffer containing 100 mg/ml proteinase K (Applied Biosystems, Foster City, CA) at 55°C with constant shaking. A subsample of digested tissue homogenate was diluted 1:10 in molecular grade water, then diluted 1:10 in MagMAX Multi-Sample DNA Lysis Buffer (Applied Biosystems, Foster City, CA) for a final dilution of 1:100. The diluted tissue homogenate was extracted in a 96 well magnetic bead sample processing system (Applied Biosystems MagMAX Express-96 Magnetic Particle Processor). Extracted DNA was stored at -20 °C until the QPCR assays were performed. Dilution series of 1ch trophont standards, extraction control and no template control (NTC) wells were included on each assay plate. Quantitative Cycle threshold ( $C_q$ ) values were calculated using SDS software (7300 SDS v 1.3.1, StepOne SDS v. 2.0 Applied Biosystems).

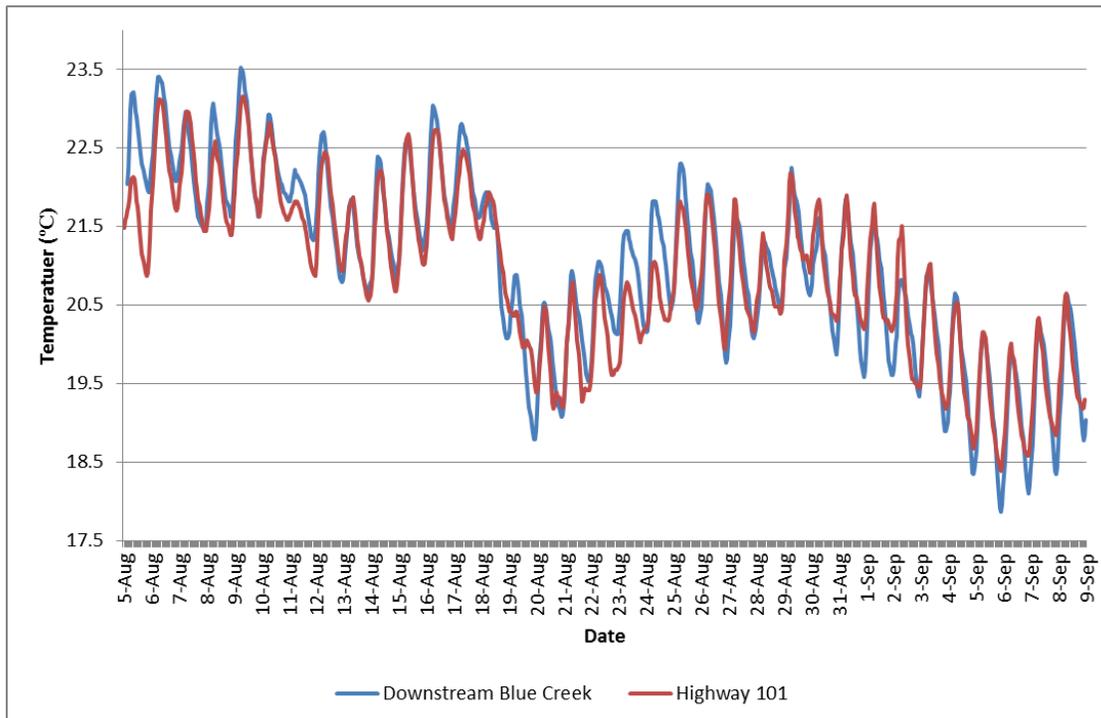
Figure 1. Seine and sentinel cage locations (HWY101 = Highway 101 bridge to Roy Rook Boat Launch, and BC = directly below confluence of Blue creek).



## Results and discussion:

*Water temperature and oxygen* – Spot measurement of dissolved oxygen, at the 2 sentinel cage sites, ranged from 7.98 – 9.18 mg/L with associated temperatures of 19 - 21°C which indicated that oxygen concentration was not a stressor for the sentinel dace. Maximum daily water temperature declined from 23-23.5°C in early August to 20°C by September 6 (Figure 2). This temperature range is within the range for replication and theront infectivity (Lom and Dykova 1992).

Figure 2. Water temperature at the sentinel cage sites over the 6 week study.



*Resident wild dace* – Speckled dace, captured by beach seine, ranged from 35 – 69mm in total length with a mean of 52mm. No moribund fish were observed in any collection. Prevalence of Ich infection was low (3% by histology and 8% by wet mount microscopy) at both sites (Table 1, micrograph 1). Only single Ich trophonts were observed on or embedded in the positive gills by either method. Other parasites were more common in the resident fish gills (Table 2, micrograph 2 – 6). Metacercaria (likely multiple species) were within gill blood vessels in 68 – 87% of all gills examined by wet mount or histological examination (Table 2, micrograph 2). Monogenetic trematodes (no identification) were seen in 1-3% of the samples (micrograph 3). Three protozoans were detected at low prevalence in histological sections (presumptive

chlamydia *Epitheliocystis* (micrograph 4), the ciliate protozoan *Trichodina* sp. (micrograph 6), and a sessile ciliate (micrograph 5). No gill hyperplasia was observed in any histological gill section. A limited number of the juvenile resident fish samples came from other species (Chinook salmon (3), three-spined stickleback (*Gasterosteus aculeatus*) (8), Klamath smallscale sucker (*Catostomus rimiculus*) (10), and sculpin (2)). The low prevalence and severity of Ich infection in this resident fish population is reflective of a normal host-parasite relationship where host immune function acts to limit parasite infection prevalence and intensity (Dickerson and Finley 2014).

Table 1. Prevalence of *Ichthyophthirius multifiliis* on **resident wild** speckled dace gills, captured in the Highway 101 bridge reach (101) or below the mouth of Blue Creek (BC), by wet mount microscopy or histology.

	BC	101	
<u>05Aug</u>			
Wet mount	0 / 11	2 / 15	
Histology	ND	ND	
<u>12Aug</u>			
Wet mount	ND	ND	
Histology	0 / 20	0 / 17	
<u>19Aug</u>			
Wetmount	1 / 9	ND	
Histology	1/15	3 / 16*	
<u>26Aug</u>			
Wetmount	4 / 7	0 / 10	
Histology	2 / 19	0 / 24	
<u>02Sep</u>			
Wetmount	0 / 8	0 / 8	
Histology	0 / 15	0 / 15	
<u>09Sep</u>			
Wetmount	0 / 8	0 / 8	
Histology	0 / 16	0 / 20	
<u>Totals</u>			
Wetmount	5 / 43 (12%)	2 / 41 (5%)	7 / 84 (8%)
Histology	3 / 85 (4%)	3 / 92 (3%)	6 / 177 (3%)

Low catch – all to histology.

ND not done

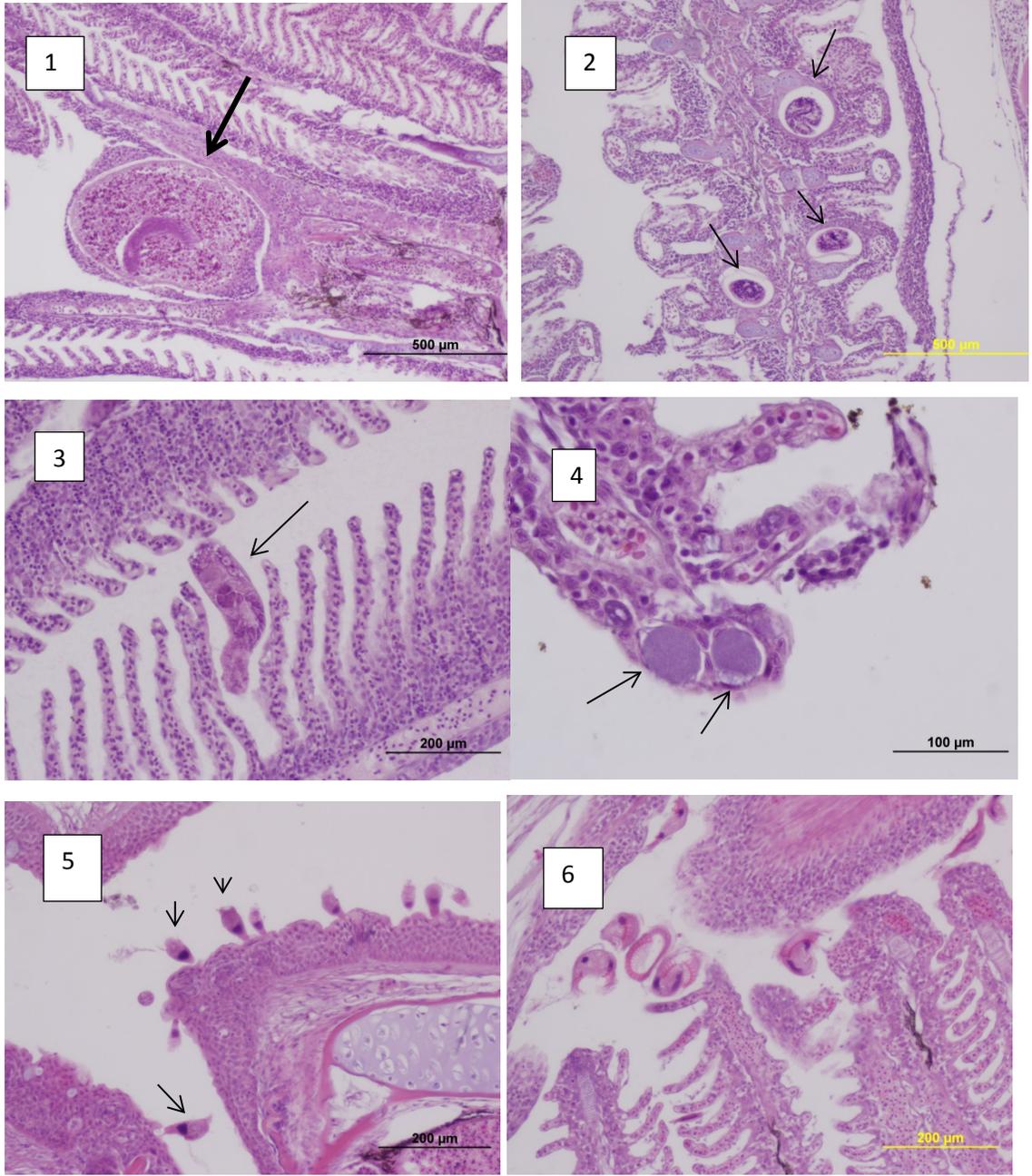
Table 2. Prevalence of infection for metacercaria stage of digenetic trematodes, epitheliocystis\*, monogenetic trematodes (Monogene), and ciliate *Trichodina sp.* in **resident wild** dace gills, captured between 05 Aug – 09 Sep. in the Highway 101 bridge reach (101) or below the mouth of Blue Creek (BC), by wet mount microscopy or histology.

	BC	101	total (%)
<b>Metacercaria</b>			
Wetmount	34 / 43	23 / 41	57 / 84 (68)
Histology	75 / 85	79 / 92	154 / 177 (87)
<b>Epitheliocystis</b>			
Wetmount	NA	NA	
Histology	5 / 85	3 / 92	8 / 177 (5)
<b>Monogene</b>			
Wetmount	3 / 43	6 / 41	9 / 84 (11)
Histology	2 / 85	3 / 92	5 / 177 (3)
<b><i>Trichodina</i></b>			
Wetmount	0 / 43	1 / 41	1 / 84 (1)
Histology	14 / 85	9 / 92	23 / 177 (13)

NA not applicable – limited ability to view in wet mount preparations

\* Presumptive identification based on morphology only

Figure 3. Parasite micrographs 1) Ich trophont within gill epithelium (note the lack of hyperplasia), 2) metacercaria within gill vessels, 3) monogene, 4) epitheliocystis cysts, 5) sessile ciliate attached to buccal cavity , and 6) *Trichodina* sp.



*Sentinel dace* – Ich trophozoites were not observed by wet mount phase microscopy in the sentinel population prior to exposure however both metacercaria and *Myxobolus* sp. cysts (Fig. 4) were observed in the gills prior to and after sentinel exposures. Similarly, Ich DNA was not detected in gill tissue from 8 dace of the wetlab population by QPCR. The captive dace fed aggressively on salmon diet. Fewer than 10 mortalities occurred in the captive population during the study and it was considered healthy. Only one sentinel mortality was observed (09 Sept Hwy 101 cage) however fewer than 20 fish were counted in 6 separate cage groups (16 – 19 fish counted). The wire mesh screen on the cages was damaged in several cages and may have led to escape. This was a particular problem at the Hwy101 bridge site due to wave action from boat wake.

The week long exposures did not result in pronounced Ich infections. Single Ich trophonts were seen by microscopy in 2 dace held at the Hwy101 site and none at Blue Creek (Table 3). QPCR analysis of the opposite gill filaments detected Ich DNA in 3 of 32 (9%) at Hwy101 and 7 of 30 (23%) at Blue Creek (Table 3). Eighteen of 80 gill samples were excluded from the QPCR data set due to their abnormal amplification curves (AAC) which could indicate inhibition by organic contaminants on the gill. There was no correlation between observed trophonts on the right side (wet mount) and PCR positive reactions on the left side of a given fish. PCR appears to be much more sensitive than on-site microscopy for surveying the resident dace.

Water samples detected consistent low concentrations of Ich DNA ( $C_q$  ranging from 25 - > 40, reflective of  $10^{-3}$  of an individual Ich trophont) at both sites throughout the summer (Figure 4). Assay specificity was addressed through the observation of single peaks in melt curve analysis of all samples with measurable DNA. Higher concentrations were observed in 4 of 5 sample dates at the downriver Hwy101 site than near Blue Creek. The DNA content of the infective stage (theront) compared to the trophont stage (the control sample) in QPCR is currently unknown. Both the tissue and water sample QPCR are to be considered a preliminary data as these are new assays with only limited field validation.

Table 3. Ich prevalence of infection (POI) in sentinel speckled dace held for 7d exposures in the Klamath River below the mouth of Blue Creek (Blue Creek) or the highway 101 bridge (Hwy 101) between 05 August – 09 September. Data reported as POI in QPCR (PCR POI) assayed gill tissue, Abnormal Amplification Curve (AAC) sample number (not included in POI data), and positive detection in left gill filaments by wet mount microscopy (WM).

sample date	Hwy101				Blue Creek		
	PCR POI	AAC	WM		PCR POI	AAC	WM
12-Aug	1 / 4	4	1 / 16		0 / 6	2	0 / 18
19-Aug	0 / 7	1	0 / 13		3 / 7	1	0 / 19
26-Aug	0 / 6	2	0 / 19		2 / 6	2	0 / 20
2-Sep	1 / 8	0	0 / 20		2 / 6	2	0 / 20
9-Sep	1 / 7	1	1 / 19		0 / 5	3	0 / 18
Prevalence	3 / 32 (9%)	8 / 40 (20%)	2 / 87 (2%)		7 / 30 (23%)	10 / 40 (25%)	0 / 95 (0%)

Figure 4. Gill cyst (white arrow), of presumptive *Myxobolus sp.* myxospores (black arrow), in Shasta River dace used for sentinel study. Low and high magnification of wet mount sample using phase microscopy.

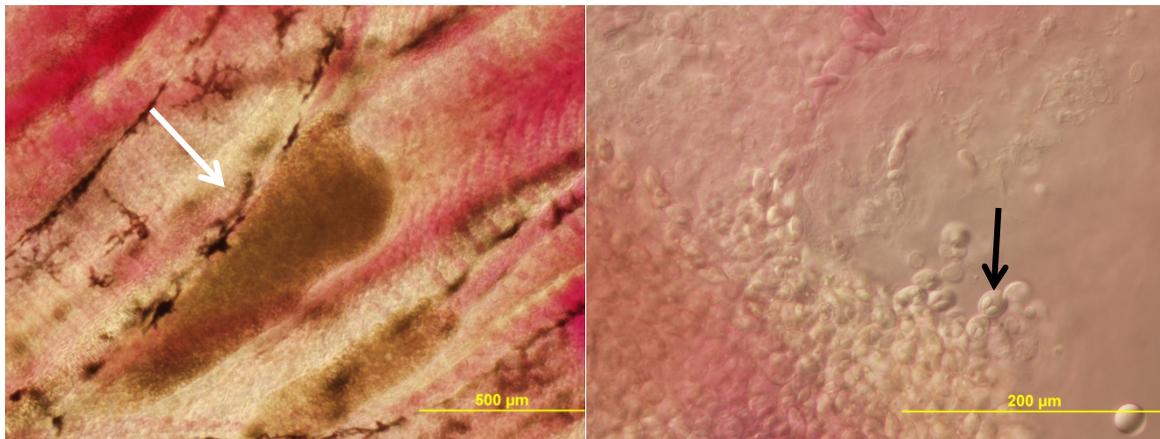
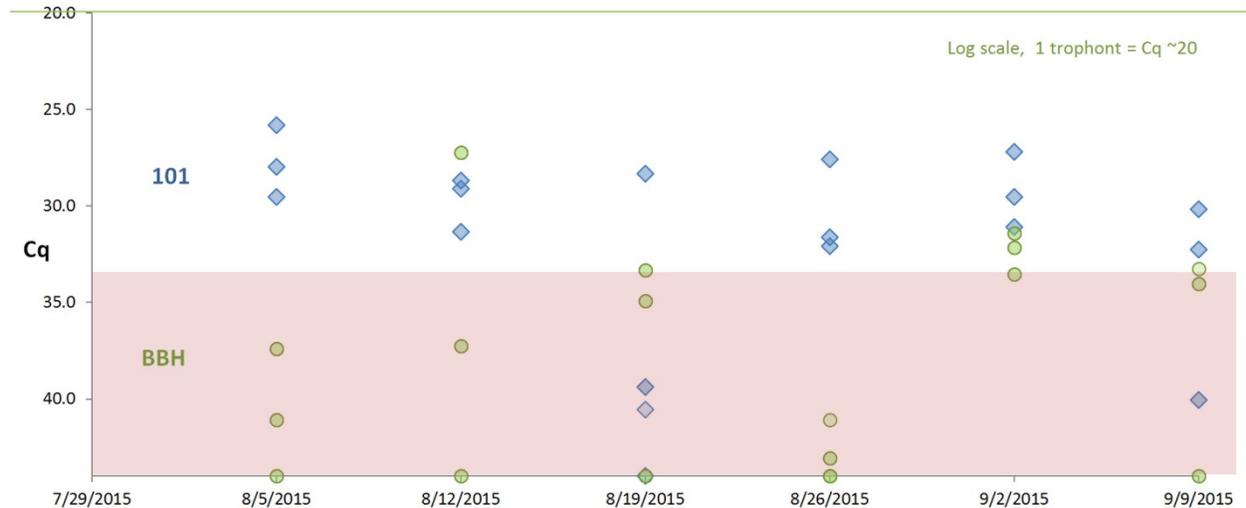


Figure 5. Ich DNA detected in triplicate 1 liter water samples collected at the beginning and end of each sentinel exposure at highway 101 (101, blue diamond) and directly below Blue Creek (BBH, green circle). Higher quantification cycle (Cq) number has an inverse relationship to target DNA concentration. Values within the red shaded region were considered below the current detection threshold. Note that 1 Ich trophont has a C<sub>q</sub> of approximately 20.



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