

## U.S. Fish & Wildlife Service

### California Nevada Fish Health Center

#### FY2006 Technical Report:

### Effects of simulated algal bloom pH on juvenile Lost River Sucker energetics and growth.

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**Summary:** Summer algal blooms, within Upper Klamath Lake of Oregon, produce diurnal pH fluctuations in excess of pH 9.5. While hypoxia and elevated ammonia can be associated with senescent blooms, high pH is the dominant water quality condition in summer for endangered Lost River Sucker (*Deltistes luxatus*) that inhabit the lake. This study examined the effects of elevated alkalinity from a simulated algal bloom (diurnally fluctuating pH of 9.5 – 10.0) on juvenile Lost River Sucker growth, energy reserves (whole body protein, lipid, triglyceride, and glycogen), tissue lysozyme activity (innate immune defense indicator), blood cell composition, and histological morphology of selected tissues. A test population was reared from eggs to 60mm fork length size, acclimated from pH 8.0 to 9.4 over 18 days(d) and held for 27 d in replicate aquaria systems that produced diurnal patterns simulating bloom (daily pH mean of 9.58) and normal spring conditions (mean pH of 8.61). Other influences (feed rate, ammonia, dissolved oxygen, temperature) were maintained at acceptable levels to isolate the effect of pH.

Hyaline deposits were observed in the kidney tubule epithelium of fish at the beginning of the diurnal fluctuation suggesting diuresis was associated with increasing pH. Subsequent kidney samples taken at 15 and 27 d were largely normal. No mortality occurred in either group and all fish continued to grow at a similar rate throughout the study. There was no difference between groups in whole body percent lipid, protein, lysozyme activity, or blood leukocyte composition. At 15 and 27 d, suckers in the bloom pH conditions had higher condition factors and lower whole body glycogen content than normal pH fish. Whole body triglyceride content was significantly lower in bloom pH fish than normal lake pH fish at 15d but was similar at 27d. Lost River sucker juveniles can acclimate to elevated pH profiles while continuing to grow and add energy reserves.

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#### Notice

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**Background-** In 1988, the US Fish and Wildlife Service listed the Lost River Sucker (*Deltistes luxatus*) as an endangered species. Factors contributing to the species 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). Poor water quality has been linked to the adult mortalities (Perkins et al. 2000) and may negatively influence juvenile survival. This fish inhabits the eutrophic waters of Upper Klamath Lake in Southern Oregon. The lake supports massive blooms of the cyanobacterium *Aphanizomenon flos-aquae* during the summer and early fall. Diurnal pH maxima of greater than 9.5 often occur for weeks during the summer as a result of CO<sub>2</sub> removal during intense photosynthesis. Elevated pH is the most dominant water quality parameter tracked to date (Wood et al. 1996). Elevated unionized ammonia (> 1.0 ppm) and periods of anoxia (dissolved oxygen < 2.0 ppm) have also been identified as water quality problems but are more transient than chronic alkalinity.

Bioassays have identified the short-term and chronic thresholds for low oxygen levels, elevated pH and ammonia in this species (Saiki et al. 1999, Meyer & Hansen 2002). The 96 h LD<sub>50</sub> values for sucker larvae and juveniles are pH 10.3 - 10.4, ammonia of 0.5 – 1.1 ppm, and dissolved oxygen of 1.3 – 2.1 ppm. Similarly, 14 and 30 d thresholds are reported as pH > 10, ammonia of 0.37 – 0.69 ppm, and dissolved oxygen of 1.5 – 2 ppm. These bioassays were run at single concentrations and not under the diurnal fluctuation profile found in the lake. Examination of fish response to diurnal fluctuation in pH has been identified as an information gap in sucker management (NRC 2004).

One consequence of chronic exposure to high pH waters is the inhibition of ammonia excretion in freshwater fish (Wilkie and Wood 1996). Other physiological responses associated with high pH exposure include ion imbalance and respiratory alkalosis. As demonstrated in the LD<sub>50</sub> work, Lost River suckers can survive relatively extreme water quality conditions. We hypothesize that there is a significant energy cost to juvenile suckers for maintaining homeostasis in the alkaline waters of Upper Klamath Lake. Both energy reserves and growth have been observed to decline in juvenile suckers collected from Upper Klamath Lake between August and September 2004 (Foott and Stone 2005). Poor over-winter survival could be a factor in low juvenile recruitment. It is essential that juveniles reach a minimum size and obtain sufficient energy reserves for adequate over-winter survival in temperate latitude lakes (Pangle et al. 2004, Kirjasniemi and Valtonen 1997, Oliver et al. 1979).

The objectives of this study were to determine the effects of a simulated algal bloom (diurnally fluctuating pH of 9.5 – 10.0) on juvenile Lost River sucker (LRS) growth, energy reserves, immune defense, and morphological tissue changes in comparison to cohorts reared in waters at pre-bloom alkalinities (pH 8.5 – 9.0).

## **Methods:**

*Diurnal pH profile* - The diurnal pH profile of the bloom and normal lake groups were based on a qualitative review of pH data from four USGS monitoring sites in Upper Klamath Lake during the June through September period for 2002 – 2004.

([http://or.water.usgs.gov/cgi-bin/grapher/graph\\_setup.pl?basin\\_id=ukl&site](http://or.water.usgs.gov/cgi-bin/grapher/graph_setup.pl?basin_id=ukl&site))

The sites were located in the northern portion of the lake:

422830121595700 UKL01	near Agency lake
422724121584600UKL02	north of Williamson R. mouth
422646121570600UKL03	south of Williamson R. mouth
422605121552700UKL04	near Modoc Point

Twelve data sets were examined for algal bloom pH characteristics (bloom conditions = pH >9.5) with the average “bloom” days being 29 (range 7- 36 d). Bloom pH conditions occurred from late June through September. Because of the removal of carbon dioxide during photosynthesis, peak alkalinity occurs mid-day for approximately 2 – 3 h with a range of 0.4 units per day. The daily pH range of the experimental bloom system was set at pH 9.5 – 9.9 with the normal lake pH ranging from 8.5 – 9.0. A water temperature of 20°C was selected to represent July lake temperature.

*Fish culture* - Gametes (1 mL milt / male, volumetric estimate of 1000 eggs/ female) were collected and pooled from 3 LRS males and females obtained by USGS during adult marking operations at Sucker Springs, Upper Klamath Lake on 18April2006. Permits for collection and transport of these fish to the FHC wet lab were obtained through Oregon Department of Fish and Wildlife, California Department of Fish and Game, and USFWS. The fertilized eggs were incubated in monolayers suspended on top of fine netting within multiple 40L aquaria supplied with 0.2 L / min flow of aerated 12°C water. Upon hatching, larval tanks were supplied 4 – 8 x / d with a 100 mL slurry of *Nannochloropsis* algae and rotifers (Reed Mariculture, [www.reed-mariculture.com](http://www.reed-mariculture.com)). This slurry imparted variable turbidity to each tank. Duplicate rotifer (*Brachionus plicatilis*) cultures were initially started at 15 ppt salinity. Cultures were fed algae and split each day. One half was reared at 5 – 7 ppt salinity and the next day netted out for sucker larval feeding. Newly hatched artemia nauplii (Artemia international LLC, [www.artemia-international.com](http://www.artemia-international.com)) were added to the tanks between 4 and 18 d post-hatch. Rotifer and algae additions were halted after 2 weeks and a mix of decysted artemia cysts and Otohime marine larvae diet B1 (200 – 360 µm) was begun (Reed Mariculture). The considerable feed addition required twice daily cleaning of the tanks with fine mesh nets and increased flows to 0.5 L / min. After 27 d, 20 mm larvae were strictly fed Otohime diet at 10 – 12 % estimated bodyweight (BW) / d levels.

*Tank system* - The two treatment groups (bloom and normal lake pH) were housed in identical 1710L recirculation systems consisting of two 625 L tanks whose effluent was pumped into an overhead 460L tank supplied with vigorous aeration for mixing purposes and containing immersion heaters (see photo on title page). Fish were held in one of the 625 L tanks per system. This

tank was divided into 2 parts by a 6 mm mesh screen which allowed for separation of marked and unmarked fish. The pH of each system was monitored and controlled by a YSI model 5200 water quality controller (YSI Inc, Yellow Springs, OH). As described below, pH targets were entered into controllers throughout the day to obtain the diurnal pH profile. Acid and base solution was introduced into the mixing tank by a diaphragm metering pump via the controller. Temperature was recorded hourly with Onset™ probes. Dissolved oxygen and pH was recorded hourly by the controllers. Spot measurements of oxygen (Hach LDO meter), pH (Beckman 12 meter), and ammonia (HACH ammonia-nitrogen low range method 8155, DR850 colorimeter, UIA calculations based on Emerson et al. 1975) were performed 4 times a day preceding the change in pH targets. Fresh water (20°C) replacement and the addition of 80g ChlorAm-X™ (sodium hydroxymethanesulfonate, AquaScience Research Group, Kansas City MO) occurred in the unoccupied 625L and 460L tanks at the 07:00 pH change. The tank with fish was isolated and held static while the freshwater was converted to the proper pH (approximately 20 min). At this point, the entire system was connected for the final pH shift. ChlorAm-X was also added if ammonia levels rose above 0.05 mg/L.

*Pilot experiment* - On July 25, two hundred suckers (40 mm FL) were placed into each system and pH raised 0.5 units every 2 d until 8.0 was reached. Fish were fed at 6% BW/d and water was exchanged twice daily. All the fish in one system were lost due to a base pump malfunction. In the surviving group, unionized ammonia concentration of >0.55 mg / L occurred after the system reached pH 8.0 on day 4. On the eighth day of the trial, 54 g (120.7mg/gal x 450 gal) of ChlorAm-X™ (sodium hydroxymethanesulfonate, AquaScience Research Group, Kansas City MO) was added to the mixing tank. The trial was ended after 9 days as fish demonstrated signs of external parasite (flashing behavior) and systemic bacterial infection (petechial hemorrhage of the epidermis). These infections were confirmed by microscopic examination of gill wet mounts and bacterial culture on Brain Heart infusion agar, respectively. A 100 mg/L formalin bath treatment was attempted but aborted after 30 – 60 s due to the excited behavior of the fish. Terramycin (2.2 mg oxytetracycline / g tank biomass) was mixed with Otohime and fed to the affected group for 10d.

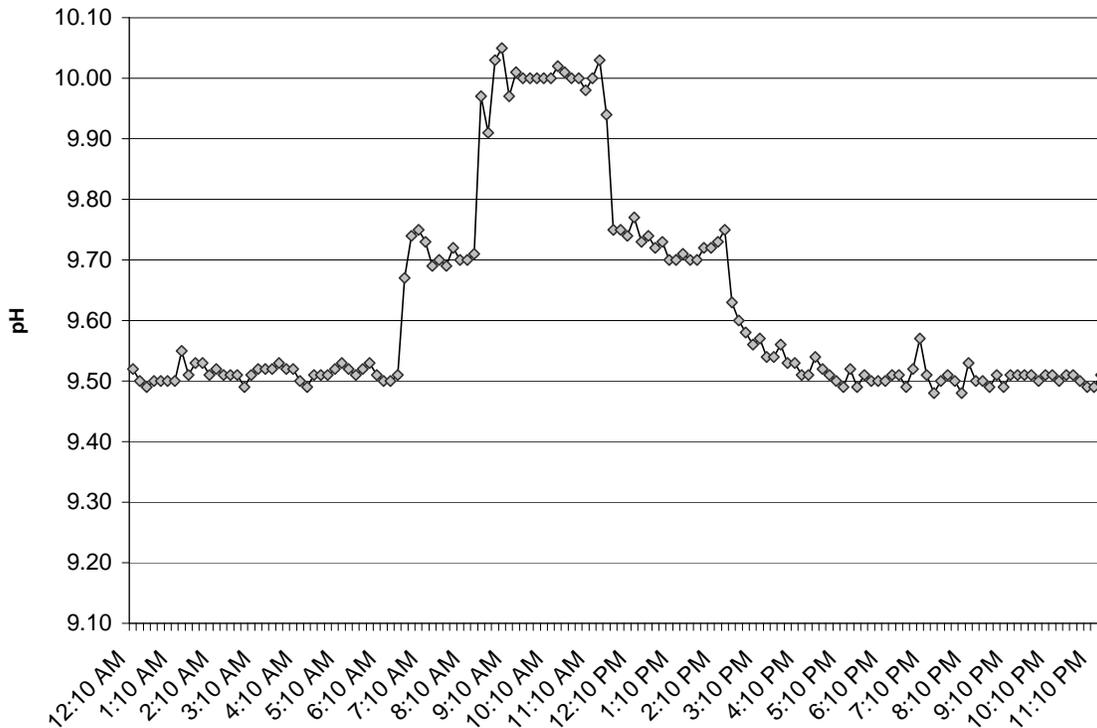
*Elastimere mark* – In order to track individual growth rates, a subset of suckers were marked by subcutaneous injection of pink, green, or orange elastimere dye (Northwest Marine Technologies Inc., Shaw Island Washington) on 3 general locations or a combination of each (left or right eye, ventral surface, dorsal surface, jaw). Marking occurred a month prior to the experiment and due to mortality, the number of marked fish was reduced to 12 in the bloom group and 17 for the normal group.

*Experiment protocol* - On August 17, both systems were loaded with 12 - 17 marked and 272 unmarked suckers (average 54 mm FL, 1.8 g). An eighteen day acclimation period preceded the 27 d diurnal fluctuation experiment. On 19August, the pH was slowly increased from the ambient 8.0 to 9.0 over 10 d. On 30August, the pH was raised to 9.4 in each system and held at this level until

diurnal fluctuations began on 7 September (referred to as 0 day post –exposure (dpe). Otohime diet (C2, 920 – 1410  $\mu\text{m}$ ) was fed at 3% BW / d.

The diurnal pH profile used in the 27 d experiment had 4 pH changes per day for each system as follows: Maximum pH for 3 h, intermediate pH for 5 h, and low pH for 16 h (Fig. 1). System pH targets were adjusted with 5N NaOH and HCL at 07:00 (bloom pH 9.7, normal pH 8.7), 09:00 (bloom pH 9.9, normal pH 9.0), 12:00 (bloom pH 9.7, normal pH 8.7), and 15:00 (bloom pH 9.5, normal pH 8.5). The systems were held at the 15:00 pH targets overnight.

Figure 1. Diurnal profile of the bloom system on 22 September showing the three daily pH levels.



*Bacterial challenge* – At 27 dpe, twenty fish from each system were lightly anesthetized with MS222, given a 0.1 mL intraperitoneal injection of a  $2.2 \times 10^6$  cells / mL suspension of *Vibrio anguillarum* and held for 7 d in 17°C flow-through aquaria (pH 8.1). The bacteria was grown for 18h in Brain Heart infusion (BHI) broth, centrifuged at 2000xg for 5 min, re-suspended in same volume of 0.85% saline, the cell / mL value was estimated by comparing the optical density of the suspension at 550 nm to a previously determined OD / cfu mL<sup>-1</sup> graph, a plate count performed, and a 1000x dilution used for the injection. Previous work had shown that the LD<sub>50</sub> dosage for these fish to be between 10<sup>5</sup> and 10<sup>6</sup> cells/ mL. (unpublished data). Kidney tissue from each mortality was inoculated onto BHI agar and the presence of *V. anguillarum* determined by the following criteria: gram-negative motile rod, cytochrome oxidase positive, sensitive to 0.1% vibriostatic agent O/ 129 disks (2,4-diamino-6,7-diisopropylpteridine phosphate).

*Sample protocols* - At 0, 15, and 27 dpe into diurnal pH fluctuations, a total of 20 suckers were collected from both the bloom and control systems. Weight (0.001 g) and fork length (mm) was measured for each fish and the condition factor calculated as  $KFL = (\text{weight} / \text{fork length}^3) \times 10^5$ . The first six fish were sampled for blood (hematocrit, bloodsmear) and histological examination. After severing the caudal peduncle, blood was collected in a heparinized microhematocrit tube, a drop of blood used to make a bloodsmear, and the remaining blood sample centrifuged in a hematocrit centrifuge for 3 min for determination of hematocrit values. Methanol fixed bloodsmears were later stained with Hemacolor stain kit (Harleco) and a differential leukocyte count performed (number of lymphocytes, thrombocytes, eosinophils, and neutrophils observed in the first 100 leukocytes) as well as an estimation of whether the smear contained > 1% immature erythrocytes (polychromatocyte or reticulocyte). The latter estimation was performed to identify individuals with elevated levels of erythropoiesis (e.g. response to anemia). Because there is poor morphological distinction between the low percentage of rounded “activated” thrombocytes (majority are spindle shaped) and the more numerous lymphocytes, the combined number of lymphocytes + thrombocytes divided by the number of granulocytes (neutrophil and eosinophil / basophils) was used to derive a L{T}:G ratio. Higher granulocyte counts result in low L{T} : G ratio values and can indicate infection, tissue damage, or seasonal blood cell changes (Modra et al. 1998). The histological samples were held in Davidson’s fixative for 48h, processed for 5µm paraffin sagittal sections, and stained with hematoxylin and eosin. The remaining 14 fish were used for energy measurements. They were placed into 20mL tubes and held on ice for up to 2 h prior to homogenization.

## Laboratory analysis

*Sample preparation* - Cold distilled water was added to the 20 mL tube containing the fish (1:1 w / v) and blended for 30 – 90s with a Biospec M133 homogenizer. Five aliquots (100- 200µl) of the homogenate (**2xWB**) were placed into tared 2mL centrifuge tubes, weighed to the nearest 0.01g to determine tissue weight (homogenate wt. divided by 2), and held on ice until assayed in this order: lysozyme activity, free glucose, triglyceride, protein and glycogen. The remaining homogenate was frozen for later analysis of lipid content (%lipid).

*Lysozyme activity*- Homogenate was centrifuged (3220xg, 5°C, 5 min) and the supernatant assayed by a turbidimetric method (Ellis 1990). Lysozyme activity (mOD / min / g tissue) of the 2xWB samples were determined from 10 µL samples. Briefly, replicate samples and hen egg white lysozyme standards used as controls (0,5,10,15 µg/mL in 0.04M phosphate buffer, pH 6.2) were added to a 96 well ELISA plate followed by 200µL of a 0.25 mg / mL suspension of freeze-dried *Micrococcus lysodeikticus* in 0.02M acetate buffer (pH 5.5). The decrease in absorption (450nm, 25°C) was immediately measured in a microplate reader at 30 s intervals for 10 min. and the maximum velocity of 15 consecutive measurements recorded (mOD / min).

*Glycogen* – Tissue glycogen was measured using the method of Murat and Serfaty (1974). Briefly, an aliquot of homogenate was assayed for free glucose and another digested with amyloglucosidase (Roche cat no. 10102857001, from *Aspergillus niger*) to liberate glucose from glycogen. The free glucose sample was diluted 3x (v/v) with a solution of cold 0.09M citrate buffer (pH 4.8) containing 2.5 mg/mL sodium fluoride (CBF), centrifuged (3220xg, 5°C, 5 min), and the supernatant assayed for free glucose content with a Pointe Scientific Glucose Oxidase Trinder kit (cat. No. G7519). The assay blank was CBF solution. A similar dilution was made with the glycogen sample except the CBF contained 0.1g amyloglucosidase / mL. After an 18h incubation at 37°C, the sample was assayed for glucose content as above. Glycogen was calculated as:

$$[\text{Digest (Glucose)} - \text{Free (Glucose)}] * 6 = \text{mg Polysaccharide / g tissue}$$

An internal control of oyster glycogen (50 mg/mL, Sigma Chemical G8751) was digested and run with each sample lot.

*Triglyceride* – 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).

*Protein* - Protein content (mg protein / g tissue) of the homogenate was assayed by a modification of the alkaline digestion method reported by Woo et al. 1978. Briefly, 0.5N NaOH was added to the homogenate (5x dilution w/v), mixed at 45°C for 120 min, centrifuged at 3220xg for 5 min, and replicate 10 µL samples of the 10x diluted supernatant assayed for total protein by the Biuret

method (Pierce BCA protein assay kit, Rockford IL). The blank consisted of 1:4 mixture of distilled water and 0.5N NaOH. Albumin diluted in the blank was used as the protein standard.

*Statistical analysis* - Analysis was performed with SigmaStat 3.1 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. Statistical significance is reported with these corresponding test statistics:

T-test	t	
Mann-Whitney U test on ranks	T	
1-ANOVA	F	(Holm-Sidak MC)
Kruskal-Wallis ANOVA on ranks	H	(Dunns MC)

## Results:

*Fish culture* - There was an estimated 90% survival to the 30 mm FL size. Three mortality events occurred prior to the diurnal pH experiment and were related to husbandry (heater malfunction in one larval tank, 100 ppm formalin bath treatment for *Ichthyoboda* (costia) infection and malfunction of base pump during July pilot experiment). As a consequence of the elevated unionized ammonia levels in the pilot experiment, the tank population had a high incidence of costia and *Aeromonas hydrophila* infections. Disease mortality responded to antibiotic treatment and increased freshwater flows. The addition of 54g of ChlorAm-X to the system during the July pilot experiment was successful in maintaining NH<sub>3</sub> levels below 0.32 mg/L. Addition of this compound was later increased to 80g for the experiment.

*Water quality:* Mean pH of the bloom system over the 27d experiment was 9.58 with a peak daily mean of 9.77 occurring on day 15 (Fig.2). The normal lake system mean daily pH was 8.61 with similar peak occurring on day 15. Fish spent an average of 165 (bloom) and 160 (normal) minutes each day at the maximum pH level. Water hardness (72 mg/L) and alkalinity (60 mg/L) were measured at the beginning of the experiment. Mean temperature for both systems was 20.3°C and dissolved oxygen levels (mean of 7.17 and 8.90 mg/L) were deemed adequate for the fish (Table 1). Mean unionized ammonia was four times higher in the bloom system than the normal lake system but at 0.016 mg/L was not considered to be stressful (Table 1).

Figure 2. Daily mean pH for algal bloom and normal lake water groups.

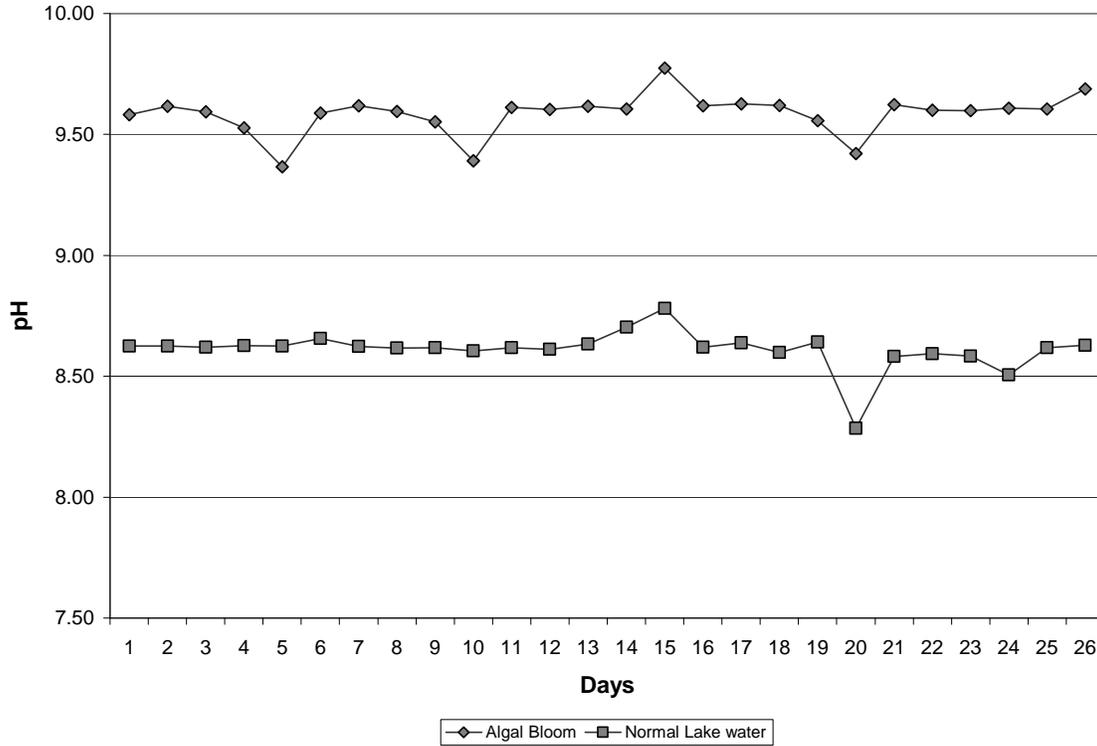


Table 1. Mean (SD) and range of unionized ammonia (mg / L, UIA), dissolved oxygen (mg/L, DO) and temperature (°C) measurements from the bloom and normal lake systems over the 27d experiment.

	BLOOM	NORMAL
UIA	0.016 (0.024) 0.00 – 0.150	0.004 (0.004) 0.00 – 0.019
DO	7.17 (0.68) 5.20 – 8.35	8.90 (1.59) 6.29 – 12.24
°C	20.3 (0.8) 18.1 – 22.7	20.3 (0.93) 18.6 – 23.3

*Growth and Morphometrics* - Growth rate of both elastimere -marked and unmarked suckers showed a similar declined 30 – 36% during the experiment. Mean growth rate of tagged fish ranged from 0.51 to 0.84 mm / d and did not differ (1<sup>st</sup> 15d : t=0.593 26 df P=0.588, last 12d: T=209 P=0.104) between treatments at any specific interval (Table 2).. Calculated growth rate of unmarked fish, using mean fork length in Table 2, was similar to the marked fish at each time period. The normal lake condition unmarked group having mean growth rate of 0.87 and 0.58 mm / d for 0-15d and 15-27d periods respectively. Similarly, the bloom condition fish had mean growth rates of 0.87 and 0.50 mm/d. Despite the reduction in growth rate, fork length increased 33% in both treatment groups over the 27d study (Table 2). Condition factor was significantly higher in the bloom pH group at 15 and 27 dpe (Table 2, P<0.001). This difference in

condition factor was driven by the 4-5% greater wet weight per mm FL in the bloom pH fish. Only one fish (bloom pH) died during the 27 d experiment.

Table 2. Fork length (FL= mm), growth rate of individual fish (mm/day), Weight (Wt= g), Condition factor (KFL = (Wt/ FL<sup>3</sup>)\*100000) of juvenile Lost River Suckers reared for 0 , 15, and 27 d under normal and bloom pH conditions. Data presented as Mean (St. Dev.) with n = 20 for FL, Wt, and KFL, and n=14 for all other parameters (except normal 0 dpe % lipid, n=8). Whole body percent lipid (%lipid), protein (mg/ g body), triglyceride (mg / g body), glycogen (mg polysaccharide / g body), and lysozyme activity (mOD / min/ g body).

	0d	15d	27d
FL			
normal	60 (3)	73 (5)	80 (6)
bloom	58 (6)	71 (4)	77 (6)
Growth rate mm/d	n / a	1 <sup>st</sup> 15d	Last 12 d
normal		0.78 (0.29)	0.51 (0.18)
bloom		0.84 (0.15)	0.59 (0.07)
Wt			
normal	2.3 (0.4)	3.8 (0.9)	5.2 (1.3)
bloom	2.2 (0.7)	3.6 (0.7)	4.9 (1.0)
KFL			
normal	1.026 (0.056)	0.962 (0.044)	0.982 (0.083)
bloom	1.068 (0.114)	1.020 (0.058)**	1.047 (0.052)**
%Lipid			
normal	4.52 (1.5)	2.97 (0.34)	3.33 (0.63)
bloom	4.22 (1.2)	3.49 (0.89)	3.32 (0.51)
Protein			
normal	57.3 (3.1)	38.9 (4.7)	41.9 (4.7)
bloom	56.4 (7.2)	38.9 (2.6)	42.5 (3.0)
Triglyceride			
normal	14.3 (1.9)	13.6 (2.3)	18.6 (1.5)
bloom	14.4 (2.8)	11.6 (2.5)*	18.7 (1.1)
Glycogen			
normal	1.87 (0.64)	1.16 (0.41)**	0.94 (0.28)**
bloom	1.67 (0.58)	0.63 (0.18)	0.39 (0.21)
Lysozyme			
normal	1539 (291)	953 (109)	878 (120)
bloom	1878 (596)	996 (121)	957 (150)

\* Significant difference P<0.05

\*\* Significant difference P< 0.001

n/a = not applicable

*Carcass constituent analysis* – Mean whole body protein concentrations declined between the 0 and 15 dpe sample but then remained steady through 27 dpe (Table 2). There was no significant difference in whole body protein between the normal and bloom pH groups at each specific time point. As mentioned above, 0 d samples were significantly different from 15 and 27 dpe fish ( $H= 58.017$ , 5df,  $P<0.01$ ). Glycogen values tended to decline over time in both treatment groups (Table 2). This initial decline was statistically significant as both normal and bloom pH fish at 0 dpe were significantly higher than their cohorts at 15 and 27 dpe ( $H=60.977$ , 5 df,  $P<0.001$ ). Normal pH fish had higher glycogen content than their bloom pH cohorts at 15 and 27 dpe (t-test,  $P<0.001$ ). Mean whole body triglyceride concentrations (TG) declined between the 0 and 15 dpe samples with the bloom pH fish experiencing a significant decline ( $F=25.366$ , 5 df,  $p<0.001$ ). Normal pH fish had higher TG than their bloom pH cohorts at 15 dpe ( $t= -2.159$ , 25 df,  $P=0.041$ ). Triglyceride content significantly increased in both bloom and normal pH fish by 27 dpe (Table 2). The percent lipid level declined in both treatment groups between 0 and 15 dpe. At 27 dpe, % lipid increased for normal lake pH fish but slightly decreased for bloom pH fish (Table 2). There was no significant difference between the treatment groups at each specific time point. Normal pH fish at 0 dpe had higher lipid values than normal pH cohorts sampled at 15 dpe ( $H= 18.532$ , 5df,  $P=0.002$ ). Lysozyme activity declined after the 0 dpe sample and was at a similar level between 15 and 27 dpe (Table 2). There was no significant difference in lysozyme activity between the normal and bloom pH groups at each specific time point.

*Blood values and bacterial challenge* – No obvious hematological impairment was detected in the juvenile suckers throughout the experiment (Table 3). No significant difference was detected in the hematocrit values of groups measured at 15 and 27 dpe ( $F=1.035$ , 3 df,  $P=0.398$ ). Rounded thrombocytes were the most prevalent leukocyte observed in bloodsmears while eosinophils were the least prevalent. The LT: G ratio remained similar throughout the experiment in both treatment groups and did not indicate any inflammatory responses. The qualitative rating of immature erythrocyte (>1% of erythrocyte being classified as polychromatocytes) suggests that normal pH fish may have had a greater level of erythropoiesis than bloom pH cohorts.

Normal pH suckers, challenged with  $10^5$  cfu / fish of *Vibrio anguillarum* on 27 dpe, showed a greater mortality rate (8 of 15 fish, 53%) than the bloom pH cohorts (1 of 15 fish, 7%).

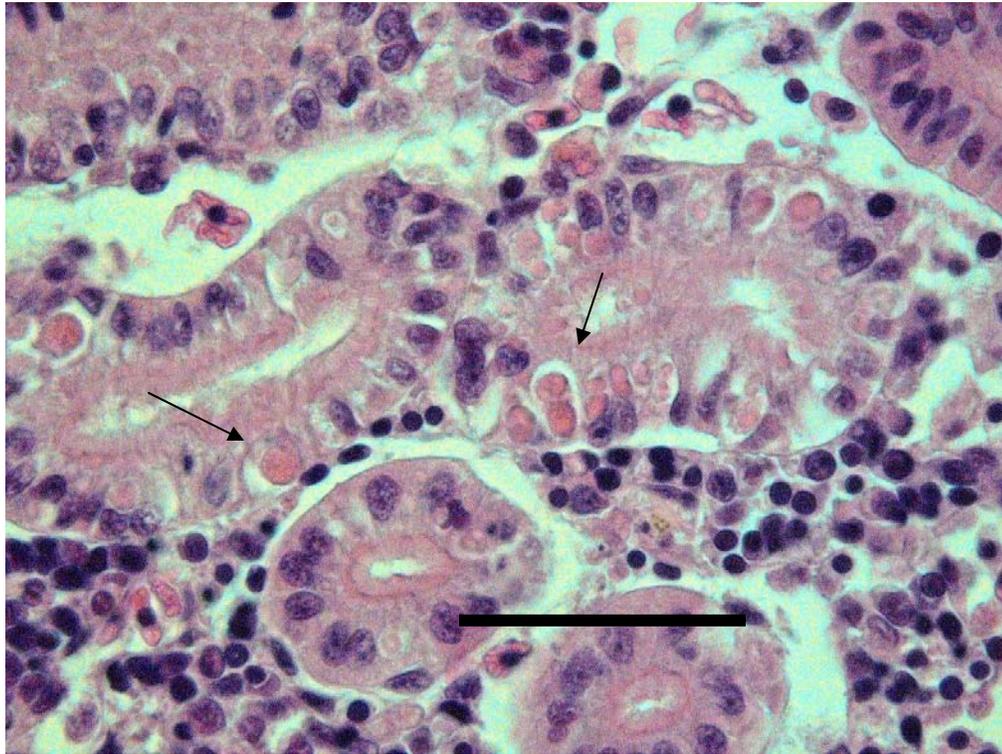
Table 3. Blood values of juvenile Lost River Suckers reared for 0, 15, and 27 d under normal and summer bloom pH conditions. Differential leukocyte count percentages of 3-6 fish samples per group (Lymphocytes, thrombocytes, neutrophils, eosinophils, and Lymphocyte+thromobocyte / Granulocyte ratio) and hematocrit values reported as mean (st.dev.). The number of samples that contained an estimated 1% or greater polychromatocytes (PC =immature erythrocyte) was also recorded.

	0d	15d	27d
Hematocrit (%)			
Normal	ND	37 (5)	29 (9)
Bloom	ND	33 (10)	34 (6)
Sample no.	3	6	6
Lymphocyte			
Normal	10 (7)	8 (8)	9 (12)
Bloom	12 (2)	10 (7)	18 (19)
Thrombocyte			
Normal	89 (7)	88 (10)	81 (22)
Bloom	86 (1)	81 (22)	80 (22)
Neutrophil			
Normal	0 (1)	3 (4)	5 (11)
Bloom	2 (2)	1 (1)	2 (5)
Eosinophil			
Normal	0	2 (2)	5 (11)
Bloom	0	1 (2)	0
LT : G ratio			
Normal	99 (1)	96 (5)	90 (13)
Bloom	98 (2)	98 (2)	98 (5)
%PC > 1%			
Normal	33%	17%	67%
Bloom	0%	0%	33%

ND not done as blood volume too small for hematocrit tube reading

*Histological examination* – Tissue morphology did not differ between the treatment groups. Eosinophilic droplets (“hyaline deposits”) were observed in the proximal tubules of suckers collected from both bloom and normal populations on day 0 (Fig. 3). No such abnormality was seen in the kidney of fish from either treatment sampled at 15 dpe. Two 27 dpe bloom pH suckers had low numbers of similarly affected tubules. Separation of lamellar epithelium (edema), swollen epithelium cells and moderate hyperplasia in the interlamellar region of the gill was seen in fish from both treatments at 15 and 27 dpe . The edematous changes in the secondary lamellae could also be fixation artifacts. No abnormalities were observed in liver, intestine, or acinar tissue.

Figure 3. Eosinophilic “hyaline” droplets (arrows) in the proximal tubular epithelial cells of suckers (bloom pH) sampled on 0 days post exposure (H&E stain, bar = 50µm).



#### Discussion:

The 27 d experiment successfully simulated the diurnal pH pattern observed in Upper Klamath Lake prior to (normal lake pH) and during *A. flos-aquae* blooms (bloom pH) during summer. The initial change in rearing conditions, from ambient pH 8.0 flow-through to pH 9.4 re-circulation, was associated with short-term diuresis. At 27 dpe, energy (lipid, protein, triglyceride) and immune (leukocyte composition, lysozyme activity) parameters were similar among bloom and normal lake pH fish. Bloom pH suckers could have experienced a greater stress level than the normal pH group as their whole body glycogen concentration was significantly lower at 15 and 27 dpe. The tertiary physiological responses of continued growth and low mortality indicate that changes in the above mentioned physiological indicators were not sufficient to significantly impair the fish (Mazeaud and Mazeaud 1981).

Alkalinity was the isolated influence in the experiment as both ammonia and dissolved oxygen were maintained at acceptable levels for the fish. As reported by Richie et al. (2006), ChlorAm-X was successful in controlling UIA in the recirculation systems. There was only one 12 h period during the 27d experiment with elevated ammonia (UIA 0.15 mg /L in the bloom system) and this level was well below the 96h LD<sub>50</sub> value of 0.5 – 1.1 mg /L (Saiki et al. 1999, Meyer & Hansen 2002). Control of ammonia was critical as 60.2% of total ammonia would

be the toxic unionized form at the mean daily pH of 9.58 (Emerson et al. 1975). Lost River suckers were successfully reared from eggs and grew rapidly in culture prior to the pH experiment.

Hyaline droplets within kidney tubule epithelium cells are typically excess protein reabsorbed from the filtrate and do not necessarily indicate dysfunction (Ferguson 1989). Hyaline droplets have been reported in rainbow trout exposed to elevated ammonia levels (Daoust and Ferguson, 1984). We have observed similar hyaline droplets in the tubules of Lost River Suckers reared for 62 d in pH 9.5 waters with elevated ammonia content (0.440 ppm NH<sub>3</sub>-N) and speculate that increase urine output, stimulated by elevated ammonia, resulted in the observed tubule changes (Foott et al. 2000). The observation of hyaline deposits in day 0 kidney samples indicates that diuresis was occurring in all fish during the 8 d acclimation period to pH 9.4. Fish appear to acclimate to alkaline conditions as hyaline droplets were not observed in 15 dpe samples and only minor amounts were seen in two 27 dpe bloom pH fish.

We had hypothesized that pH 9.5 conditions would impose a negative energy balance in juvenile suckers due in part to impaired ammonia excretion. While growth rate declined in both groups during the experiment, high pH did not differentially affect growth. Meyer and Hansen (2002) report that juvenile LRS exposed to water of pH 10 for 30d gained in bodyweight (positive growth) and had low mortality. These fish were of similar size as used in our experiment. Terwilliger et al. (2003) used models with otolith daily increment widths (daily growth estimates) and water quality data to analyze the effects of stressful water quality on juvenile sucker growth. They report no inhibition of growth associated with alkaline water conditions (> 9.0) during the summer. Suckers in the bloom pH system had significantly higher condition factors (shorter and heavier) than the normal pH fish. It is unlikely that high condition factor was a result of increased water content in the tissues as whole body protein and percent lipid were similar between the groups.

The effect of feeding a high energy diet was apparent in the higher protein, percent lipid, and triglyceride content of experimental fish compared to juvenile suckers collected from Upper Klamath Lake. Whole body protein levels of the bloom and normal lake study fish were 1.6 – 3.5x higher than observed in juvenile suckers of similar size collected from Upper Klamath Lake in August 2005 and 2008 (Foott et al. 2007, unpublished data on protein and triglyceride analysis of 25 frozen sucker carcasses submitted by USGS in 2008). Percent lipid content ranged from 0.3 – 2.0 % in juveniles from the lake while mean % lipid for study fish was 3 – 4.5 %. Similarly, the mean whole body triglyceride content tended to be higher (12 – 19 mg TG / g body) in study fish compared to the above feral fish (3 – 16 mg TG / g body). The initial declines in these somatic constituents between day 0 and 15 suggests an initial stress response by both treatment groups to experimental conditions followed by acclimation and increased energy storage. It is unclear what factors resulted in the observed lower whole body glycogen in bloom pH suckers. While early stages of the stress response promote glycolysis (catecholamine action), the effect of cortisol on carbohydrate metabolism is more complex and has not been fully reported

(Pickering and Pottinger 1995). Liver glycogen is the first energy reserve used by fish under starvation conditions followed by neutral lipids and then protein catabolism (Love 1980, Einen et al. 1998). We did not observe obvious declines in white blood cell composition indicative of overt stress (Wendelaar Bonga 1997). The significance of the 27d bacterial challenge results, in which bloom system fish had higher survival than normal lake cohorts, is limited by both small test group size (15) and lack of replication. At a minimum, this data suggests that the alkaline water in the bloom system was not overtly immunosuppressive.

We did not observe significant mortality (1 fish on day 3) or neurological symptoms associated with ammonia toxicity in the bloom pH group. Ronan et al. (2007) reports that brain monoamines (e.g. dopamine and serotonin) levels are markedly depressed in fathead minnows exposed to elevated ammonia. Alkaline waters are reported to alter ammonia excretion, sodium – chloride balance, and acid-base regulation in teleosts (Danulat 1995, Wilkie et al. 1999). In circumneutral pH (6 – 8) waters, about 90% of the fish's total nitrogenous waste is excreted from the gills as ammonia (by diffusion) with the remaining amount attributed to urea (Wilkie and Wood 1996). The hydration of excreted CO<sub>2</sub> by carbonic anhydrase generates H<sup>+</sup> ions that “trap” diffusing NH<sub>3</sub>. This acidification of the boundary layer along the outer gill membrane by carbonic anhydrase can be temporally inhibited by raising the pH or increasing the buffering capacity of surrounding water (Wilson et al. 1994). Blood to water diffusion gradients for NH<sub>3</sub> are unfavorable for branchial diffusion when ambient pH approaches the pK (9.0-9.5) of the NH<sub>3</sub> / NH<sub>4</sub><sup>+</sup> reaction (Danulat 1995). The ion transporting capacity and cell membrane permeability are also altered by alkaline waters. Wilkie et al. (1999) reports a 70% reduction in sodium and chloride influx across the gills of rainbow trout moved into pH 9.5 water. Three days post-transfer, chloride transport returned to normal but sodium transport remained 33% below normal. Branchial chloride cells (mitochondrial –rich ion transport cells) increase their exposed surface area in response to metabolic alkalosis and act in chloride transport recovery when trout are exposed to alkaline waters (Laurent et al. 2000). The calcium ion concentration of Upper Klamath lake and in this study was likely sufficient to aid in ammonia excretion (Iwama et al. 1997). Freshwater fish adapted to high pH environments show a variety of nitrogenous excretion / accommodation mechanisms (Wilkie and Wood 1996):

1. urea excretion over ammonia (ornithine-urea-cycle in marine toadfish and Lake Magadi tilapia),
2. excretion of ammonia and urea-N through the kidney (Lahontan Cutthroat trout {LCT}) and skin (marine flatfish ),
3. tolerance of elevated plasma ammonia and pH levels (Magadi tilapia, Lake Van cyprinid *Chalcalburnas tarichi*, LCT) which increase the pNH<sub>3</sub> gradient from blood to water,
4. elevated plasma pH, and
5. elevated glutamine synthetase (GS) activity in the brain to protect it from ammonia toxicity (*C tarichi*, LCT liver GS activity increased following 72 post-exposure to pH10 water).

There are no reports on the mechanism of nitrogenous waste excretion in Upper Klamath Lake suckers. The rapid elevation in ammonia observed in the July pilot experiment indicates the suckers were provided with excessive levels of dietary protein (Cai et al. 1996). The Otohime diet was reported to be 50% protein and this level of dietary protein was a likely factor in the continued growth of the 2 treatment groups. The rapid impairment of health observed during the pilot study phase indicates that elevated ammonia is stressful for juvenile LRS. Due to their small size we were unable to obtain sufficient plasma for evaluation of ions, ammonia, and cortisol. Evaluation of stress and physiological acclimation in any future study would benefit from analysis of whole body concentrations of these metabolic parameters.

Juvenile Lost R. suckers acclimated to summer lake pH conditions and maintained energy reserves while continuing to grow. This data in conjunction with other bioassay literature suggests that these fish can tolerate a wide range of water quality conditions up to critical threshold levels. If adequate food is available and other water quality parameters do not reach critical threshold levels, it is unlikely that summer alkalinity impairs growth or energy reserve development to a degree that would affect winter survival.

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