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California Nevada Fish Health Center

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Juvenile Lost River Sucker sentinel survival in Upper Klamath Lake mesocosm cages: July 2012 - March 2013.

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Summary: Captive Lost River sucker (*Deltistes luxatus*) fry were exposed as water quality sentinels for 21 days(d) at two locations in Upper Klamath Lake between 17 July and 9 October 2012. An additional group was held for 170d over the winter. A laboratory experiment was conducted to examine the role of starvation and stress on bacterial resistance of fry at summer water temperatures. Cultured sucker survival was high until pelleted diet was fed for several weeks. Gill hyperplasia affected the captive fry population in August but was not associated with water quality or infectious agents. Nutritional factors are suspected with an interesting observation of rapid recovery in fry reared over lake sediment. We hypothesize that gut microflora, associated with lake sediment, may be needed for assimilation of B-vitamins. Fry recovered and were used for sentinel deployments in September and October. Control groups had complete survival. Starvation, not handling stress events, was the primary factor associated with increased bacterial susceptibility in laboratory challenges. A delayed mortality pattern was observed in all sentinel exposures in which $\geq 77\%$ died between 7 and 14 days post exposure. Disease and starvation were not linked to mortality. Microcystin toxin was detected by immunohistochemistry in the digesta and distal intestine of all sentinel groups. Only 1 of 45 liver sections had a positive reaction for this toxin. Moderate severity of hepatocyte abnormalities (cytoplasmic protein inclusion, coagulative necrosis, apoptosis) was consistently observed in the livers of all sentinels. The first exposure group experienced both high pH (>10.0) and ammonia while low dissolved oxygen concentrations (2-4 mg/L) were associated with last 2 groups. Overwinter survival of small fry (<30mm) was only 3%.

We hypothesize that sucker fry mortality (direct and indirect) is driven by a synergism of chronic exposure to microcystin toxin and adverse water quality (high ammonia and pH and /or low dissolved oxygen concentration during energy intensive warm water conditions). Physiological exhaustion, due to the above stressors, could be the primary driver of lake-wide recruitment failure for 0+ Lost River suckers in Upper Klamath Lake.

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Introduction

In 1988, the US Fish and Wildlife Service listed the Upper Klamath basin Lost River Sucker (*Deltistes luxatus*) as an endangered species. Factors contributing to Lost River Sucker (LRS) decline include over-harvest, habitat loss, negative interaction with non-native fishes, sporadic survival of 0+ suckers, and episodic mass mortality of adults (Markle and Cooperman 2002, Terwillieger et al. 2003, Rasmussen 2011). Poor water quality has been linked to the adult mortalities (Perkin et al. 2000) and may negatively influence juvenile survival. This fish inhabits the eutrophic waters of Upper Klamath Lake in Southern Oregon that supports massive summer blooms of the cyanobacterium *Aphanizomenon flos-aquae*. These blooms are associated with chronic high alkalinity (pH > 9.5) as well as transient periods of anoxia (dissolved oxygen < 2.0 ppm) and elevated ammonia (Wood et al. 1996).

Both bacterial and parasitic pathogens have been consistently observed in asymptomatic (apparently normal) juvenile suckers collected in Upper Klamath Lake (Foott and Stone 2005, Foott et al. 2007, Foott et al. 2010). Facultative pathogenic bacteria (*Aeromonas caviae*, *A. punctata*, and *A. hydrophilia*) have been isolated from the skin and gills of asymptomatic juvenile Lost R. (LRS) suckers in Upper Klamath Lake during August of 2006 (pers. comm. C. Ottinger, USGS Leetown). Synergistic effects of elevated water temperature (rapid microbial multiplication), presence of endemic bacteria and parasites, and stressful water quality (impaired immune functions and insufficient fat reserves for overwinter survival) is a potential avenue of juvenile sucker mortality in Upper Klamath Lake.

Low recruitment success has been observed in skewed age data (Scopettone and Vinyard 1991). Similarly Janney et al. (2008) report homogeneous size structures in recent years suggestive of poor recruitment. After their first fall, however, young suckers have been rarely observed despite widespread sampling of Upper Klamath Lake with a variety of gears (Buettner and Scopettone 1990; Burdick et al. 2008; Reiser et al. 2001; Simon et al. 2009). Extremely low catches of age-1 or older adolescent suckers suggests that survival rates of age-0 fish are very poor and that recruitment failure may occur during the first year of life. Burdick et al. (2008) stated that 0+ juveniles catch declines significantly between August and October each year. Several hypotheses concerning the causes of poor age-0 to age-1 survivorship have been suggested including emigration out of the lake (Harris and Markle 1991; Gutermuth et al. 2000) and poor summer water quality conditions (Martin and Saiki 1999). In a 2011 sentinel study with LRS fry, Foott et al. (2012) reported high mortality of fry held within the Williamson R. delta. These fish experienced hypoxia and showed signs of starvation. In contrast, sentinels held in the north lake had > 48% survival and showed steady growth and triglyceride fat accumulation during the summer.

We focused this study on LRS fry survival during the late July through October time frame. One sentinel groups was held over the winter. The study design used captive reared progeny, of Upper Klamath Lake Lost R. sucker adults, held in replicate mesocosm cages. Cultured fry provided experimental fish of known age, health, similar size, and energy content. The mesocosm cage represented a link between observational field studies in the lake and controlled laboratory experiments (Sala et al. 2000).

Specific objectives were:

1. Develop early rearing techniques and identify husbandry problems.
2. Determine temporal survival of sentinel fry in the north and south lake.
3. Perform histological examination of select tissues for abnormalities and pathogen infection in sentinel fry.
4. Monitor temperature, ammonia, total dissolved gas, dissolved oxygen, and pH of water at the two sites in order to observe relationships to survival.
5. Through controlled laboratory experiments, determine the relative effects of starvation and stress on bacterial resistance.

Methods:

Fish rearing – On 15 May 2012 gametes were obtained from 4 male and 2 female Lost River suckers captured by USGS at Sucker Springs, Upper Klamath Lake. These fish were released immediately after a small portion of their gametes were collected (~36 g / female wet weight of eggs and 2 mL of semen). We estimate that approximately 20,000 eggs were collected (275 eggs/ g {pers. comm. S. Vanderkooi, USGS} x 36g samples x 2 females). This egg collection is a small percentage of the 50,000 – 100,000 eggs per female reported for 500 – 750 mm FL female LRS (Buettner and Scopettone 1990). Gametes from one female were expressed directly into a plastic Tupperware container and semen immediately added into the same container. This process was repeated with an additional female and two additional males into a separate container. Gametes were gently mixed for 10 minutes, excess fluid poured off, and the container filled with lake water then placed into an ice chest for transport to the Ca-NV Fish Health Center. Upon arrival, lake water was poured off and eggs disinfected in a 100ppm iodine solution for 5 minutes. This process was repeated once more to insure adequate surface disinfection of the fertilized eggs. Virological assay of ovarian and seminal fluid did not detect virus (USFWS and AFS-FHS 2007). Two prior attempts to obtain viable gametes were made in late April and early May. Approximately 12,000 eggs were taken at each spawn (~22g from 2 females each). The April spawn yielded no eyed eggs while attempt 2 produced < 30% eyed eggs. The majority of these eggs hatched prematurely and swim-up survival was poor. Formalin treatment (100 mg/L, 10 min bath) in a portion of spawn #2 eggs was associated with premature hatch but did reduced fungus infection.

Eggs were incubated in upwelling cylinders (5in PVC with 250µm mesh screen bottom) at 13-14° until larvae were actively swimming and observed feeding. Prior to hatch, it was necessary to treat with 1600 ppm formalin for 20min and pick fungus eggs. First hatch was observed 22 May. By 27 May, 99% of all larvae had hatched and were actively swimming. Larvae were subsequently reared in 2.8L baskets (6.5" x 5" x 5.25" frames covered by a 500µm mesh bag) suspended within aquaria. Rotifers (*Brachionus plicatilis*) were fed in a solution of Nanno Greenwater Instant Algae (Reed Mariculture®) (Nannochloropsis sp.) to provide visual contrast for the larvae. Newly hatched artemia nauplii were introduced at 4-9 days post-hatch (dph) and a larval diet (1.0mm Bozeman June Sucker diet, manufactured by Skreting USA, Longview, WA and purchased directly) started 7-12 dph. Pellet size ranged from 250µm - 900 µm over the experiment and 3 feedings were done per day (pellet at estimated 5-6% bodyweight

/d). There was an overlap of approximately 1-2 weeks for each new diet with the previous diet type due to expanding size variation. We estimate that the population experienced a 16% loss from egg to 20mm fry. No virus was detected in a 60 fry sample performed in July prior to the first movement to Upper Klamath Lake. Fry were transported to the lake within 0.67 cu ft bucket cages held in tanks containing water adjusted to pH 9.0 at ambient lake temperature. Our intent was to provide some acclimation to lake conditions during the 3 h transport to the lake.

Exposures - Three exposures were conducted between 17 July and 09 October at 2 sites (Table 1, Figure 1). The sites were 1) northern Upper Klamath Lake near Modoc point and 2) southern Upper Klamath Lake near Buck's Island. Unlike the study in 2011, bucket cages did not extend the full depth of the water column. The weighted cages were positioned vertically on the lake bottom. Total height of each cage was approximately 12 inches and contained 20 sentinel fish. Lake depth ranged from 2.0 m at both exposure sites on 17 July to 2.0 m in the south and 1.5 m at the northern sites on 09 October. Bucket cages were sampled in triplicate each week from both exposure locations.

The black bucket cages and lids used were constructed from high-density polyethylene (see photo on report cover). Overall bucket cage dimension was 11.91" diameter x 14.50" high x 10.33" diameter at the bottom with a wall thickness of 0.090" (+/- 0.005"). Each cage was outfitted with six 3 inch ports around the side, twelve 1 inch ports in the lid and one 5 inch port in the bottom (center) allowing for water exchange and foraging of benthic material. All ports were covered by 2mm square fiberglass coated mesh to eliminate escape. A 5 inch ring of schedule 40 PVC was affixed on the inside bottom of each bucket cage to protect the 5 inch port and concrete was poured around the PVC ring acting as a weight insuring the vertical alignment of each cage on the lake bottom. Stainless steel eye bolts were attached to the bucket cage lids. Quarter inch nylon rope was attached to each lid with a total length of approximately 3 m. Each rope was then attached to a high density polyethylene jug acting as a buoy. Each buoy was given a unique alpha numeric number used to identify sampling groups.

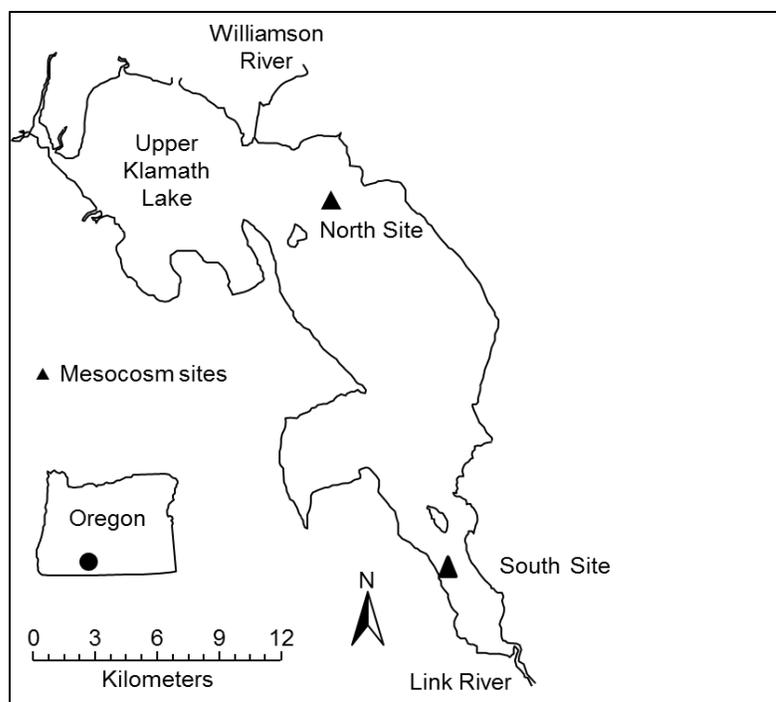


Figure 1.—Map of the study area in Upper Klamath Lake, Oregon, showing the northern and southern cage sites.

Bacterial challenge of stressed and starved fish – Four groups of 80 fry (Fed no stress, Fed stress, Unfed no stress, and Unfed stress) were placed into separate bucket cages held within separate 800 L circular tanks supplied with flow-through, aerated 20°C water. Fed groups were given June sucker diet twice daily at 4% BW/d. A standard stress event was applied to the stress groups twice daily for 3 consecutive days immediately prior to a bacterial challenge. Stress group cages were lifted into the air for 30s followed by the same action 3 h later. On the day of the challenge, 30 fry were removed from each cage. Five fry were immediately euthanized in an overdose of MS222 and frozen at -20°C for weight and length measurement as well as whole body cortisol assays. The remaining 25 fry were placed within a 15x15cm cage held in an aerated 20L aquaria. The aquarium was within a larger 18 - 20°C water bath. *Yersinia ruckeri*, grown in Brain heart infusion broth for 24h, centrifuged and re-suspended in PBS to approximately 10^9 cfu/mL (OD_{600} ~0.9 – 1.2) was added to the challenge tank as a 10x dilution (final concentration 10^8 cfu/mL). Plate counts provided the actual challenge concentration. After 24h, fry were moved to separate 40L aquaria supplied with aerated 20°C and mortality monitored for 3 – 7d. On 27July (Trial 1), fry were challenged for only 6h. Fresh dead fry were sampled for bacterial isolation. Whole body cortisol was assayed by the method of Sink et al. 2007 using an cortisol ELISA kit (Neogen Corporation, Lexington KY). Small fish weights necessitated a minimum homogenization volume (800 μ L). Briefly, fry were defrosted, weighed (0.01g) and measured with calipers (Standard length 0.1mm), placed into 20mL tubes with 800 μ L PBS, homogenized for 10-20s with a hand-held mixer (Biospec M133), 100 μ L vegetable oil added and mixed followed by 800 μ L ethyl acetate. The sample was frozen at -20°C and the liquid solvent layer carefully pipetted (mean 43% recovery of added ethyl acetate, 6-88% range) off the frozen portion. The solvent was placed into a glass tube held in a 40°C heat block and evaporated under a slow stream of nitrogen

gas. Prior to assay, extract was dissolved in 150µL of extraction buffer supplied by the ELISA kit. Data was reported as ng cortisol/g fish and condition factor ($KSL = wt/SL^3 \times 10^5$).

Laboratory analysis

Sample preparation - Standard length and weight were recorded for each frozen fish and its condition factor calculated ($KSL = WT/ SL^3 \times 10^5$). König and Borchering (2012) report that freezing has less effect on morphometrics than preservation in ethanol or formalin. Cold distilled water was added to a 20 mL tube containing each fish (800 µL each due to small fish size) and blended for 30 – 90s with a Biospec M133 homogenizer. Tissue triglyceride content (mg TG / g tissue) was assayed by a modification of Weber et al. 2003. Absolute isopropanol was added (5x dilution w/v) to an aliquot of homogenate, mixed at room temperature for 20 min, centrifuged at 3220xg for 5 min, and replicate 10 µL samples of the 10x diluted supernatant used in an enzyme assay for triglyceride (Pointe Scientific triglyceride GPO kit).

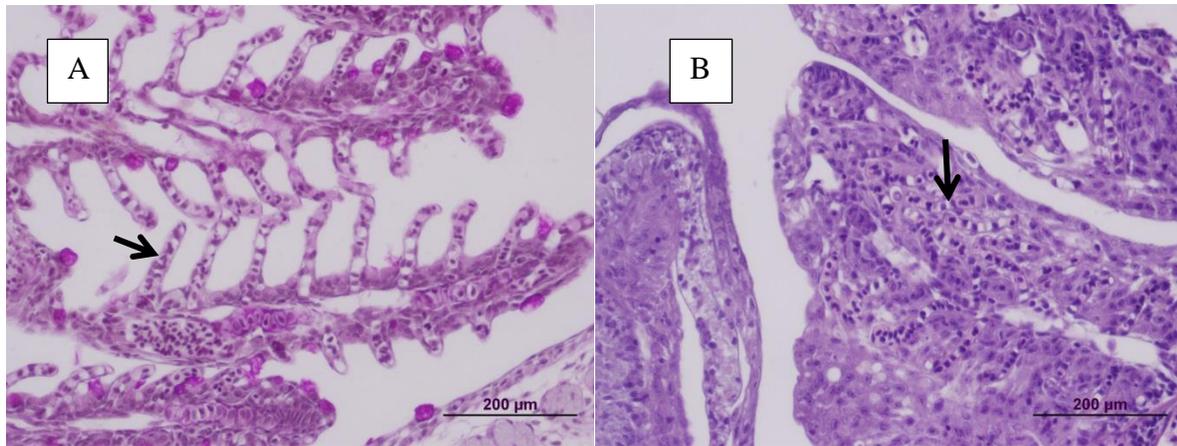
Histology - Histological samples were held in Davidson's fixative or cold zinc formalin (Zfix, Anatech Ltd, Battle Creek MI) for 24h, processed for 5µm paraffin sections, and stained with hematoxylin and eosin. Zinc formalin was chosen to accommodate immunohistochemistry. Sagittal sections were made of smaller fish with tissues examined including skin, muscle, gill, thymus, olfactory pit, eye, brain, liver, intestine, adipose tissue, acinar cells, kidney, and the peritoneal cavity. Fish larger than 70mm were dissected and specific organs (kidney, gastrointestinal tract, gill, and liver) processed for histology. Abnormalities were rated on an ordinal 0,1,2,3 scale based on distribution (none, focal, multifocal, and diffuse). A subset of zinc formalin and Davidson fixed specimens were examined for the presence of microcystin-LR antigen in the liver, kidney, and intestine by the immunocytochemistry method of Djediat et al. (2010) using a monoclonal antibody to microcystin-LR (MC10E7, Enzo Life Sciences ALX-804-320) and the universal VECTASTAIN kit (Vector laboratories). Positive (MCLR treated Chinook) and negative (wetlab LRS) control sections were processed with the sample set.

Laboratory data statistical analysis - Analysis was performed with SigmaPlot 12 software on raw data. Normality was tested by the Kolmogorov – Smirnov method at the $P = 0.05$ level. One-way ANOVA or T-test (data with normal distribution, reported with F or t value) or Kruskal-Wallis ANOVA or Mann-Whitney U test on ranks (non-parametric analysis) with subsequent multiple comparison procedures (Holm-Sidak or Dunns method respectively, $\alpha \leq 0.05$) was used to compare groups.

Permits- Permits for this project are as follows: sub-permit FWSKFFWO-7 (USFWS Klamath Falls Office) under the USFWS Region 8 regional blanket permit (TE-108507), Section 10 Federal authorization number TE007907-14 (adult brood stock, TE007907-14), Oregon Scientific Take Permit Number 16157 M2, and California DFG Standard Importation Permit number 2011-1623.

Results: *Fish culture and cage-* No cage failures were observed over the study and the weighed bottom kept the bucket cage upright on the bottom. As mentioned above, mortality of the wetlab population was minor through July. On 7 August, we observed high mortality in groups destined for transport to UKL and subsequently canceled their movement to the sentinel sites. Daily mortality began a doubling pattern in early August with a maximum cumulative mortality of 0.04% / day. Mortalities remained high (>500/d) until 13 August at which time total mortality per day fell below 100. The sentinel population saw an overall loss of approximately 8,000 fish between 27 August and 01 September with 73% (5778) occurring between 04 August – 13 August. As seen in 2011, moribund fry exhibited tetany and twirling behavior when startled. These clinical signs suggest ionic imbalance. Gill hyperplasia was the primary clinical sign associated with morbidity. While *Aeromonas sp.* was isolated from the peritoneum / kidney, the small size of the fish made aseptic collection from the kidney very difficult. Additionally, no petechial hemorrhage (internal organs or dermis) was observed nor were gram-negative bacteria seen in kidney imprints. Diagnostic laboratory samples for virus and external parasite were negative. Histological examination confirmed severe gill hyperplasia (Figure 2), normal liver cells, extensive lipid vacuolization of distal intestinal epithelium, and a moderate degree of kidney tubule hydropic change. The June sucker diet was reported by the manufacturer to contain sufficient vitamin content as well as protein and lipids from krill and fish sources. Sublots of fry were then fed 4 additional diets 1) brine shrimp- krill-spirolina flake (equal parts), 2) Orange (INVE Aquaculture, Salt Lake City, UT) a diet used for delta smelt, 3) June Sucker feed and Bio-Oregon salmon starter (Bio-Oregon, Longview, WA) mixed at a 1:1, and 4) adult Artemia with SELCO enrichment (INVE Technologies, N.V. Joint Stock Co.). No obvious improvement was noted in the chronic mortality pattern over a 14d period. Gill hyperplasia is reported to be associated with malabsorption or insufficient intake of biotin, pantothenic acid, or essential n-3/n-6 fatty acids (Lall 2010). Intestinal microflora are involved with biotin synthesis. One subplot was placed into an aquarium containing Upper Klamath benthic lake sediment and fed lightly with June sucker-salmon diet. Morbidity dropped significantly in this subplot over the next 10 days with a return of normal gill structure. Mortality declined in the wetlab population by late August and no mortality was observed in fry held for 48h in cages. Starting 20 August fish were treated with a 1% salt bath for 30 minutes for seven consecutive days. Sentinel fry were moved to the lake on 28 August and the laboratory controls for this transfer group did not experience mortality over a 14d period.

Figure 2. Normal (A) and hyperplastic (B) gill of LRS fry sampled from wetlab population in August 2012. Note lamellar artery (arrow) in both micrograph for orientation.



Sentinel survival- The pattern of mortality was chronic not acute in sentinel groups. Survival was high (>85%) at 7day post-exposure (dpe), with the exception of the 25September south cages (mean 18% survival, Figure 3). At 14 dpe, survival declined in all groups with only $\leq 23\%$ surviving to day 21. In the second and third exposures, north site groups had higher 14dpe survival (60 and 80%) than cohorts held in the south (10 and 23%). Generally, there was little variation in mortality between replicate cages at a given sample site. Five sample groups had 25 – 31% range in cage mortality which equates to a 5 to 6 fish difference in mortality (Table 1). Fourteen day post-exposure survival was over 95% in non-transported, fed controls held in bucket cages within the wet lab (see fed-stress laboratory challenge section below). Additionally, transported control fry (driven to Klamath fall boat launch, held for 5h in the transport tank and returned to the wetlab) were monitored for 21d in exposures 2 and 3. No holding mortalities were observed in these transport controls.

Table 1 – Exposure period, site location, sample dates, number (n) of mortalities observed per cage and mean percent mortality for each exposure at North and South locations.

Exposure	Site	Date	Mortality Per Cage (n)			Mean Percent Mortality
			A	B	C	
Exposure 1 17July – 06August	North Lake	24July (7 dpe)	1	1	1	5%
		31July (14 dpe)	20	20	20	100%
		07Aug. (21 dpe)	20	20	20	100%
	South Lake	24July (7 dpe)	1	5	0	10%
		31July (14 dpe)	16	15	12	72%
		07Aug. (21 dpe)	20	20	20	100%
Exposure 2 28August – 18September	North Lake	04Sept. (7 dpe)	1	2	2	8%
		11Sept. (14 dpe)	11	8	5	40%
		19Sept. (21 dpe)	19	20	18	95%
	South Lake	04Sept. (7 dpe)	1	7	1	15%
		11Sept. (14 dpe)	17	20	17	90%
		19Sept. (21 dpe)	20	20	20	100%
Exposure 3 19September - 09October	North Lake	25Sept. (7 dpe)	0	2	5	12%
		02Oct. (14 dpe)	3	5	4	20%
		09Oct. (21 dpe)	17	12	17	77%
	South Lake	25Sept. (7 dpe)	15	17	17	82%
		02Oct. (14 dpe)	16	16	15	78%
		09Oct. (21 dpe)	18	19	18	92%

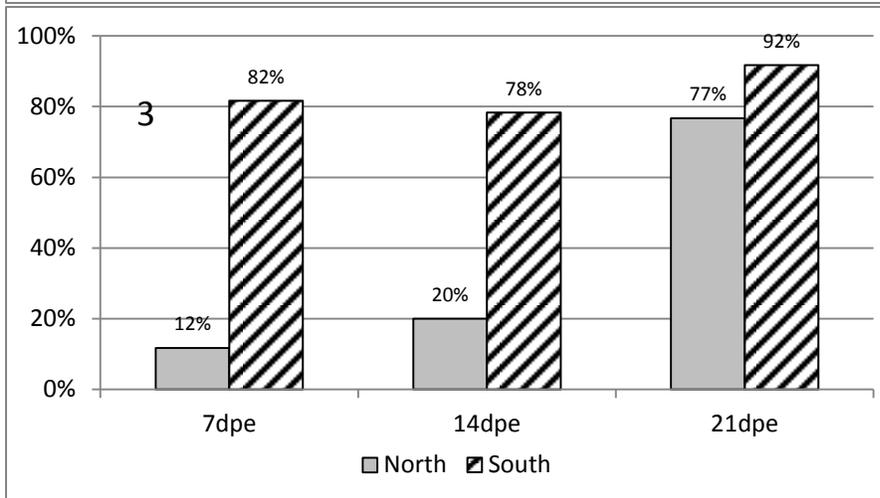
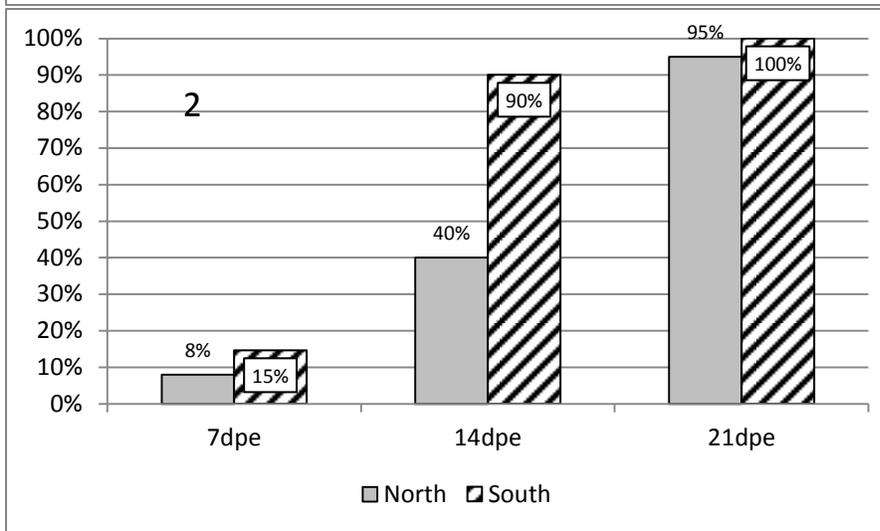
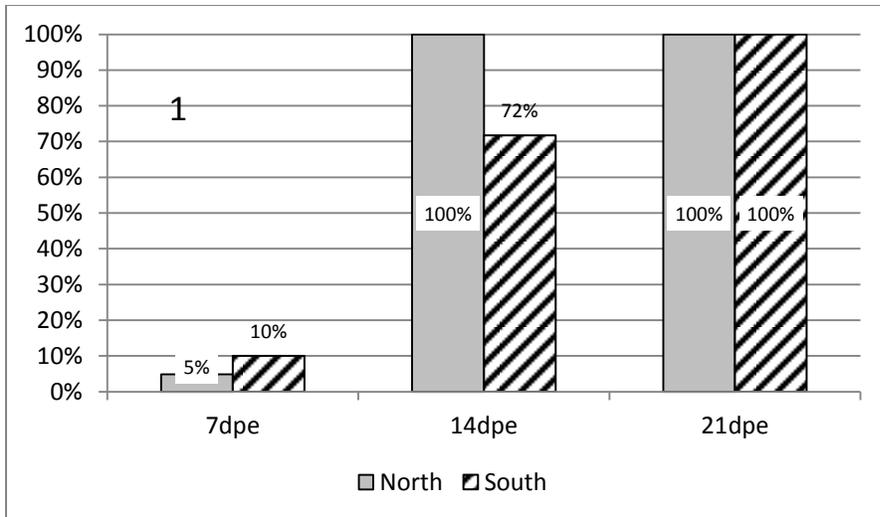


Figure 3. Mean sentinel mortality for north and south site cages sampled at 7, 14, and 21 days post exposure (dpe) for exposure 1 -3.

Condition of sentinels – Given their brief 7-14d exposure, energy analysis of sentinel fish was not part of the original study design however, a post-hoc survey of frozen carcasses was performed to determine if overt starvation could be identified as a mortality factor. Both the main laboratory population and cage survivors showed minimal growth over the summer. At the time of transport to Upper Klamath Lake, mean standard length (SL) was 18mm on 17July and fry only reached 22mm by 19September. Condition factors of the laboratory population upon movement to Upper Klamath Lake was consider normal (1.2 – 1.5 KSL). No growth in length or weight was observed in sentinels sampled at 7, 14, or 21d post-exposure (Table 2). Condition factors were greater than the emaciated threshold of ≤ 1.0 observed in fed / unfed challenge fry (Table 20) and lake sentinels in 2011 (Foott et al. 2012). One exception was the low KSL values (mean 1.0) observed in 31July (14dpe) south sentinel survivors (Table 2). Whole body triglyceride (TG) values were within the ranges seen in previous sentinel fish (Foott et al. 2012). TG values tended to decline between 7 and 14 dpe (Table 2). Small fish size likely affected the detection level of the TG assay. This assay constraint was demonstrated by the percentage of samples below negative cutoff (BNC) in the first and third exposure groups. While we expected this detection issue in samples from the first exposure (18mm SL fry), it also occurred in larger 23mm fry from the 25September and 2October south sample groups.

A “winner and loser” trend was apparent in the 3rd sentinel exposure groups with both the highest TG values observed for 2012 sentinels as well as having 30 and 60% of the samples below detection. Mann Whitney rank sum test of SL, KSL, and TG of sentinel groups held at the 2 locations for any given sample date were not significant ($P < 0.05$). Coefficient of variations ranged from 12 – 100% in TG dataset and was particularly high in south site fry. While growth and increased energy (TG) reserves did not occur during sentinel exposures, condition factors of survivors did not indicate starvation.

Only 3% of the fry (1 of twenty 21d lake survivors and 2 of 84 wetlab fry placed into the benthic cage on 9 October) held over the winter for 170d were recovered on 28March2013. Escape is unlikely as no breaks in the cages were observed however there were several large 80 – 100mm leeches in each cage. These leeches were much larger than the cage mesh and we hypothesize that they could have entered as smaller individuals in the fall, preyed upon fry, and grown. Surviving fry had grown in length (6-14mm) but depending on origin showed differences in condition factor and TG reserves (Table 2). The single survivor from the 21d exposure group (lake pool) had higher KSL and TG values than the mean of the cohort 9Oct sample while wetlab fry lost both weight (KSL) and 8-12x of their starting TG content.

Table 2. Mean (SD) standard length (SL, mm), condition factor (KSL= Wt/SL³x10⁵), and whole body triglyceride content (TG mg/g fish) of sentinel fry held for 7-20d in the north and south Upper Klamath Lake sites. Survivors of winter cage exposure also recorded. Data includes the percentage of TG samples below the negative cutoff (BNC) for the assay.

		NORTH	SOUTH	NORTH	SOUTH	NORTH	SOUTH
	DPE	SL	SL	KSL	KSL	TG	TG
24-Jul	7	17 (0.8)	18 (1.6)	0.645 (0.094)*	0.568 (0.277)*	3.9 (0.5)	8.1 (3.7)
n		6	6	6	6	4	3
%BNC						33%	50%
31-Jul	14	ND	17 (0.8)	ND	1.000 (0.107)	ND	3.1 (0.7)
n			5		5		4
%BNC							24%
4-Sep	7	22 (3.6)	20 (1.1)	1.441 (0.267)	1.169 (0.130)	2.3 (0.5)	1.9 (0.6)
n		6	5	6	5	6	6
%BNC						0%	0%
11-Sep	14	21 (2.1)	19 (1.4)	1.312 (0.425)	1.250 (0.124)	1.6 (0.5)	1.6 (0.0)
n		6	2	6	2	6	2
%BNC						0%	0%
25-Sep	6	22 (1.6)	21 (4.1)	1.302 (0.228)	1.280 (0.244)	1.5 (0.2)	4.2 (2.8)
n		6	3	6	3	6	2
%BNC						0%	30%
2-Oct	13	23 (3.8)	22 (4.9)	1.481 (0.948)	1.238 (0.459)	1.4 (0.3)	3.3 (3.3)
n		6	5	5	5	6	3
%BNC						0%	60%
9-Oct	20	lake pool		lake pool		lake pool	
n		23 (1.9)		1.383 (0.389)		0.6 (0.1)	
%BNC		5		5		3	
28-Mar	190	37		1.563		0.94	
		1		1		1	
9-Oct	na	wetlab		wetlab		wetlab	
n		25 (3.4)		1.574 (0.234)		4.7 (2.5)	
%BNC		12		12		12	
28-Mar	170	34 (3)		1.141 (0.093)		0.52 (0.17)	
		2		2		2	

* Condition factor of 24July frozen carcasses cannot be compared to other groups due to dehydration

ND No data as no live fish available to sample

na not applicable

Histological analysis – A total of 144 sagittal sections stained with hematoxylin and eosin and 45 duplicate sections assayed by immunohistochemistry for microcystin LR toxin were examined for tissue changes and infectious agents.

Eosinophilic droplets were observed in the proximal tubule epithelial cells of kidneys from both south and north site fry sampled on 24July (Table 3, Figure 4). This reversible condition is sometimes referred to as hyaline degeneration (Tubule hyaline droplet = THD in Table 3) and is due to excess lipoprotein accumulation within the epithelial cell phagolysosomes (Cotran et al. 1989). High environmental ammonia induces diuresis in freshwater fish which in turn results in excess protein within the nephron filtrate. The association of THD with elevated ammonia has been reported in juvenile LRS and other FW fish (Foott et al. 2000, Daoust and Ferguson 1984). Similar to elevated ammonia, formation of protein hyaline droplets within the proximal tubules has also been reported in fish as an initial response to hypoxia (Tervonen et al. 2006). Unfortunately, ammonia measurements were not performed on 24July however we suspect that levels were elevated as the highest ammonia readings of the study occurred on the following 2 samples dates of 31July and 7August.

Gill morphology was largely normal in the 137 tissue sample set. *Trichodina* sp. was observed on the gills of one south site sentinel fish collected on 7August however no inflammation or necrosis was associated with the parasites. Edema of the secondary lamellar epithelium (gill edema = GED in table 3) was in some samples from 3 collection groups: 1) 7, 14, and 21dpe samples at the North site during the second exposure (28August – 19September), 2) 7dpe sample at south site on 24July, and 3) 20dpe sample at north site on 9October (Table 3, Figure 5). This abnormality had been observed in low ammonia control LRS held in pH 9.5 water (Foott et al. 2000) but can also result from delayed fixation (Ferguson 1989). No obvious water quality parameter was associated with abnormality.

Intestine and acinar cell (pancreatic tissue) morphology was largely normal in the 94 tissue sample set. Large clear vacuoles (presumptively containing lipid) was seen in the distal intestine epithelium of a variable number of fish (0 – 64%) in most sample groups. We see this feature in the laboratory population and consider it normal. Single cell necrosis was also seen in the distal intestine but was considered minor given its low prevalence and distribution (<5% of the intestinal epithelial cells). No parasites or inflammatory response was seen in the sample set. It appears that the fry, at both sites over the entire study, consumed *Microcystis* sp. or material containing the MC-LR toxin. The distal intestine epithelial cells and gut content was immunohistochemistry positive for MC-LR toxin in all sample groups (Table 4, Figure 10). As mentioned above, the occurrence of the toxin was not associated with significant necrotic changes. Only one liver sample (moribund fry sampled from south site on 4September) was immunohistochemistry positive for MC-LR toxin. We do not know the sensitivity of the immunohistochemistry method to detect low levels of the toxin in liver tissue. Both fixatives were suitable for this procedure.

Hepatocyte morphology varied considerably among the sentinel groups with 3 abnormalities (cytoplasmic eosinophilic droplets, coagulative necrosis, and apoptosis).

The degree of these abnormalities was rated on a 0-3 scale (0= none, 1= ≤ 10% of tissue, 2 = 11- 30%, 3= >30%). We did not observe inflammatory cell responses, liquefactive necrosis, or presence of bacterial foci or macroparasites in the 144 fish sample set. Unlike 2011 lake cage sentinels, cytoplasmic lipid and glycogen vacuoles were rarely seen in the hepatocytes indicating a low to moderate level of energy intake by the fish. Sample groups with ≥ 30% prevalence of cytoplasmic eosinophilic droplets rated 2 or 3 (referred to as Hepatocyte Eosinophilic Droplet =HED in Table 3, Figure 6) included:

- a. Exposure 1 South 14 dpe (31 July)
- b. Exposure 2 South 7 and 14 dpe (4 & 11 September)
- c. Exposure 2 North 7 and 14 dpe (4 & 11 September)
- d. Exposure 3 North 7 and 14dpe (25September and 02October).

These eosinophilic droplets may be synthesized proteins that are inhibited from normal export from the hepatocyte and are not considered an irreversible necrotic change. Acuna et al. (2012) describe cytoplasmic inclusions in hepatocytes of Sacramento splittal fed low concentrations of microcystin and their micrograph of such lesions strongly resembled our observation of hepatocyte eosinophilic droplets (Figure 6). Similarly, Djediat et al. (2010) report “granular cytoplasm” in hepatocytes of microcystin gavaged medaka. A liver section stained with Strep-horseradish peroxidase (unsuccessful attempt to demonstrate apoptotic nuclei with Trevigen TACS-XL blue label kit 4828) may have demonstrated peroxidase reactivity of these cytoplasmic granules (Figure 8). Alternately, the granules could have a non-specific binding to the Strep-HRP reagent. Oxidative stress induced by microcystin toxins are reported to induce antioxidant defenses such as glutathione peroxidase (Wang et al. 2006, Martinez – Alvarez et al. 2005).

Coagulative necrosis of fish hepatocytes is considered a biomarker for toxic chemical exposure (including algal toxins) as well as ischemia (Hinton and Lauren 1990). Characteristics include dissolution of the nucleus, maintenance of the general cell shape, lack of inflammatory cell response, and increased eosinophilia of the cytoplasm as proteins are degraded within the cell (Cotran et al.1989). This abnormality was common in sentinel groups throughout the summer. Sample groups with ≥ 30% prevalence of hepatocyte coagulative necrosis rated 2 or 3 (referred to as Hepatocyte Coagulative Necrosis =HCN in Table 4, Figure 4) included:

- a. Exposure 1 South 7 and 14 dpe (24 and 31 July)
- b. Exposure 1 North 7dpe (24July, no survivors at 14dpe)
- c. Exposure 2 South 14 dpe (11September, 25% prevalence on 4 September)
- d. Exposure 2 North 7 and 21 dpe (4 and 19September)
- e. Exposure 3 South 7 an 14 dpe (25September and 2October)
- f. Exposure 3 North 7 an 14 dpe (25September and 2October)

Apoptotic hepatocytes were also seen in a low percentage (< 10%) of the sentinels (Figure 9). Apoptosis is characterized in H&E sections as bright uniformly stained eosin cytoplasm with condensed chromatin fragments (Cotran et al. 1989). There is a specific cascade of enzymatic triggers for this “programmed” cell death. Microcystin toxin has been shown to induce hepatocyte apoptosis in carp (Fischer and Dietrich 2000).

Table 3. Number of sagittal sections from sentinel fry collected at either the north or south site from 24July to 9October with specific tissue abnormalities (hepatocyte eosinophilic droplet HED, hepatocyte coagulative necrosis HCN, kidney tubule hyaline droplet THD, and gill edema GED) in two categories (slight to none, 0-1 score) and (moderate to severe, 2-3 score).

		HED	HED	HCN	HCN	THD	THD	GED	GED
		0-1	2-3	0-1	2-3	0-1	2-3	0-1	2-3
24-Jul	North	10	1	7	4	1	10	11	1
24-Jul	South	10	2	5	7	5	6	13	3
31-Jul	North	na	na	na	na	na	na	8	0
31-Jul	South	9	7	11	5	13	0	9	0
7-Aug	North	na							
7-Aug	South	0	1	1	0	1	0	1	0
4-Sep	North	10	5	9	6	15	0	11	4
4-Sep	South	2	14	12	4	16	0	16	0
11-Sep	North	0	2	2	0	2	0	3	5
11-Sep	South	1	4	3	2	5	0	4	0
19-Sep	North	4	3	2	5	6	0	2	5
19-Sep	South	na							
25-Sep	North	10	6	10	6	6	0	6	0
25-Sep	South	5	0	1	4	4	0	4	0
2-Oct	North	10	7	7	9	17	0	13	0
2-Oct	South	5	2	4	3	7	0	7	0
9-Oct	North	11	0	10	1	11	0	8	3
9-Oct	South	3	0	3	0	3	0	2	1

Na not applicable as there were no samples

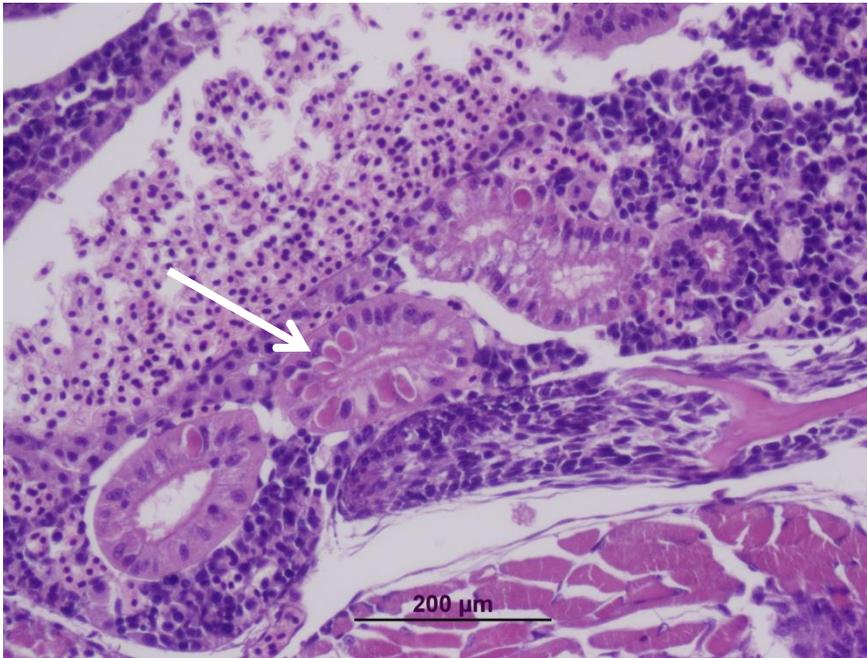


Figure 4. Eosinophilic hyaline inclusions (arrow) in kidney proximal convoluted tubule of fry sampled on 24 July north site. It is likely due to excess protein reabsorption and referred in text as tubule hyaline droplet (THD).

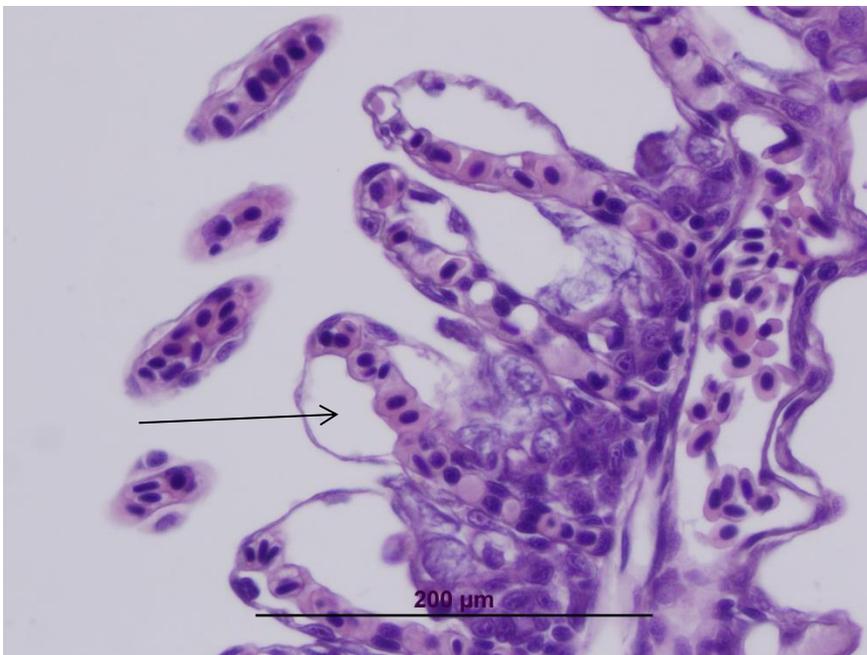


Figure 5. Gill edema (arrow), within secondary lamellae epithelium, from fry sampled on 11 September north site. Referred in text as gill edema (GED).

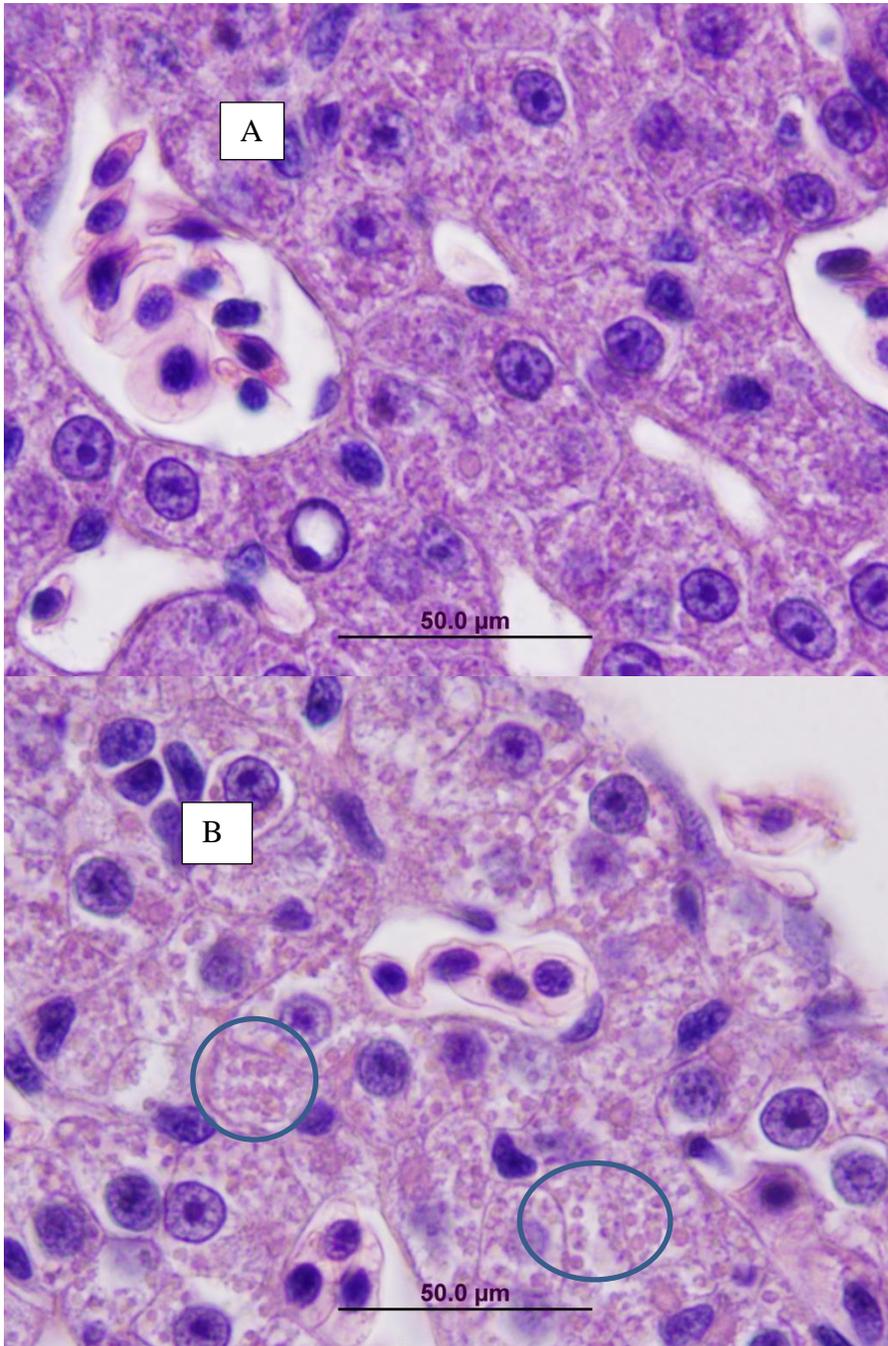


Figure 6. Eosinophilic granules within cytoplasm of hepatocytes (H&E) from sentinel fry: A) category 0 H&E with uniform fine granules considered normal, B) category 2 H&E with condensed granules (circled).

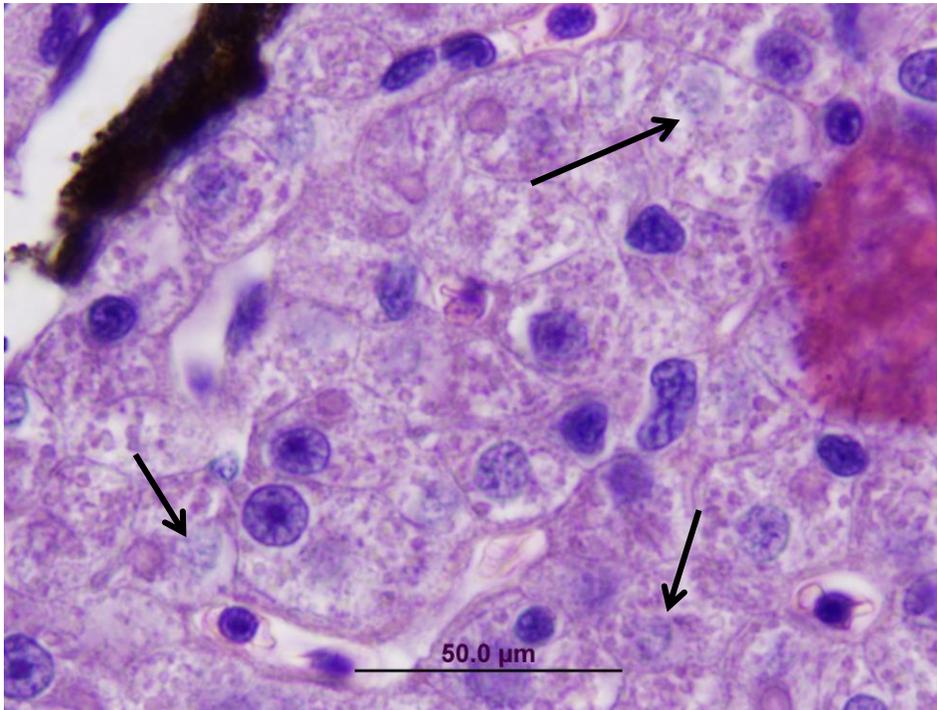


Figure 7. Hepatocyte coagulative necrosis (HCN) from sentinel fry liver sampled 4 September north site. Note intact cell membrane and missing or dissolved nuclei (arrows) of affected hepatocytes as well as lack of inflammatory cells.

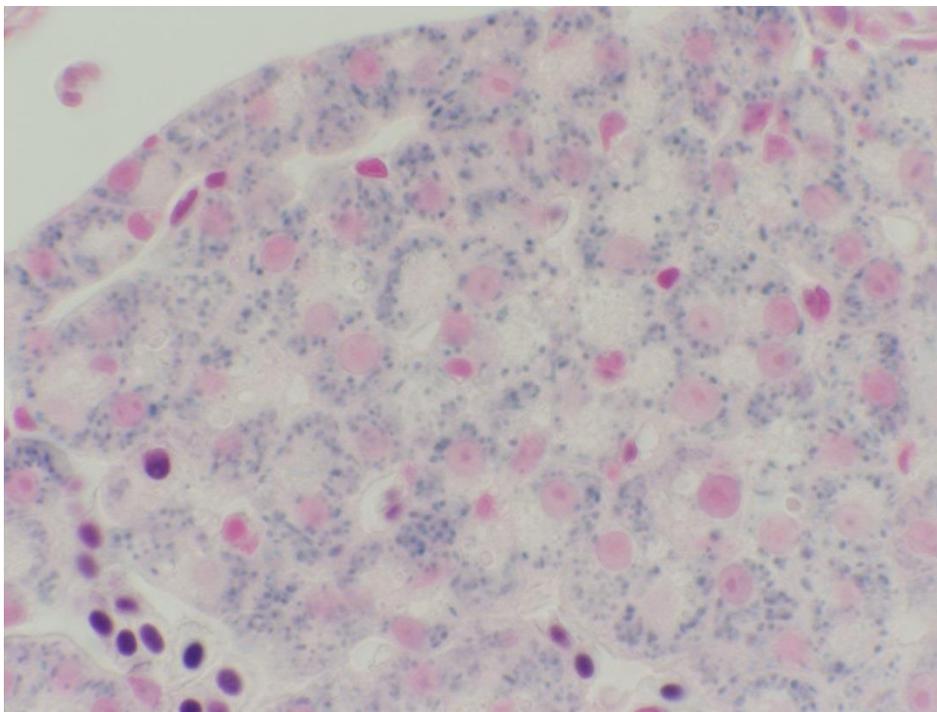


Figure 8. Peroxidase positive (blue) cytoplasmic granules within hepatocyte cytoplasm of 24 July south site sucker. Compare to eosinophilic granules seen in hepatocyte cytoplasm of Figure 7.

Table 4. Prevalence of MC-LRa immunohistochemistry positive reactions in distal intestine epithelium of sentinel fry from the north and south sites sampled between 24July and 9October 2012.

	North	South
24-Jul	2 / 2	5 / 5
31-Jul	ND	1 / 4
7-Aug	ND	0 / 1
4-Sep	2 / 2	3 / 4++
11-Sep	2 / 2	2 / 2
19-Sep	1 / 1	ND
25-Sep	2 / 2	4 / 4
2-Oct	2 / 2	2 / 3
9-Oct	4 / 4	2 / 5

++ Both liver and distal intestine positive
 ND not done as no live fry to sample

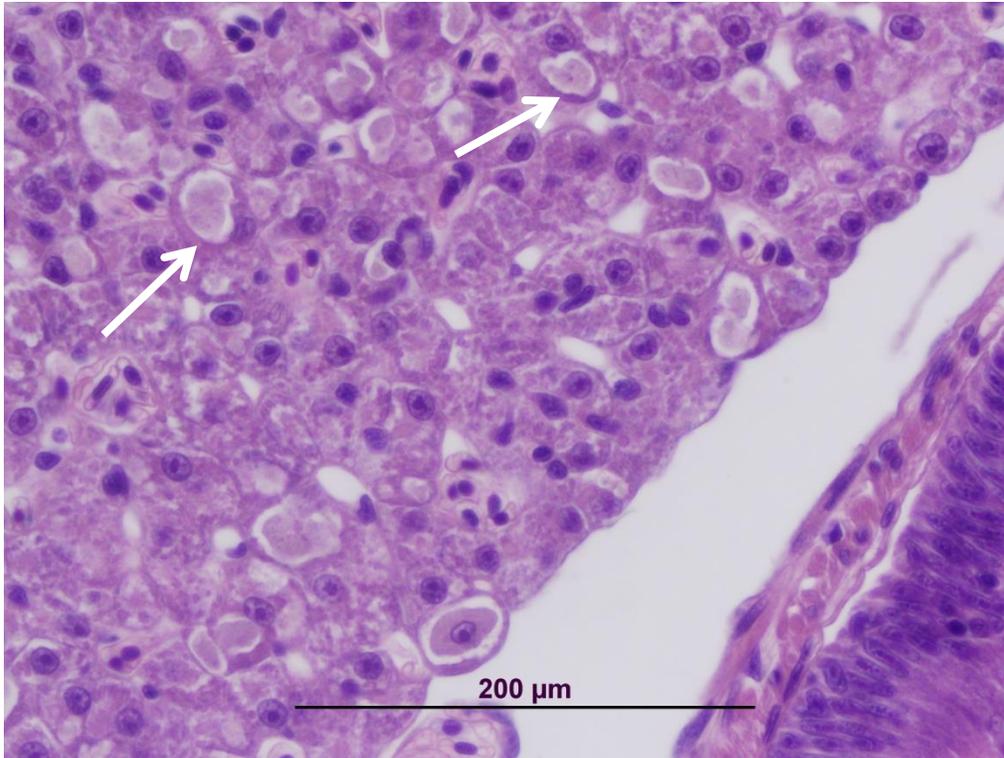


Figure 9. Sentinel liver of 2October south site fry showing distinct cytoplasmic vacuoles (arrow) suggestive of apoptosis.

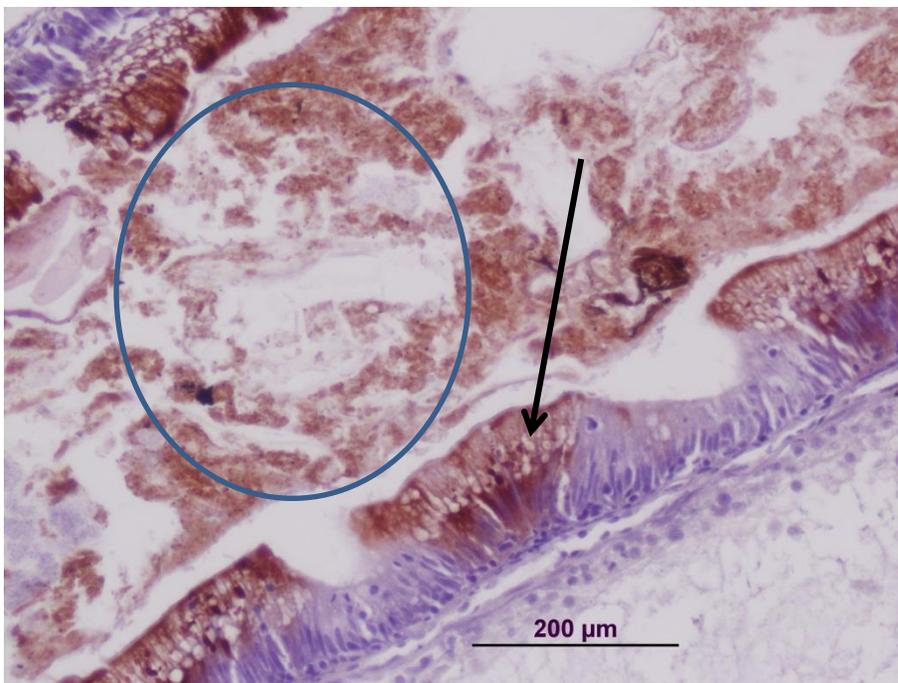


Figure10. Distal intestine showing immunoreactivity of microcystin LR antibody to epithelium (arrow) and gut content (circle).

Lake water quality- Continuous monitors recorded temperature, dissolved oxygen concentration (DO), and pH over the entire study. Highest water temperature (24°C) occurred in early August at the south site (Table 5, Figure 12). Mean 21d exposure temperatures ranged from 16 - 21°C (Table 5). The wide DO range (1.4 – 15.5 mg/L) at both sites is reflective of blue-green algal photosynthesis (Table 5, Figures 11-16). Supersaturated DO values were associated with 107% and 112% total gas saturation maxima observed at both sites on 24 July (Table 14). Other %TGS point measurements ranged from 92 – 106%. None of the TGS values, taken near the bottom (2.0 meters), were in the 115 – 120% range associated with gas bubble trauma in salmonids (Jensen et al. 1986, Mesa et al. 2000). Highest pH values, of 10.5 and 10.7, occurred during the first exposure at both the north and south sites, respectively (Table 5). No point measurements of total ammonia were done before 31 July due to lack of reagents. The highest estimate unionized ammonia (UIA) values occurred on 31 July and 7 August (Table 12 and 13). On 31 July, UIA of 0.334 mg/L was measured at the north site followed on 7 August with 0.214 mg/L. The next highest UIA measurement at the north site was on 11 September (0.067 mg/L). South site UIA values of 0.057, 0.118, and 0.050 were measured on 31 July, 7 August, and 28 August. All other point UIA measurements were ≤ 0.027 mg/L. It is likely that high environmental ammonia was a stressor during most of July and August.

Hourly data (pH, temperature, and dissolved oxygen) from benthic monitors was examined at several threshold points. The impaired health threshold range selected for elevated pH (10 – 10.5), dissolved oxygen (<2, 2 – 4 mg/L), and high ammonia (>0.5 mg/L NH₃-N) are based on the 48 h LC50 values reported in the literature (Table W5). Dissolved oxygen threshold of 4 mg/L was selected as it represents approximately 50% saturation value for the lake during the summer (water at equilibrium = 7.8 mg/L dissolved oxygen at 4,000 ft elevation and 21°C, Lietritz and Lewis 1976). Water temperature during the 2012 exposures did not reach the 96h LC50 value (30.5°C) reported by Saiki et al. (1999) and no threshold analysis was performed on this parameter however the lake was in the 23-26°C range during August (north 55h, south 136h exposure 1). Stressful pH values occurred in both sites during 17-31 July period with 197h above pH 10.5 at the south site (Figure 17). Both north and south sites experienced long periods (52 and 79h, respectively) of low 2-4 mg/L dissolved oxygen during the initial 2 weeks of exposure 2 (28 August – 11 September, Figure 18). Similarly, low 2-4 mg/L DO occurred for 16h and 31h at the north and south sites between 19 September and 2 October. The longest time (104h) at the 2-4 mg/L level was recorded in August at the north site when no sentinel fry were deployed in the lake.

Table 5 – Maximum, mean, minimum and standard error for temperature (C), dissolved oxygen (mg/L) and pH at the Northern and Southern exposure sites during the three exposure periods 2012. Monitors were hung from buoys located in the center of the bucket cage array at an approximate depth of 2.0m. n = number of measurements taken during each exposure period.

Constituent	Site		Exposure period		
			Exp. 1 July 17 - Aug 6	Exp. 2 Aug 28 - Sept 18	Exp. 3 Sept 19 - Oct 9
Temperature (C)	North Lake	max	23.08	20.77	19.62
		mean	21.06	18.59	16.14
		min	19.24	15.37	8.91
		SE	0.04	0.04	0.11
		n	491	528	490
	South Lake	max	23.99	21.19	20.75
		mean	21.32	18.63	16.28
		min	19.46	15.39	10.37
		SE	0.04	0.05	0.13
		n	491	528	490
Dissolved oxygen (mg/L)	North Lake	max	14.27	12.99	9.86
		mean	7.72	6.97	5.85
		min	2.10	1.41	2.86
		SE	0.13	0.10	0.06
		n	491	528	490
	South Lake	max	15.52	11.64	10.18
		mean	9.71	5.16	6.55
		min	4.50	1.43	2.01
		SE	0.08	0.07	0.07
		n	491	528	490
pH	North Lake	max	10.51	9.76	8.52
		mean	9.83	8.55	7.44
		min	8.93	7.36	7.13
		SE	0.02	0.02	0.01
		n	491	528	490
	South Lake	max	10.67	9.32	8.21
		mean	10.22	8.24	7.55
		min	9.55	7.14	7.15
		SE	0.01	0.02	0.01
		n	491	528	490

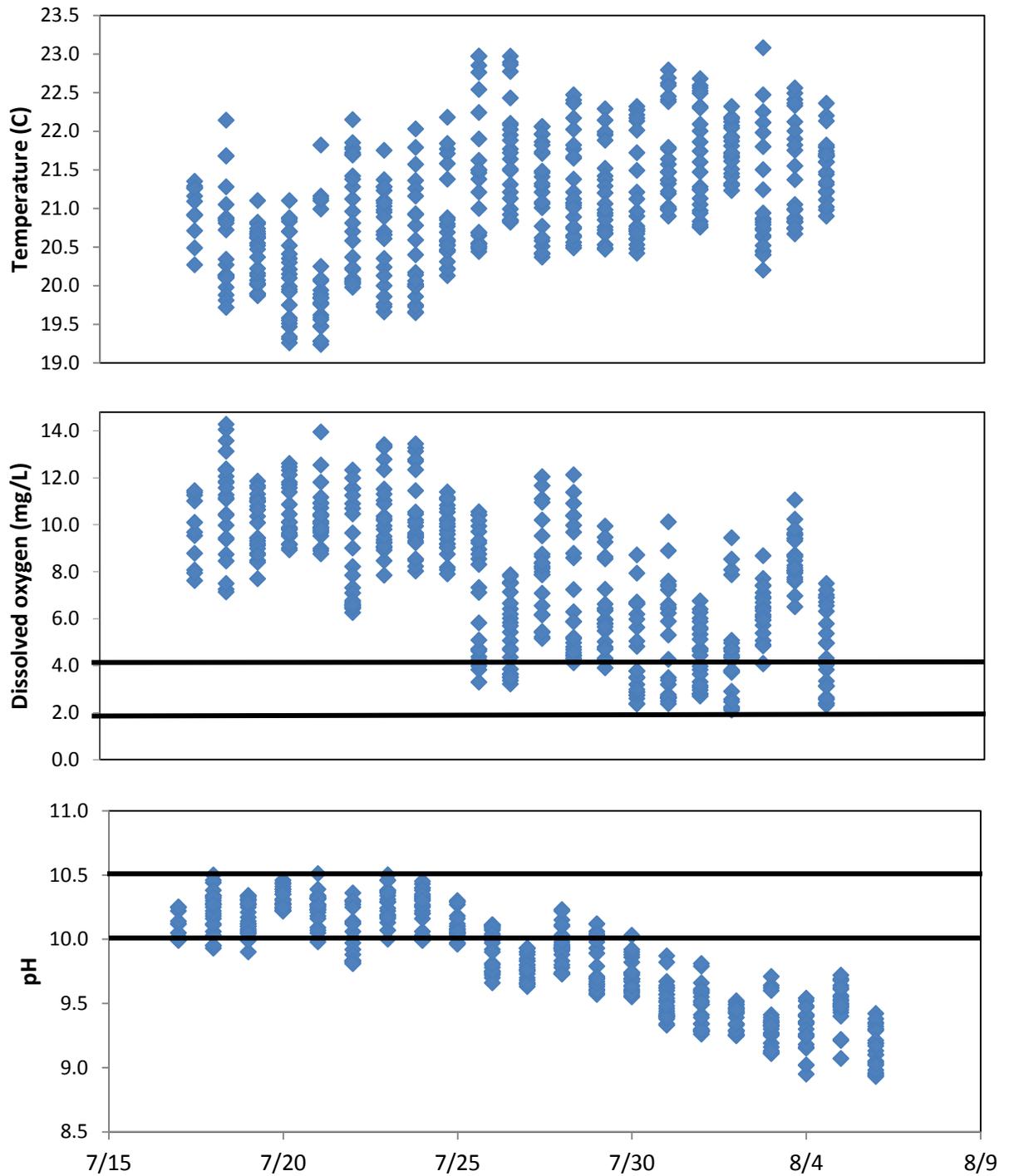


Figure 11. - Water temperature, dissolved oxygen concentration, and pH at the **Northern** exposure site in Upper Klamath Lake **17 July through 08 August** 2012. Water quality measurements were obtained hourly using continuous monitors deployed at a depth of approximately 2.0 m from a buoy located in the center of the bucket cage array.

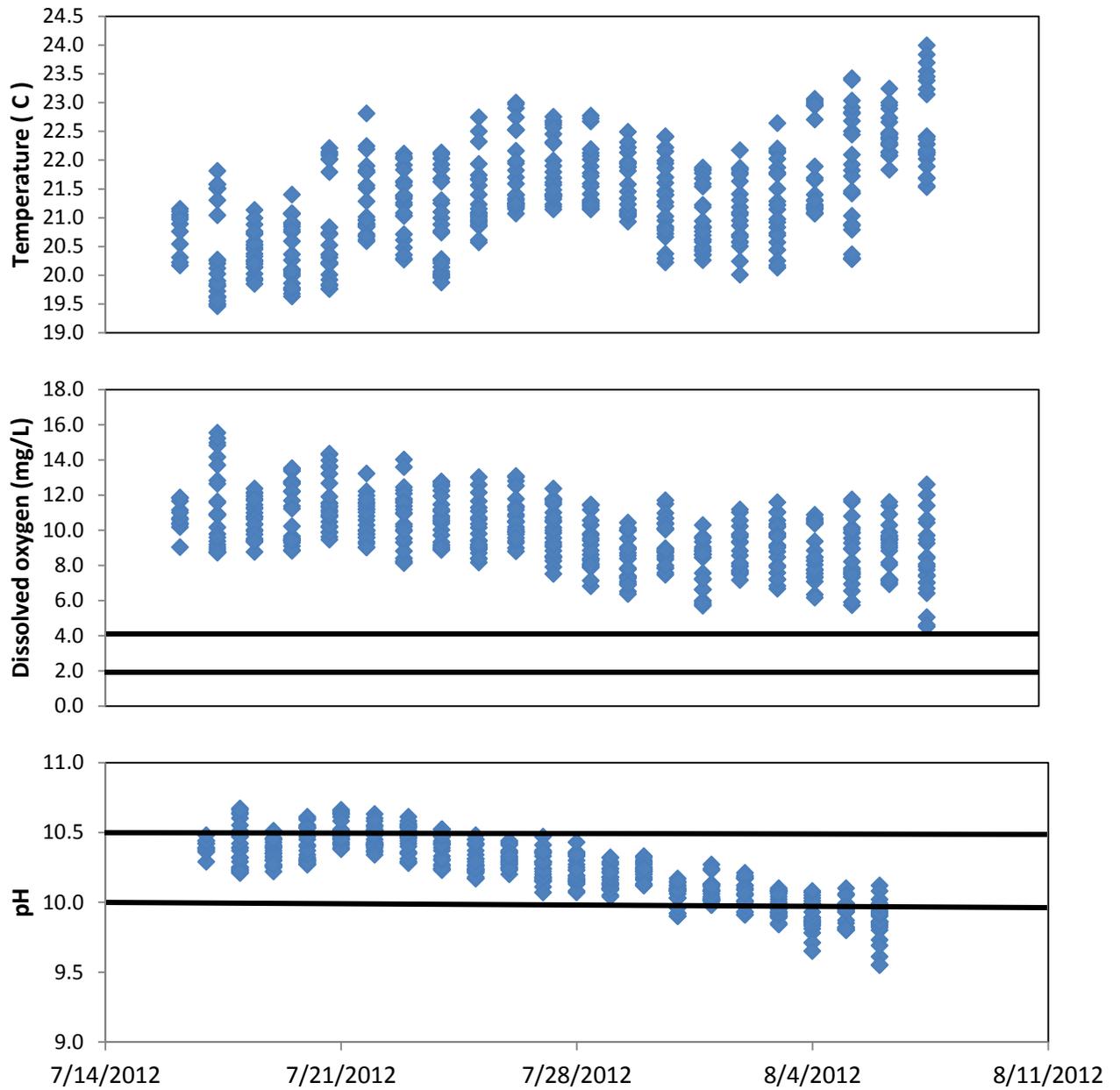


Figure 12. - Water temperature, dissolved oxygen concentration, and pH at the **Southern** exposure site in Upper Klamath Lake **17 July through 06 August** 2012. Water quality measurements were obtained hourly using continuous monitors deployed at a depth of approximately 2.0 m from a buoy located in the center of the bucket cage array.

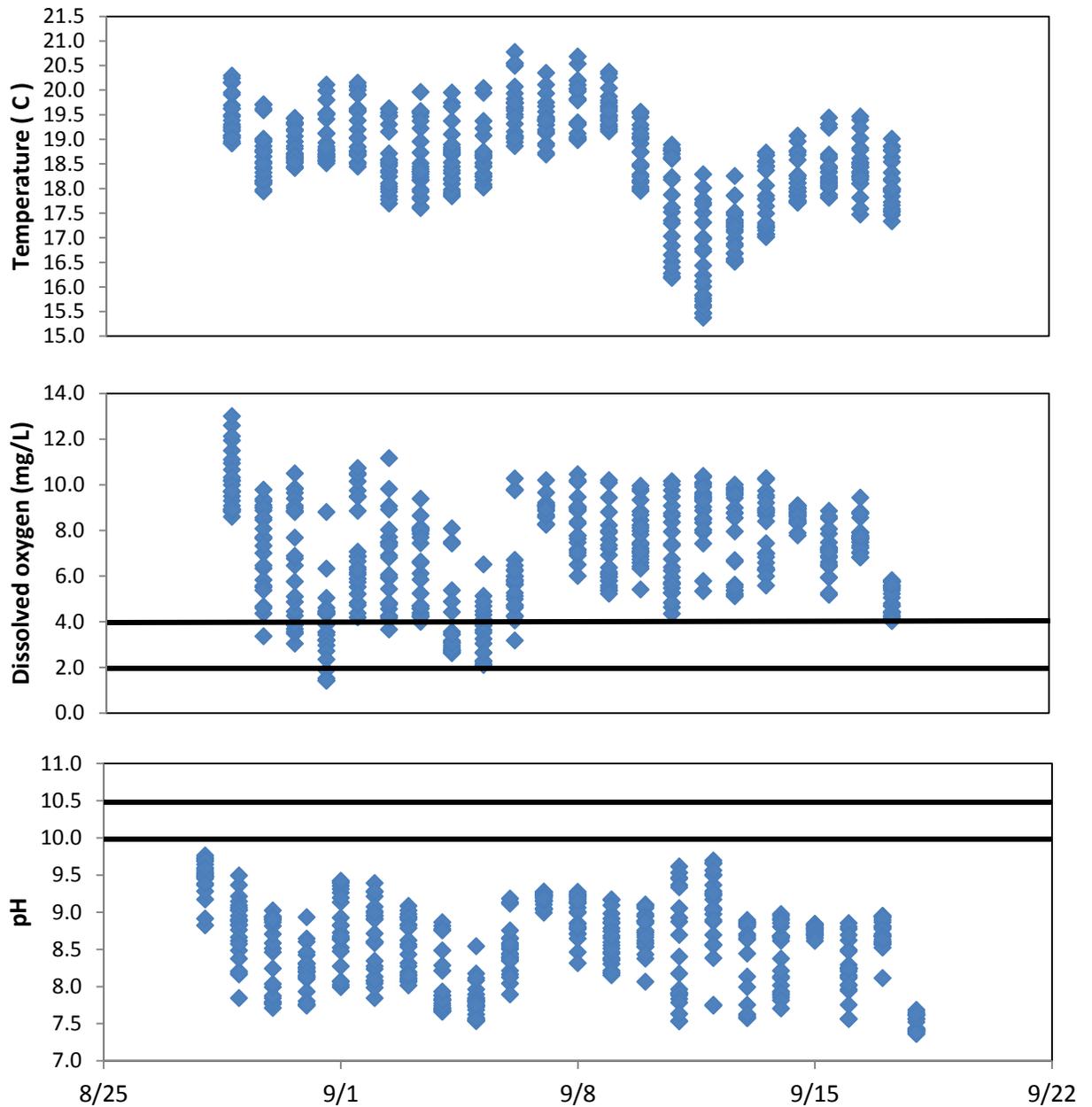


Figure 13. - Water temperature, dissolved oxygen concentration, and pH at the **Northern** exposure site in Upper Klamath Lake **28August through 18September** 2012. Water quality measurements were obtained hourly using continuous monitors deployed at a depth of approximately 2.0 m from a buoy located in the center of the bucket cage array.

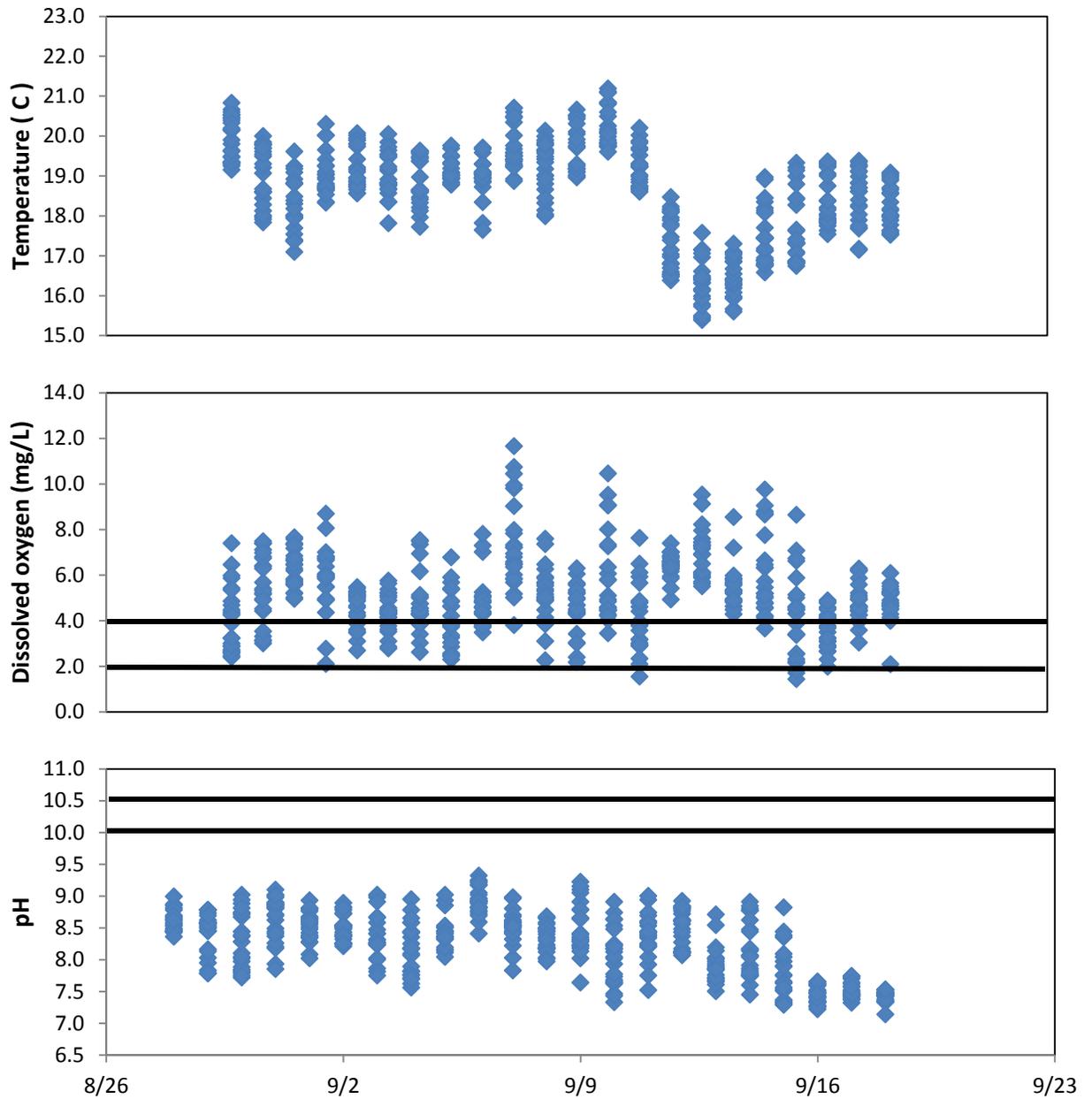


Figure 14. - Water temperature, dissolved oxygen concentration, and pH at the **Southern** exposure site in Upper Klamath Lake **28August -18September** 2012. Water quality measurements were obtained hourly using continuous monitors deployed at a depth of approximately 2.0 m from a buoy located in the center of the bucket cage array.

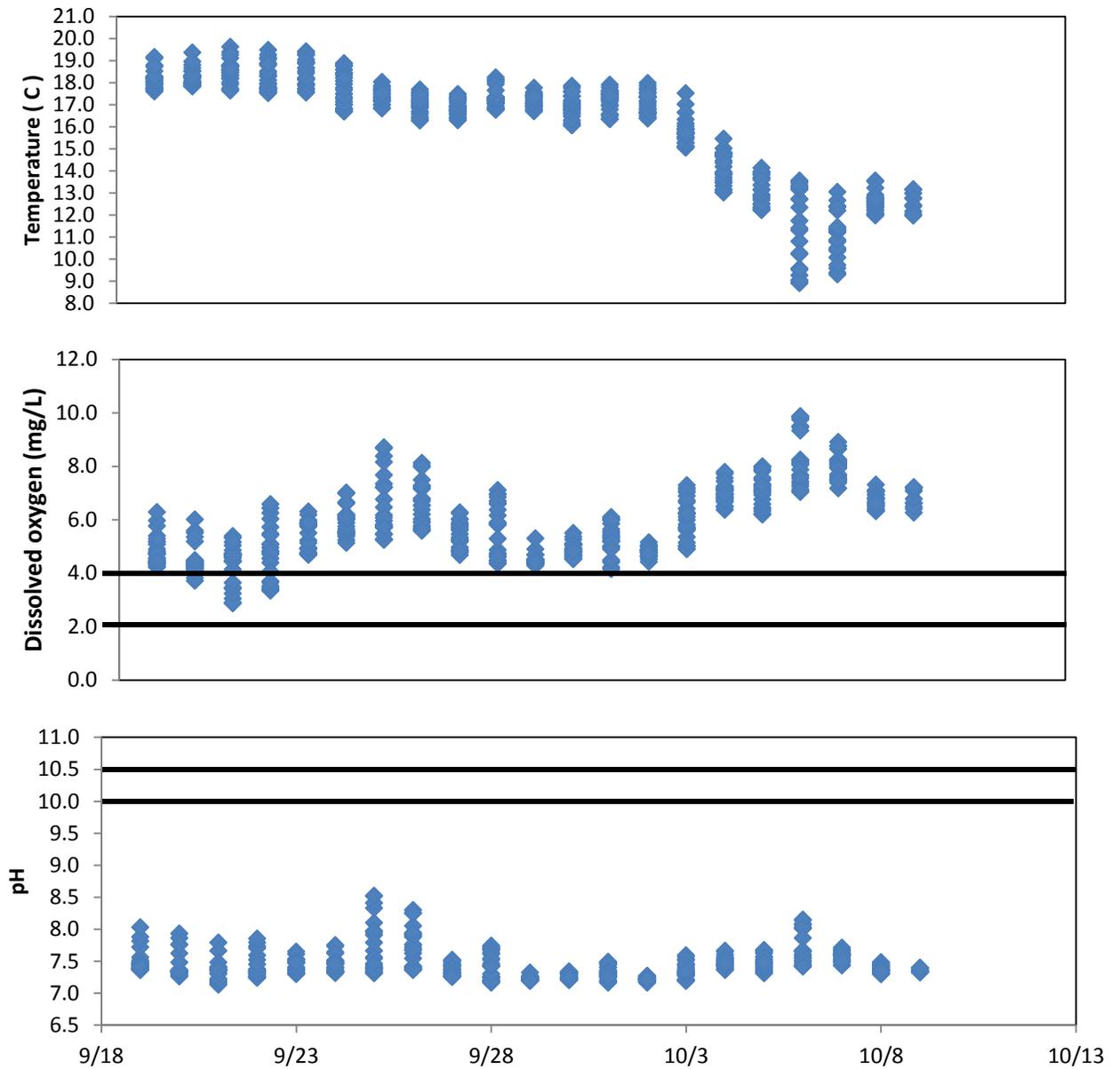


Figure 15. - Water temperature, dissolved oxygen concentration, and pH at the **Northern** exposure site in Upper Klamath Lake 19September - 09October 2012. Water quality measurements were obtained hourly using continuous monitors deployed at a depth of approximately 2.0 m from a buoy located in the center of the bucket cage array.

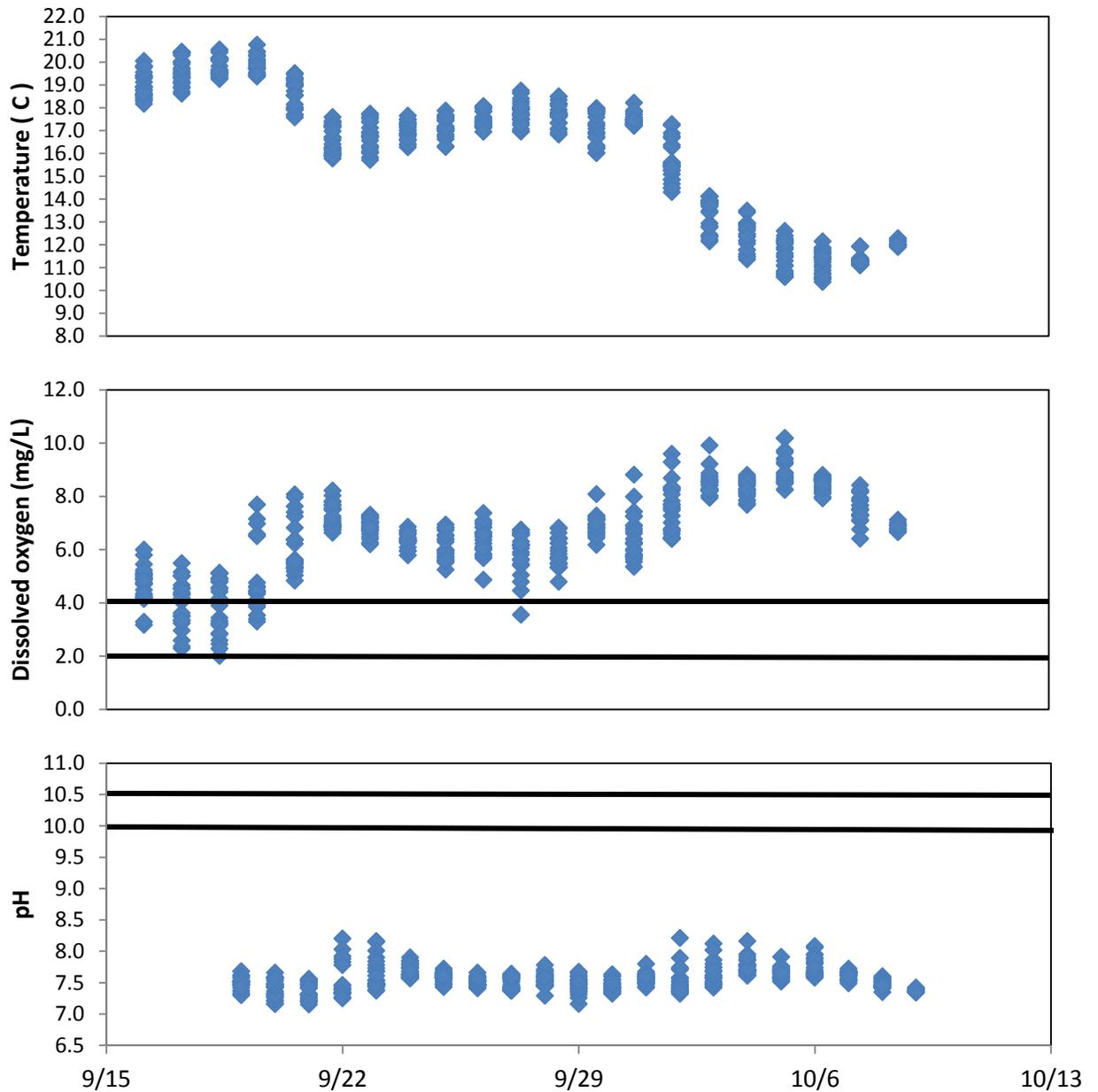


Figure 16. - Water temperature, dissolved oxygen concentration, and pH at the **Southern** exposure site in Upper Klamath Lake **19September - 09October** 2012. Water quality measurements were obtained hourly using continuous monitors deployed at a depth of approximately 2.0 m from a buoy located in the center of the bucket cage array.

Table 6 – Benthic point measurements taken at the **Northern** exposure site of total ammonia NH_4^+ (mg/L), temperature ($^{\circ}\text{C}$) and pH as well as temperature ($^{\circ}\text{C}$), pH and percent unionized ammonia NH_3 in aqueous solution values used to calculate unionized ammonia concentrations.

Date	NH_4^+ (mg/L)	Actual Temp ($^{\circ}\text{C}$)	Actual pH	Temp Used ($^{\circ}\text{C}$)	pH Used	% NH_3 Used mg/L	Calculated UIA
31July	0.60	20.3	9.53	20.0	9.5	55.70%	0.334
07Aug	0.68	21.8	9.15	22.0	9.0	31.50%	0.214
28Aug	0.05	19.1	9.41	19.0	9.5	53.90%	0.027
04Sept	0.50	18.0	7.76	18.0	8.0	3.31%	0.017
11Sept	0.28	16.6	9.08	17.0	9.0	24.10%	0.067
19Sept	0.30	17.8	7.45	18.0	7.5	1.07%	0.003
25Sept	0.17	17.0	7.50	17.0	7.5	0.996%	0.002
02Oct	0.08	16.4	7.39	16.0	7.5	0.925%	0.001
09Oct	0.18	11.9	7.48	12.0	7.5	0.684%	0.001

Table 7.– Benthic point measurements taken at the **Southern** exposure site of total ammonia NH_4^+ (mg/L), temperature ($^{\circ}\text{C}$) and pH as well as temperature ($^{\circ}\text{C}$), pH and percent unionized ammonia NH_3 in aqueous solution values used to calculate unionized ammonia concentrations.

Date	NH_4^+ (mg/L)	Actual Temp ($^{\circ}\text{C}$)	Actual pH	Temp Used ($^{\circ}\text{C}$)	pH Used	% NH_3 Used mg/L	Calculated UIA
31July	0.07	20.60	9.98	21.0	10.0	81.00%	0.057
07Aug	0.2	22.4	9.6	22.0	9.5	59.20%	0.118
28Aug	0.48	19.4	8.53	19.0	8.5	10.50%	0.050
04Sept	0.15	19	7.89	19.0	8.0	3.56%	0.005
11Sept	0.15	16.9	7.88	17.0	8.0	3.08%	0.005
19Sept	0.09	18.8	7.64	19.0	7.5	1.15%	0.001
25Sept	0.11	16.1	7.75	16.0	8.0	2.87%	0.003
02Oct	0.05	17.4	7.51	17.0	7.5	0.996%	0.000
09Oct	0.11	12.1	7.54	12.0	7.5	0.684%	0.001

Table 8. – Benthic point measurements for total gas saturation obtained from Northern and Southern exposure locations at each sampling event.

Constituent	Date	Site	Values
Total Gas Saturation	July 24 th	North Lake	107%*
		South Lake	112%*
	July 31 st	North Lake	96%
		South Lake	101%
	August 7 th	North Lake	99%
		South Lake	106%
	August 28 th	North Lake	106%
		South Lake	96%
	September 4 th	North Lake	95%
		South Lake	92%
	September 11 th	North Lake	104%
		South Lake	98%
	September 19 th	North Lake	98%
		South Lake	100%
	September 25 th	North Lake	99%
		South Lake	100%
	October 2 nd	North Lake	99%
		South Lake	100%
	October 9 th	North Lake	103%
		South Lake	101%

* July 24th values obtain from inside a Van Dorn water sampler.

Table 9. Median upper lethal concentrations(LC₅₀) for pH, un-ionized ammonia (mg/L, UIA), and temperature (°C), and lower LC₅₀ for dissolved oxygen (mg/L, DO)for juvenile 0+ Lost River suckers exposed for 48 and 96h as reported by (A) Meyer and Hanson (2002) and (B & C) Saiki et al. 1999.

	(A) <u>48 h</u>	(B) <u>48h</u>	(C) <u>96h</u>
pH	10.39	10.62	10.30
UIA	0.51	0.92	0.78
DO	1.58	1.58	1.62
°C	nd	30.8	30.5

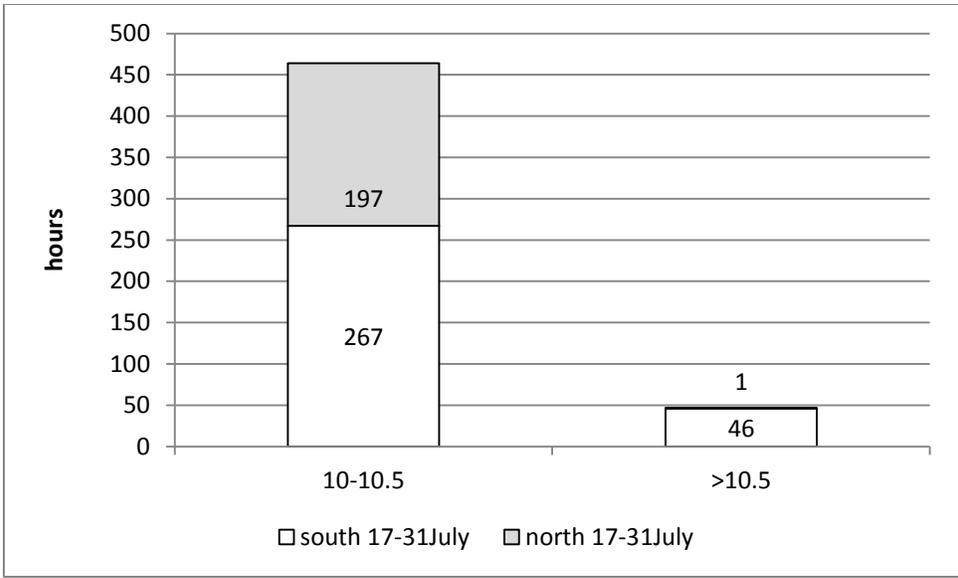


Figure 17. Hours at pH 10 -10.5 and > 10.5 measured at the bottom of the south and north cage site in the first 2 weeks of exposure 1.

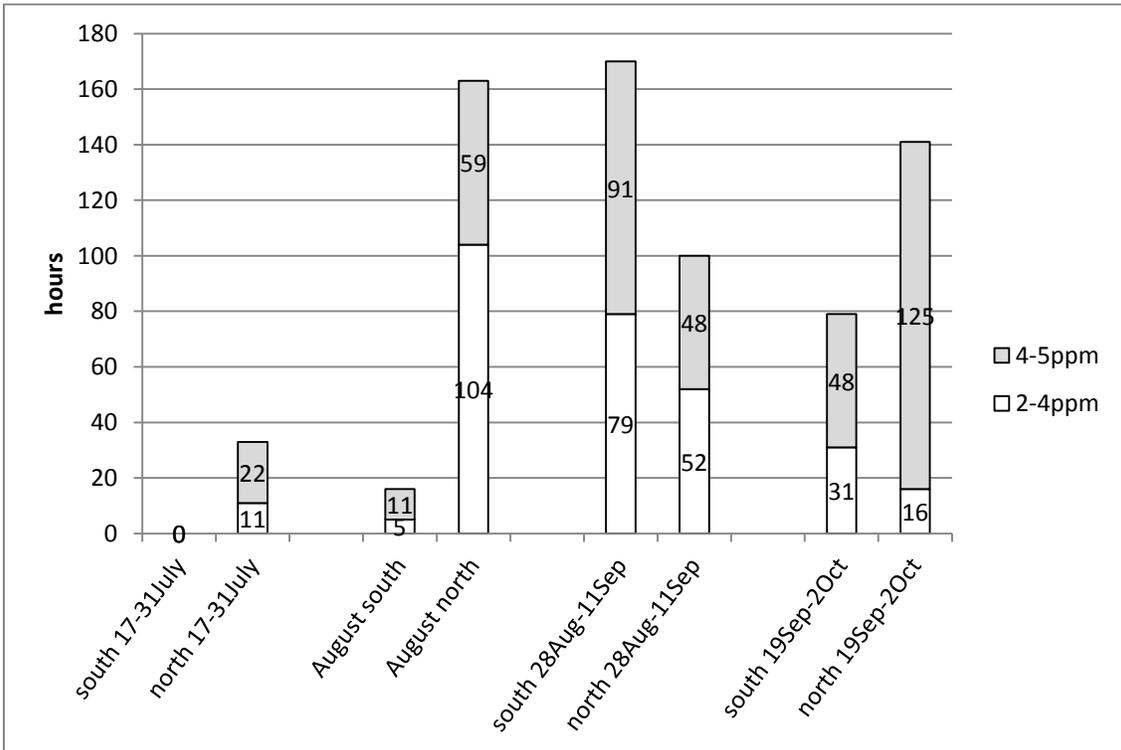


Figure 18. Hours at 2-4 (3.99) mg/L and 4 – 5 mg/L dissolved oxygen at the bottom of the south and north cage sites during the first 2 weeks of the 3 exposures and during August when no sentinels were present.

Bacterial challenges - Starvation, not handling stress, was the primary factor associated with increased bacterial susceptibility. The first trial was conducted between 16 July (initial loading of cages within treatment tanks) and 7 August (Figure 19). On 25 July, the first *Y.ruckeri* challenge was unsuccessful as all fry were killed by an unintended chlorine residual on a PCV tube used to increase water height within the challenge aquarium. It was repeated on 27 July (8×10^8 cfu/mL for 6h) for an abbreviated 3 d observation period. Mortality was low in all treatment groups over the 3d observation period (Table 10). In contrast, the day15 challenge on 31 July (1.1×10^8 cfu/mL for 24h) resulted in high mortality of unfed fry from both stressed (30%) and unstressed (67%) groups (Table 10.). When comparing mortality between the 2 challenges, the effect of starvation is apparent at 3 day post-challenge (challenge 1 = 5 and 10%, challenge 2 = 58 and 17%). Stressed unfed fry had significantly higher 7d survival than the unfed no stress fry in the day 15 challenge (Chi-square 4.805, $P=0.028$). *Y.ruckeri* was isolated from 4 of 4 fresh mortalities in the unfed – no stress group of the 31 July challenge. Unfed fry were emaciated by day 15 with mean condition factor (KSL) of 0.681 compared to 1.149 in fed cohorts (Table 11).

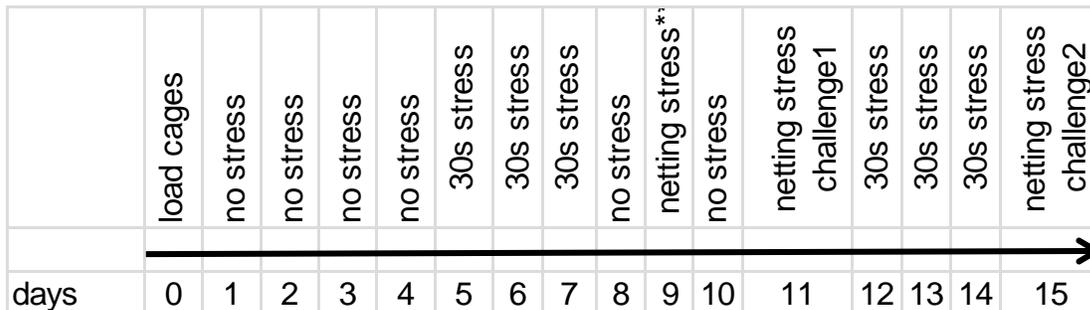


Figure 19. Flow chart of handling events for stressed groups in bacterial trial 1.
** Challenge failed due to toxicity and repeated on day11.

Table 10. Percent mortality (total mortality / total population, %) of *Yersinia ruckeri* challenged sucker fry in the 4 fed and stress level groups of trail 1. Two *Yersinia ruckeri* challenges occurred during the 21d trial. For comparison, 3 day post-challenge (dpc) mortality of the 2 challenges is listed as well as the final 7dpc cumulative mortality in the 2nd challenge.

Dates	DPC	Fed-no stress	Fed stress	Unfed-no stress	Unfed stress
27-30 July**	3	0/20 (0)	1 / 20 (5)	1 / 20 (5)	2/20 (10)
31 July –	3	0 / 21 (0)	0/20 (0)	14/24 (58)	4 / 23 (17)
7 August	7	2 / 21 (10)	0/20 (0)	16/24 (67)	7/23 (30)

** On 27 July a 6h challenge was conducted in response to the complete mortality experienced by the 25 July challenge fish due to chlorine toxicity.

Table 11. Mean (SD) condition factor of fed and unfed fry in trial 1 and 2.

	<u>Fed</u>	<u>Unfed</u>
25-Jul	1.203 (0.236)	1.181 (0.292)
31-Jul	1.149 (0.166)	0.681 (0.062)
5-Sep	1.302 (0.094)	0.967 (0.039)
11-Sep	na	na

na carcasses dehydrated, mean fed (0.492) and starve (0.394) cannot be compared to other samples

Trial 2 was conducted between 28 August and 20 September similar to trial 1 except that the first challenge occurred at 9 dpe (Figure 19). Bacterial challenges were started on 5 September (1.1×10^8 cfu/mL) and 11 September (7.2×10^7 cfu/mL). Unfed fry had significantly higher mortality than fed cohorts of the same stress treatment in both trials (Chi-square with Yates correction, $P \leq 0.028$). No significant difference was detected between unfed fish of either stress treatment in either challenge (Chi-square, $P > 0.05$). *Y. ruckeri* was isolated from only 1 of 6 mortalities sampled in the 2 challenges. The aquatic bacterium *Aeromonas hydrophilia* was detected in 5 samples however the size of the emaciated unfed fry made stab needle inoculation from the kidney suspect for surface contamination. As in the first trial, unfed fry were emaciated with a 26% lower mean condition factor than the fed fry (Table 11). Mortality in the cage bucket populations was higher in unfed fry. Between 11 and 15 dpe, unfed groups had 22 mortalities compared to 4 for fed groups. Similarly, unfed fry had mortality after only 24h in the challenge.

Table 12. Percent mortality (total mortality / total population, %) of sucker fry in the 4 fed and stress level groups in bacterial trail 2. Two *Yersinia ruckeri* challenges occurred during the 22d trial.

Dates	Fed-no stress	Fed stress	Unfed-no stress	Unfed stress
5-11Sept.	1/26 (4)	3/25 (12)	13/26 (50)	10/23 (43)
11-17Sept.	3/23 (13)	2/26 (8)	24/25 (96)*	22/24 (92)*

*Mortality within 24h considered due to non-specific handling stress. If these mortalities are removed from the 6d total cumulative mortality for unfed-no stress was 84% (21/25) and 63% (15/24) for unfed stress fry.

No consistent trend in whole body cortisol content was observed in the sample set (Table 13). There was considerable variability in each sample group that could be related to poor extraction efficiency and high sample dilution. This dilution effect was related to low fry weights (mean 0.063g trial1, 0.113g trial 2). No significant difference between treatment groups was detected by ANOVA ($P > 0.05$). When 31 July fry are pooled by feeding treatment, fed fry showed higher cortisol levels than unfed cohorts (Mann-Whitney rank sum test, $P = 0.008$).

Table 13. Mean (SE) whole body cortisol (ng/g fish) in sucker fry of the 4 fed and stress level groups from trial 1 (25Jul,31Jul) and 2 (5Sep,11Sep).

	<u>Fed-Stress</u>	<u>Fed-No Stress</u>	<u>Starve-Stress</u>	<u>Starve-no stress</u>
25-Jul	2.1 (0.7)	4.1 (3.1)	2.1 (1.5)	4.2 (3.6)
31-Jul	1.8 (0.6)	4.6 (0.5)	0.5 (0.1)	0.6 (0.1)
5-Sep	3.5 (1.7)	1.2 (0.4)	1.1 (0.2)	1.8 (1.1)
11-Sep	14.2 (4.6)	0.6 (0.1)	1.0 (0.7)	0.7 (0.2)

Discussion:

Adverse water quality appears to be the primary influence on low survival for all sentinel groups in 2012. Mortality was delayed with the majority of loss occurring between the 7 and 14 dpe. One notable exception was the high mortality (82%) observed at the south site on 25September (7dpe). These survival results were much lower than the 2011 study (Foott et al. 2012). In 2011, 21d or greater survival was $\geq 48\%$ at the north lake site with fish reaching a mean of 72mm SL by October and having good condition factors. Both water temperature and pH were similar for both summers. One distinct difference between the summers was that dissolved oxygen was largely above 4 mg/L (except for $< 3h$ on 3 days) in 2011 while sentinels in 2012 experienced 2-4 mg/L DO between 11 and 79h during the first 14dpe. No ammonia measurements were made in 2011. Winter survival was also markedly lower in 2012 (3% in 170d in 2012-13 compared to 97% over 67d in 2011) however fish size and rearing histories were different between years. This data suggests a minimum size ($> 30mm$) and condition factor ($KSL \geq 1.4$) for lake juveniles to have the necessary energy reserves for winter survival.

Similar to 2011, movement of sentinels to Upper Klamath Lake was interrupted due elevated mortality of the laboratory population in August. We hypothesize that the gill hyperplasia associated with this occurrence was related to a nutritional imbalance. We have no evidence for infectious disease or adverse water quality. Recovery of fry exposed to lake sediment suggests a missing micronutrient and / or gut microflora deficiency. Despite a health problem with the laboratory population, all sentinel groups sent to the lake were deemed adequate for the study. This view is based on the 100% survival of transported controls held in the laboratory as well as the high 7d survival of the lake sentinel.

Another difference between the 2011 and 2012 studies was the use of benthic bucket cages instead of a full water column netpen. While we were able to document water quality near the bottom, the inability of the sentinels to move throughout the water column could have negatively influenced their 14 dpe

survival. The 15 May spawn date resulted in sentinel fry < 20mm in length at the initial late July lake exposure. Fry of this size could not be held in our original netpens (3/16 in mesh) and necessitated the benthic buckets with screen sizes of 2mm.

Potential mortality (loss) factors include infectious disease, escape or predation within the cage, starvation within 14d of exposure, toxicity from algal toxins, and adverse water quality. It is unlikely that infectious disease was a significant factor as we did not observe clinical signs of infection in either histological specimens or fry directly sampled from the cages. Only one sentinel gill section contained an asymptomatic infection by *Trichodina* sp. Escape from the cages or predation within a cage is not a likely mortality (loss) factor. No broken cage screens were observed nor were predatory fish or large invertebrates found in any cages during the summer.

The role of low energy intake is less clear. Our laboratory experiments comparing fed to unfed fry clearly demonstrated that starvation effects occur by 11d at the summer temperature of 22°C. Feed status also had much stronger effect on resistance to bacterial challenge than net stress. In 2011, we determined that a condition factor (KSL) of 1.4 was associated with good survival and growth (“healthy”) while suckers with a KSL of 1.0 were emaciated. Only one 2012 sample group (south site 31 July) had a condition factor of 1.00 or less however, other sentinel sample groups from the south site had mean KSL values of less than the “healthy” 1.4 threshold. Another indicator of low energy reserves are the number of sentinels with whole body triglyceride contents below assay detection limit. This phenomenon occurred in fry at both sites from the first exposure however their small size (18 – 20 mm) was close to the limit of the assay. A “winner and loser” trend was apparent in sentinels from the south site in late September. This group had both the highest TG values as well as a large number of fish with below detection limit values. In conclusion, starvation can occur within 2 weeks at summer temperatures but was not consistently associated with sentinel mortality in 2012.

Microcystin toxins are produced by cyanobacteria, such as *Microcystin aeruginosa*, and can produce a number of overt and subclinical pathological effects in fish that are influenced by toxin concentration and type, exposure route (gastric, respiratory), host capacity to uptake, excrete or detoxify and general nutritional condition, and environmental conditions such as elevated water temperature or ammonia (Wiegand and Pflugmacher 2005, Sahin et al. 1996, Malbrouck and Kestemont 2006, He et al. 2012, Chen et al. 2011, Zhang et al. 2011). *Microcystin aeruginosa* and microcystin toxin has been documented in Upper Klamath lake (Gilroy et al. 2000, VanderKooi et al. 2009). Microcystins are cyclic heptapeptide toxins that bind and inactivate protein phosphatases (PP-1,2A,2B) resulting in hyperphosphorylation of cytoskeletal proteins (Boelsterli 2003). Programmed cell death, or apoptosis, is a typical outcome of microcystin exposure. Other responses include genotoxicity and apoptosis (da Silva et al. 2011), impaired gill Na-K-ATPase activity in carp (Zambrano and Canelo 1996),

immune disorders through lymphocyte apoptosis (Rymuszka et al. 2010), and oxidative stress and lipid peroxidation (Chen et al. 2011). Microcystins are considered hepatoxins and this organ is affected in fish (Fischer and Dietrich 1999, Acuna et al. 2012). All sentinel groups tested positive for MC-LRa toxin in their distal intestine by immunohistochemistry. Overt necrosis was not observed in this tissue. The high prevalence of moderate hepatocyte abnormalities (cytoplasmic protein droplets, apoptosis, coagulative necrosis) may represent a response by sentinel fry to chronic exposure of ingested toxin and could be a contributing factor in their chronic mortality. This hypothesis is complicated by similar hepatocyte responses to hypoxia.

Adverse water quality, driven by blue-green algae dynamics, has been identified by numerous workers as a significant stressor for Upper Klamath Lake suckers (Martin and Saiki 1999, Wood et al. 1996, Rasmussen 2011). Demands of this variable yet adverse water quality environment (warm alkaline water with variable ammonia and oxygen levels) should invoke a number of energy-intensive compensatory responses in the suckers. High temperature increases metabolic rate and oxygen demand while decreasing hemoglobin-O₂ binding affinity (Castleberry and Cech 1992). Lease et al. (2003) reported that LRS fry exposed for 30d to ammonia (0.3 mg NH₃-N/L) in water of pH 9.5 increased gill lamellar thickness and associated oxygen diffusion distance. While this response was not linked to mortality, impaired gas and ion regulation would act to reduce a sucker's scope of activity and its ability to withstand hypoxia. Similarly, Magaud et al. (1997) describes the synergistic effect of ammonia and hypoxia on LC₅₀ responses of rainbow trout. They state that the physiological effects of ammonia on blood, such as acidosis and reduce oxygen binding of hemoglobin, are the likely drivers of this synergism. Active migration of juvenile suckers to regions of higher oxygen concentration (congregating in microhabitats or to the surface) could also increase bird predation rates during the summer. Fish respond to hypoxia by first increasing delivery (ventilation, erythrocyte numbers), then conserving energy through metabolic depression, and finally enhanced supply of limited anaerobic energy sources (Wu 2002). Small fry will have much lower glycogen stores than older suckers to use for an anaerobic energy source during hypoxic conditions (Nilsson et al. 2008).

All exposure groups had several factors in common: 1) presence of MC-LRa toxin in the lower intestine epithelium and digesta, 2) presence of varying degrees of abnormal hepatocytes (apoptosis, coagulative necrosis), and 3) rearing in water temperature reaching 19°C or greater for some period during their 14 d exposures (Table 14). Specific combinations of adverse pH, dissolved oxygen, and ammonia were also associated with mortality between 7 and 14dpe (Table 14). Exposure 1 (17July – 31July) fry experienced high ammonia and pH conditions. The presence of hyaline droplets in the kidney tubules provides some evidence for this water quality stress (Foott and Harmon 2000, Meyers and Hansen 2002). This group also had individuals with low condition factors and TG values at 14dpe indicative of starvation. Fry in exposures 2 (28Aug-11Sept) and

3 (19Sept-2Oct) were exposed to low DO concentrations for 16 – 79 h during the first 14dpe.

Table 14. Factors associated with sentinel mortality over the first 14dpe. Dates listed include initial exposure to 14 dpe. Factors included high ammonia (HEA= 0.067 – 0.334 mg/L UIA), low dissolved oxygen concentrations (LDO = 2-4 mg/L), alkaline pH (≥ 10.3), signs of starvation (STR = KSL ≤ 1.0), low whole body triglyceride concentration below assay detection limit in $\geq 30\%$ of sample group (TGB), presence of microcystin LRA toxin in the distal intestine (MCL), eosinophilic droplets within cytoplasm of $\geq 20\%$ hepatocytes in $\geq 30\%$ of sample group (HED), coagulative necrosis of $\geq 20\%$ hepatocytes in $\geq 30\%$ of sample group (HCN), and hyaline droplets in kidney tubule epithelium (THD).

	NORTH	SOUTH	NORTH	SOUTH	NORTH	SOUTH
	<u>17-31July</u>	<u>17-31July</u>	<u>28Aug-11Sept</u>	<u>28Aug-11Sept</u>	<u>19Sept-2Oct</u>	<u>19Sept-2Oct</u>
HEA	+	-	+ / -	-	-	-
LDO	+	-	+	+	+	+
APH	+	+ / -	-	-	-	-
STR	?	+	-	-	-	-
TGB	+	+	-	-	-	+
MCL	+	+	+	+	+	+
HED	-	+	+	+	+	-
HCN	+	+	+	+	+	+
THD	+	+	-	-	-	-

We hypothesize that sucker fry mortality (direct and indirect), during the summer, is driven by a synergism of chronic microcystin toxin exposure and adverse water quality (high ammonia and pH and /or low dissolved oxygen concentration during energy intensive warm water conditions). Physiological exhaustion, due to the above stressors, could be the primary driver of lake-wide recruitment failure for 0+ Lost River suckers in Upper Klamath Lake.

Future research needs to link sentinel survival and tissue changes to cohorts experimentally reared under simulated lake conditions of high summer temperature, alkaline pH, a range of ammonia concentrations, and low dissolved oxygen. Other research goals should include an evaluation of liver phosphatase activity of sentinel fry to document the effect of M-LR toxin exposure. Sentinel should be housed in full water column netpens to better represent feral fry and be examined at 3, 7, 10, and 14dpe in order to narrow down peak mortality.

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