

Laboratory Challenges with the Bacterial Pathogen, *Flavobacterium columnare* and
Infection of Juvenile Lost River Suckers (*Deltistes Luxatus*) During Their Exposure to
Sublethal Ammonia Concentrations at pH 9.5

Completion Report
Order Number 10181-9m577

Submitted by

Richard A. Holt
220 Nash Hall
Corvallis, Oregon 97331

Prepared for

Tony Hawkes
Environmental Contaminant Specialist
U.S. Fish and Wildlife Service
6610 Washburn Way
Klamath Falls, Oregon 97603

June 28, 2001

Probably part of
1 F24
1 998/0003

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Summary

1. Three *Flavobacterium columnare* isolates which had been obtained from dying adult suckers at Upper Klamath Lake during a fish kill in August, 1996 and stored by lyophilization were chosen for use in bacterial challenge experiments. Two of the isolates were from gill lesions on an adult Lost River sucker and a shortnose sucker (*Chasmistes brevirostris*). The third *F. columnare* isolate was obtained from lesions in the mouth of an adult Lost River sucker.
2. Preliminary *F. columnare* challenge experiments were conducted in September and October, 1999 at the Corvallis Salmon Disease Laboratory. These experiments were designed to determine an effective bacterial exposure method for infecting juvenile Lost River suckers that could be used for the University of Wyoming challenges of suckers exposed to water with four sublethal ammonia concentrations. In the first challenge, the juvenile suckers, held in water at 22°C, were removed from a stock tank, stressed by netting them into a bacterial exposure container and exposed to one of the *F. columnare* strains at an 1:20 dilution of a 0.1 absorbance culture ($3.8 - 5.0 \times 10^6$ cfu/ml) for 10 minutes. In this test, *F. columnare* infections were detected and fish losses ranged from 44-76% depending on tank and *F. columnare* strain. In the second preliminary challenge, a tank draw-down procedure was done in glass aquaria containing 8 L of water with a 0.5 L inflow similar to those being used at the University of Wyoming Red Buttes laboratory. In this test, the water was lowered and the bacterial culture added excluding the stressing of the fish by netting into a separate container for exposure. The fish were exposed to an equal mixture of the three strains with the same concentration of cells as in the first test for 10 minutes. In this test very few fish died and there was no recovery of *F. columnare*.
3. The *F. columnare* cultures and supplies to conduct the challenges and to assay the fish for infections were transported to the University of Wyoming, Red Buttes Environmental Biology Laboratory, October 27-28, 1999. There were 24 aquaria in this experiment containing 25 juvenile Lost River suckers each. Fish in six aquaria were exposed to one of four sublethal ammonia concentrations. The bacterial challenge of the fish in 12 aquaria using the aquaria draw-down method was begun October 30, 1999. The draw-down method was considered the only practical challenge method that could be used in this test at the Wyoming laboratory. Fish in twelve other aquaria were exposed to sterile broth using the same method. See the report by University of Wyoming cooperators for more

details on experimental design and fish loss results. In this experiment, survival of the fish was increased in those tanks receiving elevated ammonia concentrations. *Flavobacterium columnare* was recovered from 16 of 17 dead or moribund fish sampled from the skin, gills and kidney in the experiment. The *F. columnare* recovery was greatest from the gills (16 positive of 17), followed by kidney (13 positive of 17) and skin surfaces (4 positive of 10). Recovery of the *F. columnare* was enhanced by use of a selective medium at 94.1% versus 70.6% in non-selective culture medium. Upon termination of the experiment on December 1, 1999, 10 killed fish from each tank were examined by inoculation of bacteriological media and no *F. columnare* was detected.

4. A concurrent side experiment involving the netting stress challenge method was conducted at the University of Wyoming Red Buttes Laboratory in conjunction with the main sublethal ammonia experiment. In this test, a few fish died in the *F. columnare* exposed groups but losses never approached those observed in the first preliminary test at Corvallis, Oregon. These results add to the evidence that the *F. columnare* strains from the Upper Klamath Lake fish loss in 1996 are of low virulence.
5. The survival of *F. columnare* cells in water of elevated pH and ammonia, elevated pH and "zero" ammonia, and pH 7.3 and "zero" ammonia was examined. This test was conducted to determine if water quality affected *F. columnare* viability during the bacterial challenge of the Lost River suckers at the University of Wyoming Laboratory. Results of this test showed the colony-forming units (cfu) per ml of *F. columnare* decreased slightly at 10 and 60 minutes of exposure to the elevated pH and ammonia water, or elevated pH and "zero" ammonia water but began to increase in all three water quality conditions after 3 and 15 hours. The greatest increase in *F. columnare* was observed in the water at pH 7.3 and "zero" ammonia. No dramatic decrease in viability of *F. columnare* in any of the water qualities tested was observed.
6. The effect of water temperatures of 22, 25 and 27°C was compared in a *F. columnare* challenge conducted on juvenile Lost River suckers at the Corvallis Salmon Disease Laboratory. The same three strains of *F. columnare* used in previous challenges were used with the tank draw-down challenge method. After 34 days, no losses had occurred in any of the duplicate tanks in which the fish were exposed to sterile broth or in the bacterial challenged aquaria receiving 22°C water. At 25°C, 10% of the bacterial challenged fish had died and 5% at 27°C. Higher losses with associated *F. columnare* infections were not observed at 27°C as had been expected. Once again it appears the *F. columnare* strains from the 1996 fish kill are of low virulence.

7. The growth rate of the Upper Klamath Lake *F. columnare* isolates was evaluated in tryptone yeast infusion broth at 20, 22, 24, 27, and 30°C. Growth was measured by changes in absorbance and percent transmission with a Spectronic 21. Most rapid growth occurred at 27 and 30°C for the first 24-30 hours but after 30 hours growth at the 20-24°C incubation temperatures began to reach or exceed that of growth at the higher temperatures. The most rapid growth appears to be nearest 27°C. When TYES agar plates were inoculated and incubated at each of the temperatures along with a test at 35°C, most rapid appearance of colonies occurred at 27, 30, and 35°C. Good growth of these Upper Klamath Lake *F. columnare* isolates occurred at 35°C.
8. A *F. columnare* challenge method comparison was conducted at the Corvallis Salmon Disease Laboratory at a water temperature of 25°C. Groups of Lost River suckers (3.3 g) were challenge by the handling net stress method involving transfer to a separate exposure container and compared to the aquaria water level draw-down procedure. A mixture of the same three *F. columnare* strains were used at the same previously used concentration for 10 minutes. In this test, after 28 days, only one challenge (draw-down) fish died with *F. columnare* infection, again demonstrating that under these challenge conditions the *F. columnare* strains from Upper Klamath Lake are of low virulence. Perhaps the presence of parasites such as copepods and leeches on the adult suckers dying in the lake in 1996 provide an entry for these low virulent strains to invade the fish tissue.

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Laboratory Challenges with the Bacterial Pathogen, *Flavobacterium columnare*, and Infection of Juvenile Lost River Suckers (*Deltistes Luxatus*) During Their Exposure to Sublethal Ammonia Concentrations at pH 9.5

Introduction

A series of severe fish kills have occurred in recent years in Upper Klamath Lake in south central Oregon among two federally listed endangered suckers, the Lost River sucker (*Deltistes Luxatus*) and the shortnose sucker (*Chasmistes brevirostris*). During August-October, 1996 the largest kill was documented with 60,000 to more than 240,000 adult suckers estimated to have died (Perkins et al. 1996). The fish kill was preceded by various environmental conditions including high water temperatures (due to prolonged warm weather), algal blooms, low oxygen conditions, high water pH and elevated concentrations of un-ionized ammonia. Dying suckers were submitted to the Oregon Department of Fish and Wildlife Fish Pathology Laboratory at Corvallis for necropsy. These fish were found to be infected with the bacterial fish pathogen, *Flavobacterium columnare* (Bernardet et al. 1996) and to have concurrent infestations of leeches, anchor worms, protozoan parasites such as Ichthyobodo, fungi and aeromonad or pseudomonad opportunistic bacteria.

Flavobacterium columnare is reported to cause high losses in many species of fish when they experience high water temperatures (Pacha and Ordal 1970; Holt et al. 1975, Becker and Fujihara 1978). Also, a wide variation in virulence has been reported among different strains of this bacterium isolated from fish (Rucker et al. 1953, Pacha and Ordal, 1967, 1970). Strains of low virulence produced slow progressive infections at water temperature above 21°C with massive tissue damage before death while high virulent strains caused fulminating infections, killing young salmon in 12-24 hours at 20°C. Fish infected with high virulent strains showed no gross tissue damage at the time of death.

The virulence of strains of *F. columnare* obtained from diseased suckers from the Upper Klamath Lake is unknown, and their impact along with the effects of sublethal ammonia exposures to these fish. This project was begun to provide assistance in conducting a laboratory experiment at the Fish Physiology and Toxicology Laboratory, University of Wyoming, Laramie, Wyoming. This experiment was designed to test the hypothesis that strains of *F. columnare* obtained from the 1996 sucker kill either alone or in combination with prior exposure to adverse water-quality conditions such as elevated ammonia concentrations and pH would result in losses in juvenile Lost River suckers. Little is known about the virulence of these *F. columnare* strains from Upper Klamath Lake suckers and perhaps the test would help to explain the 1996 sucker kill as well as other fish kills in the Klamath Basin.

The experimental design, water quality monitoring and all other details of this experiment were developed by cooperators including the principal investigator, Dr. Elaine Snyder-Conn (at the initiation of this study was Contaminant Specialist, Klamath Basin, USFWS) and cooperators at the University of Wyoming including Dr. J. Meyer, J. Morris, M. Suedkamp, S. Clearwater, and H. Lease. For more detail on facilities, water quality monitoring and methods, contact the aforementioned or refer to their report of this study. Dr. Scott Foote of the California-Nevada Fish Health Center provided histological and hematological evaluation in this study.

Scope of Work

The following tasks were selected for this project to be provided by Richard A. Holt, Corvallis, Oregon. Order No. 10181-9M577.

1. Select and transport sufficient viable cultures of *Flavobacterium columnare* from the 1996 Upper Klamath Lake fish kill to perform bacterial challenges on Lost River suckers at the University of Wyoming in twelve aquaria stocked with up to 25 fish/aquarium. Conduct preliminary challenge tests at the Salmon Disease Laboratory, Corvallis, Oregon to determine appropriate exposure protocols.
2. Perform bacterial challenges in the aquaria stocked with Lost River suckers at the University of Wyoming Fish Physiology & Toxicology Laboratory, 30 days after exposure of the suckers to sublethal ammonia concentrations.
3. Culture the bacteria from the suckers and evaluate infection rates on suckers from the exposed aquaria and from an equal number of control aquaria containing suckers.
4. Rank the severity of pathogenic lesions on each sucker, and determine the presence/absence of *Flavobacterium columnare* on the skins and gills of each fish through cultures of the bacterium. Train University of Wyoming personnel in standard methods to culture the bacterium, if additional help is needed.
5. Present the results of these findings by aquarium and/or ammonia treatment and accompanying tables or spreadsheets. Provide the data in spreadsheet format on a diskette or through electronic mail. Include in the report a complete description of methods, and full citations for any previous work cited.

Additional work identified during the course of the challenge experiment: Modification #1

6. Test the viability of *F. columnare* bacteria exposed to water at pH 9.5 and elevated and "zero" ammonia concentrations and in a pH 7.3 "zero" ammonia holding tank.
7. Conduct a columnaris challenge on Lost River sucker juveniles exposed to water temperatures of 22°, 25° and 27°C at the Salmon Disease Laboratory, Corvallis, Oregon.

Selection of *Flavobacterium columnare* Strains for the Challenge Study.

During the 1996 fish kill in Upper Klamath Lake, moribund and fresh dead adult shortnose and Lost River suckers were collected on August 29, 1996 by Mike Green, Bureau of Reclamation. He transported these fish to the Corvallis Fish Pathology Laboratory at the Department of Microbiology, Oregon State University. Classic columnaris gill lesions were evident on 19 of 26 fish sampled. Lesions on the gills and mouth were noted and sampled along with kidney tissue by inoculation onto a modification of TYES agar (Holt et al 1989) and cytophaga agar (Anacker and Ordal 1959). The modified TYES agar consists of agar 1.0%, tryprone 0.4%, yeast extract 0.04%, Mg504.7H₂O 0.05%, CaCl₂ 2H₂O 0.02% adjusted to pH 7.1. A sterile milk solution (0.2g/ml) is added to the cooled medium just prior to pouring plates at 1.0 ml per 100 ml medium. The agar plates were incubated at 18°C for 4-7 days before being evaluated for growth. Typical *F. columnare* colonies were isolated from 25 of 26 adult suckers. Identification of the bacteria was based on observing the typical greenish yellow, flat rhizoid colonies that adhere tenaciously to the agar. A sample of isolates was identified by the procedures described by Griffin (1992). After a limited 2 or 3 transfers on agar medium, the *F. columnare* strains were preserved by lyophilization in skim milk and stored at -20°C.

Three strains of the preserved *F. columnare* from the August 29, 1996 examination were chosen for this project to be used in challenges at the Red Buttes Laboratory, University of Wyoming, each of which, were confirmed as *F. columnare* by procedures described by Griffin (1992). Details regarding each strain are as follows:

Strain Designation: KlsuM17-96, Klsu65-96, and Klsu61-96,

#KlsuM17-96. This isolate was obtained from a Lost River sucker adult (no length recorded). This fish had no gill lesions but was hemorrhaged in the mouth with leeches and ulcers in the buccal cavity. This isolate was obtained from the mouth but columnaris bacteria were also grown from the gill and kidney. Some fungi and opportunistic bacteria were found on the gills.

KlsuG5-96. This stain was isolated from the gill lesion of a Lost River sucker (565 mm total length), sex not recorded. This fish had a large fungal lesion on the left upper area of the gill, ulcers in the mouth and 23 leeches attached in the mouth. Also, there was an anchor worm at the dorsal fin base. Columnaris bacteria were isolated from the gill lesion and kidney. Also, some opportunistic bacteria (aeromonads and pseudomonads) were isolated from the gill and kidney.

KlsuG1-96. This isolate came from the gill of a shortnose sucker female (435 mm total length). This fish had a typical columnaris gill lesion. There were several leeches in the buccal cavity and columnaris bacteria observed in the mouth along with fungal hyphae. Much *Ichthyobodo* was observed on the gills. There was a hole in the skin at the base of a pectoral fin with an anchor worm attached. Columnaris bacterial were isolated from the buccal cavity, gill, and kidney. Some opportunistic bacteria were isolated from buccal cavity, kidney and gill.

Preliminary *F. columnare* Challenge Tests on Lost River Suckers at the Corvallis Salmon Disease Laboratory, Corvallis, Oregon

Purpose – Conduct bath challenges with the selected *F. columnare* isolates on juvenile Lost River suckers to determine appropriate exposure protocols necessary to best produce *F. columnare* infections in the subsequent challenges at the University of Wyoming laboratory. Also, to “pass” the *F. columnare* isolates through fish and preserve the strains for subsequent challenges.

Test Fish – The Lost River juvenile suckers at 0.6-0.8g each were received at the Corvallis Salmon Disease Laboratory from the Klamath Tribes Native Fish Hatchery, Chiloquin, Oregon, September 13, 1999. Mr. Larry Dunsmoor shipped 200 fish by United Parcel Service. Upon arrival, these fish were held in a 100 L stock tank receiving 2L pathogen-free well water at 13°C. The water temperature was raised slowly to 20°C during the day of arrival. These fish received combination of two different diets as recommended by Mr. Dunsmoor. Twice a day they received shavings of frozen brine shrimp and a two component gel mixture fed at appropriately 8% body weight per day. The gel mixture consisted of 40% Argent Cyclop-eeze (Redmond, Washington) and 60% Gel fish food of Aquatic Eco-systems, Inc., Apopka, Florida. No fish died prior to starting the challenge experiment.

Water quality-The Salmon Disease Laboratory well water has a hardness (Ca) of 27.28, total hardness of 110-125, Alkalinity 72-77, conductivity 0.268, TDS 135, pH 6.81-7.2 and ambient water temperature of 12.8°C.

Preliminary Challenge Test #1

Methods - A modification of the contact method of exposure described by Pacha and Ordal (1967) was used in the first preliminary challenge test. A challenge bath for each *F. columnare* isolate was prepared as follows: the three *F. columnare* isolates were removed from lyophilized storage by inoculating 100 ml tryptone yeast infusion (TYI) broth (Pacha and Ordal 1967) and incubating at 18°C. After 48 hours, about 50-75 ml of each actively growing culture was poured into each of three prescription bottles containing 600 ml of TYI broth. These were held stationary at room temperatures for about 16 hours, then put on a reciprocal shaker for about 4 hours. Each culture was then adjusted to 0.1 absorbance at 525 nm on a Spectronic 20 using sterile TYI broth for dilution. The total viable count of each bacterial strain was then estimated using a spread plate method (Manual of Methods for General Bacteriology, 1981). Ten-fold dilutions

were prepared in tubes containing 0.5% NaCl in distilled water and 0.1 ml of each dilution inoculated on duplicate plates of TYES or cytophaga agar. Colonies of growth (colony forming units) were counted with a dissection microscope after three days incubation at room temperature.

Once the absorbance had been adjusted to 0.1 at 525 nm for each culture and the spread plate method conducted on one of the adjusted culture (Klsu65-96) then all three adjusted cultures were transported to the Salmon Disease Laboratory and the fish bath challenges completed within 90 minutes. Upon completion of the challenges of the fish, the plate counts were performed on the other two cultures which had been adjusted to 0.1 absorbance.

Fish bath challenges – The challenge was conducted on September 22, 1999. A challenge bath for each isolate of *F. columnare* was prepared as follows: in a 9.5L plastic container, 5,700 ml of tank water at 22°C and 300 ml of bacterial culture at 0.10 D were mixed; this provides a 1:20 dilution of the culture. The stock tank water level was lowered and 25 fish netted into a 4.0L beaker, the fish were poured out of the beaker into a net and promptly placed in the challenge bath for 10 minutes. Then, the fish were netted out of the plastic tub and placed in separate 100L tanks receiving 22°C water. One group of 25 fish were exposed to each *F. columnare* bath.

The fish were monitored for loss twice per day and dead fish removed and sampled for bacteria. Gills, skin, snout, if hemorrhaged, and kidney tissue were sampled by running a sterile inoculating loop over the surface or penetrating the tissues in the case of the kidney. Samples were placed on TYES or cytophaga agar plates. Plates were incubated at room temperature for 4 – 7 days and examined for the typical greenish, yellow rhizoid colonies of *F. columnare* that adhere to the agar.

Results and Discussion – (Preliminary Challenge Test #1)

Plate count of adjusted broth cultures –The plate count results for Isolate Klsu65-96 was 7.7×10^7 colony forming units (CFU)/ml of broth. In the challenge bath this was diluted 1 part culture to 19 parts water so fish were exposed to 3.8×10^6 CFU/ml for 10 minutes. For isolate KlsuG1-96 the count was 8.2×10^7 CFU/ml of the adjusted culture, and the fish were exposed to 4.1×10^6 CFU/ml. For isolate KlsuM17-96, the count was 1.0×10^8 CFU/ml of adjusted broth culture and the fish were exposed to 5.0×10^6 CFU/ml.

Daily loss that occurred after the bath challenge September 22, 1999 is shown in Table 1. The experiment was terminated on October 9, 11 days after the last death occurred. For fish in tank C10 exposed to *F. columnare* strain Klsu6.5-96, 19 of 25 fish died or 76%. Total loss of fish in tank C11 exposed to strain KlsuG1-96 was 11 or 44% and loss of fish in tank C12, exposed to strain KlsuM17-96 was 16 or 64%. The survivors in each tank were counted to account for the 25 fish total per tanks when the test was initiated. This experiment is limited by the lack of controls. The stock tank fish were held at the same

water temperature and in similar 100 L tank with no loss of fish but this was not a valid control to measure the effect of netting and handling. Under the conditions of the challenge with netting and handling stress before and after the exposures, each of the three isolates of *F. columnare* appeared to cause infections and were suspected to be involved in the deaths. Some of the dying fish were observed to have severe skin hemorrhaging on the snout and head region. No evidence of gill lesions could be seen on these small fish.

Table 2 shows a summary of the culture results of the fish that died in each tank during experiment. The recovery of *F. columnare* from dead fish was 57.9% for tank C10, 63.6% for tank C11, and 31.2% for tank C12. In all cases where the culture results were negative for *F. columnare*, large overgrowth of opportunistic bacteria (aeromonads and pseudomonads) was found on the culture plate. Due to time constraints, the dead fish could only be removed from the tanks twice per day and at 22°C water temperature opportunistic bacteria developed rapidly on dead fish and probably masked the presence of *F. columnare*. It is obvious from this test that a selective medium that would inhibit other bacteria would enhance our chances of detecting *F. columnare*. Therefore, it was decided a medium such as selective cytophage agar (SCA) described by Hawke and Thune (1992) should be used in sampling dead fish in the experiment planned at the University of Wyoming.

Preliminary Challenge Test #2

Purpose- A second preliminary challenge test at Corvallis was determined to be necessary after discussions with co-investigators, Elaine Snyder-Conn, USFWS and Joe Meyer, University of Wyoming. This was because the methods of fish handling and bath exposure employed in test #1 were not going to be practical or safe to prevent contamination with the number of tanks (24) required in the experiment planned at the University of Wyoming Red Buttes laboratory.

Methods- The challenge protocol proposed for the test #2 was as follows: this was a challenge method similar to one devised by Holt et al. (1975) in which the fish remained in their tanks during the exposure, the water level lowered, influent water supply removed from the tanks, bacterial culture added to achieve a 1:20 dilution of a 0.1 absorbance for 10 minutes, then the inflow water re-introduced to dilute the challenge bath. This method eliminates the handling of the fish and associated contamination concerns.

The Preliminary Challenge Test #2 was begun at the Salmon Disease laboratory at Corvallis on October 14, 1999 and was designed to determine if these challenge procedures would result in *F. columnare* infections in juvenile Lost River suckers. Six 20L glass aquaria (total water volume 8 L) similar to those used at the University of Wyoming laboratory were installed with an influent water supply at 22°C of 0.5 L/min. Because there was a limited number of juvenile suckers available for the test. Three tanks contained 25 fish each and three tanks had 15 fish each. The fish were distributed into the tanks in 10 and 5 fish groups until all tanks had the appropriate numbers of fish.

On October 15, 24 hours later the challenges were initiated. The treatments were as follows: two control tanks C10 with 25 fish, C14 with 15 fish; two tanks receiving the 1:20 dilution of 0.1 absorbance bacterial culture (100ml/tank) C12 with 25 fish and C15 with 15 fish; and two tanks receiving 1/4 challenge dose (25ml/tank) tank C11 with 25 fish and C13 with 14 fish. The treatment each group received within the 25 fish tanks and 15 fish tanks were randomized. The tank water level was lowered to 1900 ml,

Table 1. Results of Preliminary Challenge Test #1 including loss information and results of bacteriological examination of mortality.

Tank C10 (Klsu65-96)			Culture Results*			
Date	Time	Morts	Gill	Kidney	Other	<i>F. columnare</i>
9/22	4:30-6PM	Experiment Started				
9/23	6:00PM	1	-	-		
9/24	7:30 AM	2	-	-		
		3	-	-		
9/24	6:45PM	4	-	-		
		5	-	-	skin +	+
9/25	9:00Am	6	-	+		+
9/25	7:00PM	7	+	-	snout +	+
		8	+	-	snout -	+
		9	+	-		+
		10	+	+	snout +	+
9/26	6:10AM	11	+	-		+
		12	+	-		+
9/26	8:30PM	13	+	-		+
		14	-	-		
		15	+	+		+
9/27	8:00AM	16	-	-		
		17	+	+		+
9/27	7:30PM	18	-	-		
9/28	7:30AM	19	-	-		
		19/25	9	4	3	+11/19
		76%				(57.9%)

+ = Presence of typical *F. columnare* colonies, color, morphology and adhere to agar.

- = In all cases APS (opportunistic bacteria) overgrowth very likely prevented isolation of *F. columnare*.

*Cultures on Cytophaga agar and TYES agar.

Table 1. continued
C11 (KlsuG1-96)

Date	Time	Morts	Gill	Culture Results		<i>F.columnare</i>
				Kidney	Other	
9/23	2:00PM	1	+	+	mouth -	+
		2	+	+	mouth +	+
9/23	6:00PM	3	-	-		-
9/24	7:30AM	4	-	-	skin -*	-
9/24	6:45AM	5	-	-		-
		6	-	+		+
		7	+	-		+
9/25	9:00AM	8	-	-		-
		9	+	+		+
		10	+	+		+
9/26	6:10AM	11	+	-		+
		<hr/> 11/25 44%	<hr/> 6	<hr/> 5	<hr/> 1	<hr/> 7/11 63.6%

- Wet mount of skin typical *F. columnre* cells

C12 (KlsuM17-96)

Date	Time	Morts	Gill	Culture Results		<i>F.columnare</i>
				Kidney	Other	
9/23	6:00PM	1	-	-		-
		2	-	-		-
9/24	6:45PM	3	-	-		-
		4	-	-	skin +	+
9/25	7:00PM	5	-	-		-
9/26	6:10AM	6	+	-		+
		7	-	-		-
9/26	8:30PM	8	-	-		-
		9	+	-		+
		10	-	-		-
		11	-	-		-

9/27	8:00AM	12	+	+	+
		13	-	-	-
		14	-	-	-
9/27	7:30PM	15	-	-	-
9/28	7:30AM	16	+	-	+
			16/25	4	1
			(64%)		5/16

(31.2%)

influent water line removed, and 100ml of either bacterial culture or sterile broth (control) was added, mixed around by swirling a net 3 times in the tank and after 10 minutes the inflow resumed. The 1/4 challenge dose consisted of 25 ml bacterial culture 0.1 absorbance and 75ml sterile broth. The bacterial challenge culture consisted of an equal mix of (200ml of each) of the three strains grown as described in test #1, adjusted to 0.1 absorbance. A plate count of viable *F. columnare* cells in the mixed culture was done just prior to the challenge. The fish were maintained on feed as described in test #1 and tanks examined for losses twice per day.

Results and Discussion- Plate count of the 0.1 absorbance mixed challenge bacterial culture was 9.8×10^6 CFU/ml. Fish in C12 and C15 were exposed to an approximate dose of 4.9×10^6 CFU/ml and those that received 1/4 dose, C11 and C13, were exposed to 1.2×10^6 CFU/ml.

In contrast to Preliminary Challenge Test #1 in which fish began dying within 24 hours of exposure, only three fish died in the first four days in this test. One control fish (C14) died on October 16, one day after challenge and much opportunistic motile bacteria were found on the gills but the kidney was negative. Also, two fish died in tank C11, a bacterial challenged group, one on 10/17 and one on 10/19. However, much opportunistic bacteria and no *F. columnare* was recovered (1/4 dose) from these two fish.

Because there was very low loss in the bacterial challenge groups of this experiment by the 4th day, a second enhanced bacterial challenge was conducted on these fish in tanks initially exposed to 0.1 absorbance culture (C12 and C15). Fish in these tanks were again exposed to a challenge of bacterial culture in a similar manner tank level draw-down with a 1:20 dilution except with a 0.2 absorbance for 20 minutes. Fish in control tanks C10 and C14 were each exposed to 100 ml of sterile broth in 1900 ml for 20 minutes. The plate count of the 0.2 absorbance mix of three strains culture was 1.18×10^8 CFU/ml. Fish in the challenged tanks were exposed to 5.9×10^6 CFU/ml for 20 minutes, then the 0.5L/min inflow was introduced to diluted out the challenge dose after the 20 minutes. From October 19-Oct. 25 (6 days) no losses occurred in any of the tanks. All groups were terminated October 25th.

Under the condition of this experiment, when juvenile Lost River suckers were exposed to a mixture of the three *F. columnare* isolates, at either 2.1×10^6 , 4.9×10^6 and 5.9×10^6 CFU for 10min, 10 min and 20 min respectively, no fish losses occurred within 6 days after exposure and no *F. columnare* was recovered. When comparing Preliminary Challenge Tests #1 and #2, it appears the netting and handling of the first challenge was necessary for infections of *F. columnare* to occur. Apparently these *F. columnare* strains from the 1996 Upper Klamath Lake fish kill are not highly virulent when used in a challenge under the conditions at the Salmon Disease Laboratory exposure of 22°C water temperature, pH 6.8-7.2, and hardness (Ca) of 27.3. According to Pacha and Ordal (1970) a low virulent *F. columnare* strain requires over 96 hours to kill 100% of the experimental fish (in their case it was juvenile salmon).

Based on the results of these preliminary challenge tests, we were uncertain that the aquaria water level draw-down challenge method would provide adequate infections of *F. columnare* in the Lost River suckers involved in the sublethal ammonia exposure experiment at the University of Wyoming. The challenge at the University of Wyoming was scheduled to start October 30. Perhaps the sublethal exposure of juvenile suckers for 30 days to elevated ammonia concentrations and pH 9.5 water would predispose these fish to infections from these low virulent *F. columnare* strains.

These preliminary challenge tests did demonstrate the need to use selective media such as selective cytophaga agar (Hawke and Thune 1992) to enhance the detection of *F. columnare* from fish that had died and may have laid in the bottom of the aquaria in 22°C water for a few hours.

***Flavobacterium columnare* Challenge of Juvenile Lost River Suckers Exposed to Sublethal Ammonia Concentrations at the University of Wyoming Red Buttes Environmental Biology Laboratory, Laramie, Wyoming.**

Purpose- The cooperators in this study developed the hypothesis that prior exposure to adverse water quality conditions increases the susceptibility of Lost River suckers to *F. columnare* infections. This experiment conducted at the University of Wyoming Red Buttes Laboratory was designed to test if high pH and elevated sublethal ammonia concentrations in combination with the fish pathogen *F. columnare* were factors that would help explain the large fish kills observed in Upper Klamath Lake, Klamath, Oregon.

Methods- The experimental design, water quality monitoring and all other details of this experiment were developed by cooperators including the principal investigator, Dr. Elaine Snyder-Conn (at the initiation of this study was Contaminant Specialist, Klamath Basin, USFWS) and cooperators at the University of Wyoming including Dr. J. Meyer, J. Morris, M. Suedkamp, S. Clearwater, and H. Lease. For more detail on facilities, water quality monitoring and methods, contact the aforementioned or refer to their report of this study. Dr. Scott Foote of the California-Nevada Fish Health Center provided histological and hematological evaluation in this study.

Experiment design- Juvenile Lost River suckers were exposed for 30 days to four sublethal ammonia concentrations at pH 9.5 in six replicate aquaria per concentration or 24 total aquaria. After 30 days, fish in 12 of the aquaria (three aquaria from each of the four ammonia concentrations) were exposed to *F. columnare* culture for 10 minutes. Following the bacterial challenge, the fish continued to be exposed to the same ammonia concentrations for 30 additional days. Aquaria were monitored for loss and dead fish tested for the presence of *F. columnare*.

Test Animals-The Lost River suckers used in this study were obtained from the Klamath Tribes Native Fish Hatchery Located in Chiloquin, OR. These fish were shipped by air - carrier as five month old larvae on September 8, 1999; three weeks prior to the initiation of the 30 day chronic ammonia bioassays. The fish were held in tanks receiving 22°C, pH 7.9 water at 110 mg/L hardness. On September 15, the fish were acclimated to 21.5°C, at pH 9.5, and hardness of 50 mg/L. Fish were fed refrigerated Argent (Redmond, WA) Cyclop-eze, a freeze-dried copepod product, at a rate of 2.6% of body weight/feeding per day, with three daily feedings supplemented by refrigerated *Artemia* eggs at a rate of 3% body weight at an additional daily feeding.

Experimental Aquaria and Water Quality Conditions-On the start date of the 30 day chronic exposure, 25 fish averaging 0.69g (47.2mm) were placed into each of the 24 experimental aquaria, each holding 8 L of water. On the same day, the temperature was increased 0.5 °C and thereafter maintained at 22-23 °C until day 6 after the bacterial challenge when the water temperature was slowly raised to 24°C.

Inflow water was delivered from the diluter to each aquarium at a rate of 0.5 L/min. This water was prepared from a mixture of Red Buttes well water and reverse-osmosis/deionized (RO/DI) water, producing a hardness of 50 mg/L as calcium carbonate. Adjustments of hardness were made by adjusting the ratio of RO/DI water as needed and pH was adjusted either by addition of potassium hydroxide or sulfuric acid. Each aquarium was equipped with a lid to limit aerial contamination of *F. columnare* once bacterial challenges were performed.

One of four ammonia concentrations was maintained in six aquaria each using proportional, flow-through diluters. Target levels were less than 0.1, 0.125, 0.25, and 0.5 mg/L NH₃-N as unionized ammonia based on the temperature and pH of the tanks. These levels were established previously as sublethal during 30-day chronic bioassays with larval Lost River suckers at this laboratory. Measured exposure concentrations of unionized ammonia were 0.006, 0.117, 0.220, or 0.433 mg NH₃-N/L) at pH 9.5 (information provided by J.M. Morris, University of Wyoming).

Bacterial Challenge-*Flavobacterium columnare* strains- The challenge mixture used in this experiment was a composite of equal portions of the same three strains used in Preliminary Challenge Test #1. The isolate designation codes of the strains used are KlsuM17-96, KlsuG5-96 and KlsuG1-96. These columnaris strains were isolated from lesions on the gills and mouth of two Lost River and one shortnose adult suckers which were affected during the 1996 fish kill in Upper Klamath Lake, Oregon

The challenge solution of *F. columnare* used in this experiment was a combination of cultures grown from each of the three original lyophilized strains plus a representative isolate from each of these strains obtained from dying juvenile suckers in the Preliminary challenge Test #1. These isolates were recovered on cytophaga agar or TYES agar and lyophilized in skim milk. Basically, the challenge solution for the experiment at University of Wyoming was a composite of six equal portions, three from the original isolates from the 1996 fish kill and three from the same isolate passed once in the Preliminary Challenge Test #1 of juvenile suckers at Corvallis, Oregon.

Preparation of the Challenge Solution-The six lyophil ampoules containing the three original *F. columnare* isolates and three stored after recovery from juvenile suckers in the Preliminary Challenge Test #1 were opened and inoculated into prescription bottles containing 100 ml of TYI broth. The one-time passed strains were taken out of lyophilized storage and inoculated on October 26, 1999 prior to transport to the Red Buttes Laboratory, then transferred to new bottles of TYI on October 28. The original three isolates were taken out of lyophilized storage on October 28 and inoculated into TYI bottles at the Wyoming laboratory. Samples from each lyophil tube were placed on cytophaga or TYES Agar plates to observe typical colony morphology and check culture purity. Cultures were incubated stationary at 22-23°C. On October 29, smears of each broth culture were prepared on slides, heat fixed and stained using the Gram method and observed microscopically at 1000 times magnification for Gram negative long thin rods typical of *F. columnare*. After the check for purity, 35-50 ml of each culture was inoculated into 900 ml prescription bottles containing 600 ml of TYI broth. The cultures were incubated at 23°C stationary for 15 hr, then placed on a New Brunswick Scientific C24 Incubator Shaker at 120 rev./min. for five hours at 23°C. The initial stationary incubation was a method suggested by Pacha and Ordal(1967) to avoid or reduce the formation of aggregate clumps of cells.

On October 30, 1999, 3:30 PM the cultures were removed from the shaker, samples taken for smears, Gram stained and the purity checked microscopically. Then each culture was adjusted to a 0.1 absorbance at 525 nm using a Spectronic 20 and sterile TYI broth for dilution. Four hundred ml of each of the adjusted six cultures were combined to provide sufficient challenge culture. The combined cultures with an absorbance of 0.11 at 525 nm was sampled to obtain a viable cell count using the spread plate method (Manual of Methods for General Bacteriology, 1981). Sterile dilution blanks (9 ml) of 0.5% saline were used to make 10 fold dilutions of the culture for inoculation onto cytophaga agar plates in quadruplicate and TYES agar in duplicate. Glass sterile pasteur pipets made into "hockey sticks" were used to spread the sample over the surface of each plate. Plates were incubated at 23°C for 48 hr, the colonies counted using a low power dissection microscope and recounted at 72 hr.

The plate counts for the composite culture on cytophaga agar were 93.25×10^6 CFU/ml and for TYES Agar 105.5×10^6 CFU/ml. The challenge dose used for the fish was 100 ml culture to 1900 ml of aquarium water. Therefore, the culture count was divided by 20 to obtain the exposure per ml or 4.7×10^6 to 5.3×10^6 CFU/ ml.

Challenge Method- On October 30, 1999, at about 5PM the challenge procedure was begun on the 24 aquaria. This was day 30 of the exposure for the suckers to the various ammonia levels. The exposures were performed by first preventing the inflow tubes from spilling into the aquaria, then concurrently draining the 12 designated control tanks (previously selected randomly) by 4,100 ml, then draining all 12 aquaria an additional 2000 ml. These draw-downs were completed in 13 minutes. Immediately after drainage to 1900 ml, each aquarium received 100 ml of sterile TYI broth that had been pre-measured into beakers. Each aquarium then received three slow figure eight stirs with an aquarium net to mix the water and broth. After 10 minutes, flow into the aquaria was resumed. Next the challenge bacterial culture was measured into 100 ml quantities in beakers and a similar draw-down procedure and exposure described for the control tanks was applied to the 12 aquaria selected for challenge with *F. columnare*. After 10 minutes the 0.5 L/min flow to the aquaria was reintroduced and gradual dilution of the challenge culture began. Beginning immediately after the challenge, all aquaria were monitored hourly for dying fish for the first three days and hourly from 8AM to 8PM thereafter.

Examination of Moribund and Dead Fish-Dying or dead fish were observed to assess for gross external anomalies including columnaris gill or skin lesions, hemorrhaging and other signs of disease. Selected moribund fish were preserved in Davidson's fixative for histology examination of the gills, kidney and liver. Selected moribund or dead fish from each aquarium in which losses were occurring were examined for the presence of *F. columnare* indicating possible infection. Skin, mucus, gills and kidney tissue samples were collected with a flame sterilized inoculation loop and placed on three media cytophaga agar, selective cytophaga agar (SCA) and TYES agar plates to enhance recovery of this bacterium. Skin mucus was streaked for isolation on a full SCA plate. Gill and kidney tissue inoculated on a full cytophaga agar plate and 1/2 plate of SCA and TYES agar. On November 6, on the seventh day after the challenge, the sampling for bacterial infection in dying fish was simplified to inoculating gill and kidney samples on cytophaga agar and SCA plates.

Inoculated plates were incubated at 22°C and evaluated for bacterial growth after three to seven days. Beginning November 6, cultures plates inoculated with fish tissues from fish that had died were prepared by Jeffrey Morris and Michael Suedkamp of the University of Wyoming Red Buttes Laboratory and were sent by next day air carrier to Rich Holt at the Corvallis Fish Pathology Laboratory.

Upon termination of the experiment on December 1, 1999, fish were killed with anesthetic (MS222) and a 10 fish sample obtained for bacterial culture from each tank to assess for presence of *F. columnare*. Fish were individually wrapped in a paper towel, placed in plastic bag, kept cool in an ice chest and shipped on December 2 by Federal Express to Corvallis, Oregon. Each fish was sampled by placing gill and kidney tissue on SCA and cytophaga agar and the plates incubated at 21-22°C. Plates were examined in 4-7 days for bacterial growth.

All inoculated plates were examined for bacterial growth and the results recorded. Typical *F. columnare* colonies were identified by the pale yellow-green color, adherence to the agar, and spreading of the colony with a convoluted center and rhizoid edge. To

further confirm the identification as *F. columnare*, sample colonies with typical characteristics were transferred to a medium described by Griffin (1992) and tested according to the methods described. Growth was obtained from plates from 11 suspected positive fish. All 11 were positive for chondroitin lyase and hydrolysis of gelatin. Using this medium, the identification scheme involved five cultural and biochemical characteristics of *F. columnare*: (1) ability to grow on a medium containing neomycin sulfate and polymyxin B (2) colony color and morphology consistent with typical *F. columnare*; (3) production of a diffusible gelatin degrading enzyme; (4) binding of aqueous Congo red dye in the surface secretions of suspected colonies; and (5) production of a diffusible enzyme (chondroitin AC lyase) that degrades chondroitin sulfate A.

Results and Discussion-

Complete details of the results of survival and growth of the juvenile Lost River suckers exposed to four sublethal ammonia concentrations for 30 days prior to the *F. columnare* challenge and 30 days thereafter are available from the co-investigators in this study at the University of Wyoming Red Buttes Laboratory. Survival of the suckers decreased in aquaria inoculated with *F. columnare*, however, contrary to our hypothesis, survival increased as the concentration of unionized ammonia increased (J.M. Morris et al. 2000).

Recovery of *F. columnare* from Moribund or Dead Fish- The results of the examination of dead or moribund fish for *F. columnare* are shown in Table 2. Columnaris was detected only in the bacterial challenged groups. Of 17 total fish available for bacterial sampling, *F. columnare* was detected from 16 or 94.1%. The recovery of *F. columnare* was greatest from the gills (16 of 17 or 94.1%); 13 of 17 or 76.5% from the kidney and 4 of 10 or 40% from the skin surface. This is characteristic of *F. columnare* to attach and be detected in gill tissue and found less often in internal tissues. Detection of *F. columnare* was enhanced with the selective medium SCA, resulting in a 94.1% isolation of the pathogen compared to 70.6% (12 of 17 fish) using cytophaga or TYES agar. A few fish showed hemorrhaging on the snout or head and some had a slight yellowish color in the skin.

On December 1, 1999 (32 days after bacterial challenge) all fish were killed and 10 fish were examined from each aquarium for *F. columnare* infection by placing gill and kidney samples on SCA and cytophaga agar. No *F. columnare* was detected in fish from any group. Opportunistic bacteria, probably aeromonads or pseudomonads, and a yellow-pigmented bacterium were isolated from the gills, and few of the same recovered from many of the kidney samples from all groups. The yellow-pigmented colony type had none of the typical *F. columnare* colony characteristics and was recovered from both bacterial challenged and sterile broth challenged groups. It did have the ability to grow on the selective medium containing neomycin and polymyxin B.

In this test, co-investigators found the poorest survival at 85% in the lowest ammonia exposed groups. According to Pacha and Ordal (1970) who developed the contact method of infection to compare the virulence of columnaris strains, highly virulent strains

should cause 100% death in 24 hr while low virulence strains required more than 96 hr to kill 100% of the experimental fish. The contact method used in this study is a modification of their method. In this study the water level is lowered in the aquarium and the bacterial culture added to produce a 1:20 dilution of 0.1 absorbance at 525 nm and exposure is for 10 min while in the Pacha and Ordal method (1970) fish are netted into a container and exposed for 2 min, then poured into the tank. This modified method has been used to expose salmon and steelhead and losses at 20.5°C or higher resulted in nearly 100% losses in 48 hr. In this test on juvenile Lost River suckers it would appear either the suckers are not very susceptible to *F. columnare* infection by the exposure method or the strains used in this test are of very low virulence. Pacha and Ordal (1967, 1970) also stated the route of infection was found to be important in determining the disease producing capacity of strains of *F. columnare* of high virulence. These strains produce infections more readily when exposure was by the contact method than by either intramuscular(IM) or intraperitoneal(IP) injection. With strains of low virulence, IM or IP injection was found to be more effective than the contact method of infection. Difference between high and low virulence might be their ability to attack particular susceptible tissues. The presence of copepods (*Lernaea sp.*) and leeches on Upper Klamath Lake adult suckers may provide openings for infection by the low virulent strains.

Another possible effect of the elevated ammonia and pH could be on the attachment mechanism of *F. columnare* to gill tissue. Virulence of *F. columnare* has been correlated with the ability to adhere to the gill tissue (Decostere et al.1998). Evidence has been presented that suggests a lectin mediated interaction between a high virulence *F. columnare* strain and the gill tissue (Decostere et al. 1999). The water quality conditions in this experiment may greatly impact the ability of even a low virulent strain of this bacterium to attach. Another affect of elevated pH could be on the invasive enzymes possessed by *F. columnare*. Bertolini and Rohovec (1992) found that zinc metalloproteases constitute a major component of the extracellular proteases of *F. columnare*. There is much uniformity among the *F. columnare* strains in regard to these extracellular proteases. In the case of the chondroitin lyase which may also aid invasion in *F. columnare* strains, pH greatly affected the activity of this enzyme which had an optimum pH near 6 and declined in activity above or below the optimum (Griffin 1991). Work at the University of Wyoming has suggested there may be a compensatory response of the suckers to the ammonia including increased numbers of mucus cells and/ or infiltration of white blood cells into the lymphatic spaces (J.M.Morris et al. 2000; H. Lease, M.S. Thesis University of Wyoming).

Table 3 Recovery of *Fl. Columnare**on bacteriological media from the tissues of moribund or dead Lost River suckers in the Wyoming Red Buttes laboratory experiment involving challenge of fish exposed to water with four sublethal levels of ammonia.

<u>Treatment</u>	<u>Tank/fish No.</u>	<u>Time/Date</u>	<u>Tissue sampled</u>		
		<u>Fish Died</u>	<u>Skin</u>	<u>Gill</u>	<u>Kidney</u>

"0" ammonia

(0.006 mg NH ₃ -N/L)	13/F02	10/31	1720	+		+		+
	13/F04		11/05	0700	-		+	+
	14/F01		11/04	1200	-		+	+
	14/F04		11/07	0800	ND		+	+
	14/F05		11/12	0900	ND		+	+
	14/F06		11/12	0915	ND		+	-
	19/F01		10/31	1317	-		+	-
	19/F02		11/05	1700	+		+	+

Summary: 8+ for *F. columnare*/ 8 fish sampled

*= *F. columnare* identification based on presence of typical colony morphology, adherence to agar and Congo red stain positive.

Table 3. continued

#1 ammonia (0.117 mg NH ₃ -N/L)	Tank/fish No.			<u>Skin</u>	<u>Gill</u>	<u>Kidney</u>
	1/F01	11/05	1317	-	+	+
	1/F02	11/14	1100	ND	-	-
	1/F03	11/17	1415	ND	+	+
	5/F01	11/04	0800	-	+	+
	20/F01	11/05	0800	+	+	+

Summary: 4+for *F. columnare* /5 fish sampled

<u>Treatment</u>	<u>Tank/fish No.</u>	<u>Time/Date</u> <u>Fish Died</u>		<u>Skin</u>	<u>Gill</u>	<u>Kidney</u>
#2 ammonia (0.220 mg NH ₃ -N/L)	7/F01	11/04 0800		-	+	+
	15 no cultures					
	16/F01	11/06 0315	ND		+	-
	16/F02	11/12 0845	+		+	+
	16/F03	11/17	ND		+	+

Summary: 4+for *F. columnare* /4 fish sampled.

#3 ammonia Tanks 3, 21,24 = no cultures received
(0.433 mg

NH₃-N/L)

Effect of Handling Stress from Netting on Mortality of Juvenile Lost River Suckers Challenged with *F. columnare* at the University of Wyoming Red Buttes Laboratory.

Purpose- Determine if juvenile suckers held in the same water quality conditions as the sublethal ammonia challenged groups but challenged with *F. columnare* by a method involving added stress of handling by netting into and back from a separate exposure container. Results of the preliminary challenge tests #1 and #2 at the Corvallis Salmon Disease Laboratory indicated stress from netting before and after challenge with *F. columnare* resulted in greater mortality. This experiment was initiated at the University of Wyoming to determine if similar results would be observed when the juvenile suckers were held in the same sublethal ammonia and elevated pH water used in the main test at this laboratory.

Methods- Twenty-five juvenile Lost River suckers were distributed into each of four separate aquaria (same as main challenge) containing 8L of water. These fish were rapidly adjusted to the experiment water quality conditions of pH 9.5, water temperature of 22-23°C and hardness of 50 mg/L. They had previously been maintained in water at 22°C, PH 7.9 and 110 mg/L hardness. In addition to the net stress, fish in the four tanks were allowed to adjust to their tanks and water quality changes only 10 hr prior to challenge. The fish in two of the tanks received the high ammonia containing water as well as being netted into separate plastic containers for challenge. Two of the aquaria received the high ammonia concentration water and two received "no" ammonia water.

The challenge dose was at the same preparation and concentration of *F. columnare* bacteria ($4.7-5.3 \times 10^6$ CFU/ml) prepared for the main experiment. The fish were exposed to this dose for 10 min in two of the four tanks. Fish from the four tanks were netted into separate plastic containers holding 1900 ml of water from the respective tank. Two of the containers received 100 ml of the challenge culture of *F. columnare* and the other two simultaneously received 100 ml of sterile media. Fish were netted from their tanks and placed in the challenge bath for 10 min, then netted back to their appropriate aquaria. Fish in tank #25 and 26 were exposed to sterile broth and tanks #27 and 28 received the *F. columnare* challenge.

After the challenge on October 30, 1999, the aquaria were monitored hourly (24 hr/day) for loss as in the main experiment for the first three days; then hourly for 12 hr/day thereafter. Skin, gills and kidney of selected dead fish were sampled for *F. columnare* as described previously for the main experiment. Suspect colonies were confirmed using the methods described by Griffin (1992). After day six, Jeffrey Morris and Michael Suedkamp, Department of Zoology and Physiology, University of Wyoming performed all monitoring of the experiment including performing necropsies of fish, and shipping of inoculated plates to Rich Holt, Corvallis, Oregon for incubation and interpretation.

The experiment was terminated on November 16, 1999 after 17 days. A sample of five fish from each tank was killed with anesthetic and tissue samples (gill and kidney) inoculated onto cytophaga agar and SCA media. The bacteriological culture plates were shipped air carrier to the Corvallis laboratory, incubated at 18°C for up to seven days then examined for growth.

Results and Discussion

The fish loss records for this experiment which was conducted for 17 days are as follows:
Sterile broth exposed groups:

Tank #25- no loss

Tank #26- 1 dead

F. columnare challenged groups:

Tank #27- 4 (2 dead and 2 moribund)

Tank #28- 1 dead

If we assume all five fish that died in the *F. columnare* challenge tanks were infected with *F. columnare*, this would be five of 50 or 10% associated *F. columnare* loss.

Unfortunately, an accident occurred early in the test for tank #28. A plate of glass that was part of the aquaria cover fell into the tank and injured three fish which were removed.

Table 4. Bacteriological examination results for fish that died in the test involving the netting-stress *F. columnare* challenge method at the University of Wyoming Red Buttes Laboratory.

F. columnare

Challenge Groups/fish no.	Tissue sampling results (recovery of <i>F. columnare</i> *)		
	Skin	Gill	Kidney
Tk 27FO3	+	+	+
TK 28/FO1	+	+	+
Sterile broth exposure groups			
TK 26FO1	ND	-	-
TK 25	no cultures done on this group		

* += detection of *F. columnare*.

Flavobacterium columnare was detected in 2 of 2 challenged fish examined but not in the one dead fish from tank #26 exposed to sterile broth.

Because the accident occurred early in the test resulting in injury and removal of fish, very limited conclusions can be drawn. However, *F. columnare* was recovered from both

challenged fish that died and were cultured for bacteria. Contrary to the results of the first Preliminary Challenge Test #1 conducted at Corvallis, Oregon, high losses of 44-76% were not observed in the test at Wyoming. Despite the netting stress, water quality conditions of high pH, water temperatures of 22-23°C and high ammonia, losses in the bacterial challenged fish were low. These results add more evidence that the *F. columnare* strains used in this study from Upper Klamath Lake adult suckers are not highly virulent strains.

Bacteriological examination results of suckers killed at termination on November 16, 1999 are as follows: Of the five fish sampled from each tank, no *F. columnare* was recovered from fish gill and kidney in any group. It is interesting that no *F. columnare* carrier fish were detected 17 days after exposure.

Survival of *F. columnare* Exposed to Water of Elevated pH and Ammonia

Purpose- Determine if water quality such as elevated pH at 9.33 and/or elevated ammonia levels in water in which the suckers were held would affect survival of *F. columnare* and perhaps influence the *F. columnare* exposure method used to challenge the juvenile Lost River suckers.

In the *F. columnare* challenge of suckers exposed to sublethal ammonia concentrations, there was some concern expressed early in the test that the lack of mortality of the bacterial challenge suckers may be caused by detrimental effects of the water quality on the bacterium. An experiment was initiated to answer this concern.

Method- This study began on November 1, 1999 at the University of Wyoming Red Buttes Laboratory where the columnaris challenge of suckers sub lethally exposed to ammonia high pH water was in progress. *Flavobacterium columnare* isolate Klsum17-96 was chosen for this test and inoculated from TYI broth that had been previously inoculated for the challenge of suckers on October 29, 1999.

The culture was started in 200 ml prescription bottles on November 1 at 1200 incubated stationary at 22-23°C until November 2 and after about 24 hr was placed on a New Brunswick model C24 incubator shaker at 120 revolutions/minute at 23°C. After about 6 hours the culture was removed from the shaker, and the absorbance at 525 nm adjusted to 0.11 using a Spectronic 20. A plate count of the adjusted culture was performed using the spread plate method on TYES agar each time just before adding cells to the different quality water samples.

Water from the influent water supply to the experimental aquaria containing the Lost River suckers was collected from the following sources: (Michael Suedkamp tested the pH and water temperature at the time of collection).

1. Bank #3 water with elevated ammonia (4.333 mg NH₃-N/L) and pH 9.33 at 22.1°C.
2. Bank #0 water with elevated pH 9.38, no ammonia, 22.2°C.
3. Influent to suckers stock tank pH 7.3, 21.4°C.

As each water sample was collected in a 300ml erlenmeyer flask, 1 ml of the culture was added and sampling for viable CFU of *F. columnare* began using the spread plate method. The counts began one minute after addition of the bacterial culture, then at 10 minutes, 60 minutes, 3 hours, and 15 hours.

Dilutions of the water were prepared and plated in duplicate. The plates were incubated at room temperature at 18.5-21°C for 48-72 hours and counted under a dissection microscope. Immediately after the one-minute sample was collected, the flasks of water with *F. columnare* cells were placed on the shaker at a slow 50 rev/min and this was continued until the 3-hour sample. After the 3 hr sample, the flasks were stored stationary up to the 15-hour sample due to problems with the shaker. By the 15 hour in the morning, the temperature had decreased only slightly from 23 down to 18.5°C.

For the first water sample, 95ml of water was mixed with 1ml of bacterial suspension. For the other two samples, 100ml of water was mixed with 1ml of inoculum.

Results and Discussion

Sample 1 (elevated pH and ammonia water)

Culture count at 0.11 O.D. was 1.4×10^8 CFU/ml.

1 ml into 95ml water results in a 1.46×10^6 CFU/ml suspension.

Sample 2 (elevated pH, no ammonia water)

Culture count at 0.11 O.D. was 1.11×10^8 CFU/ml

1 ml added to 100 ml water resulted in a 1.1×10^6 CFU/ml suspension.

Sample 3 (pH 7.3, no ammonia influent to stock tank)

Culture count of 0.11 O. D. was 1.4×10^8 CFU/ml.

1ml of bacterial culture added to 100ml water resulted in a 1.40×10^6 CFU/ml

Bacterial counts of *F. columnare* in the different water qualities at each sampling time are shown in Table 5. For samples 1 & 2 which had elevated pH in common, there was a slight depression of CFU/ml at 10 and 60 min with less of this affect in the water at pH 7.3 (#3). But in all cases, by the 3rd hour all water samples exhibited an increase in *F. columnare* CFU/ml and this increased substantially by the 15 hour; all increasing about 10 times.

Figure 1. Shows the CFU/ml counts over time for each of the water samples. The *F. columnare* isolate increased in the water of pH 7.3 more rapidly than the two water samples at elevated pH. The #1 sample of elevated ammonia and pH appeared no different than just elevated pH. However, it appears the high pH did affect the rate of increase for *F. columnare*. Fijan (1968) studied the survival of *F. columnare* in waters of different quality. He found pH 6 had profound effect on survival, resulting in a large decrease by 17 hr. At pH 9.5 and 10, *F. columnare* was viable in water for up to 8 days but had decreased in viability by 16 days. This study was done at 25°C. Soft water especially if acidic was not favorable for columnaris. In contrast to Fijan's work, Chowdhury and Wakabayashi (1988) found that best survival occurred in tap water in the total hardness range of 33.3-73.7 mg/L and was equivalent when concentrations of four cations including 0.03% NaCl, 0.01% KCl, 0.002% CaCl₂·2H₂O and 0.004% MgCl₂·6H₂O were formulated. They found *F. columnare* had extended good survival in tap water that contained Ca, Mg, Na and K. From the results of our *F. columnare* study in waters of elevated pH and ammonia, we can conclude there was at least no drastic decrease in viability of the challenge solution when introduced into the aquaria.

Table 5. Survival of *F. columnare* exposed to water with elevated pH and ammonia Levels (CFU/ml)

	(A) High pH 9.33 High NH ₄	(B) High pH 9.38	(C) pH 7.3 2NH ₄
Inoculum	1.40 X 10 ⁸	1.10 X 10 ⁸	1.44 X 10 ⁸
Water Suspension Estimate	1.46 X 10 ⁶	1.10 X 10 ⁶	1.40 X 10 ⁶
<u>Time</u>			
1 min.	1.32 X 10 ⁶	1.58 X 10 ⁶	1.76 X 10 ⁶
10 min.	1.22 X 10 ⁶	1.38 X 10 ⁶	1.64 X 10 ⁶
60 min.	1.10 X 10 ⁶	1.32 X 10 ⁶	2.34 X 10 ⁶
180 min.	2.07 X 10 ⁶	2.82 X 10 ⁶	4.70 X 10 ⁶
15 hours	2.20 X 10 ⁷	2.34 X 10 ⁷	4.65 X 10 ⁷

Effect of Water Temperature on *F. columnare* Infection of Juvenile Lost River Suckers Challenged in the Laboratory.

Purpose- Determine if water temperature influences infection rate of *F. columnare* in juvenile Lost River suckers exposed using the aquarium draw-down challenge procedure.

Methods- This study was conducted at the Oregon State University Salmon Disease Laboratory in March 2000. Juvenile Lost River suckers for this test were received from Mr. Larry Duns Moor, Klamath Tribes Native Fish Hatchery. These fish were received January 20, 2000 and maintained on the diet previously described in the preliminary challenge tests conducted in the fall of 1999. The fish were maintained at 20°C in well water until distributed to 20L glass aquaria receiving 22°C inflow water at 600 ml/min. on February 26, 2000. Twenty suckers at 1.2 g each were placed in each of 12 aquaria. Two days later the water temperature for those tanks to be held at 25 and 27°C were raised to 25°C. On March 1, the water temperature was raised from 25°C to 27°C in four tanks. Fish were maintained at these temperatures i.e. 22, 25 and 27°C for two days, then challenged with *F. columnare* or sterile broth by the same method used in previous experiments of aquaria draw-down to 1900 ml of water and addition of 100 ml of culture or sterile broth added for 10 min. This is followed by three figure eight swirls with a net

to mix the culture and aquaria water. After the 10 min, the inflow was added and the challenge solution diluted at 600 ml/min. Aquarium treatments had been randomized. The bacterial challenge was prepared as described in the Preliminary Challenge Test #2 and the main challenge at University of Wyoming laboratory. The three strains KlsuM17-96, KlsuG5-96 and KlsuG1-96 were combined in equal amounts and adjusted at 0.1 absorbance at 525 nm. KlsuG1-96 and KlsuG5-96 were removed from lyophilization storage and KlsuM17-96 was obtained from storage in a cytophaga agar deep at 4°C since it's use for the main challenge at the Wyoming Red Buttes laboratory. At each temperature, two tanks of fish were exposed to sterile TYI broth and two tanks were challenged with 4.4×10^6 *F. columnare* CFU/ml for 10 min. The spread plate method was used to determine viable cell numbers just prior to exposure of the fish. Tanks were examined two to three times per day for dead fish and fish were examined for *F. columnare* by inoculation of skin, gills and kidney on SCA and cytophaga agar. Plates were observed for growth after seven days.

Results and Discussion

The challenge was begun on March 3, 2000 and terminated April 6, 2000 after 34 days. The fish loss and examination information are shown in Tables 6, 7 and 8. No losses occurred in control groups at each temperature or in any aquarium receiving 22°C influent. At 25°C, four (10%) of the fish died when loss was combined for the two aquaria. At 27°C, two fish (5 %) died in one aquarium. No fish died after March 16,2000 and until termination of the test.

In this experiment, six fish died in bacterial challenged aquaria and *F. columnare* was recovered from 5 of 6 fish. At termination, 10 survivors from each tank were sampled for *F. columnare* from the gills and kidney. No *F. columnare* was detected from any of the 12 groups.

The total loss of *F. columnare* challenged fish in this test again demonstrated these three isolates of *F. columnare* from the 1996 Upper Klamath Lake fish kill appear to be of low virulence. The combined greatest loss of 10% was in the 25°C aquaria followed by 5% at 27°C. With *F. columnare* strains, we have examined from salmon, the percent loss increased as water temperature increased above 15°C. This was not the case for the isolates in the Lost River suckers. Too few fish died with detection of *F. columnare*; 3 at 25°C and two at 27°C to determine if there was a difference in effect between 25 and 27°C. Interestingly, more fish did not succumb at the higher water temperature of 27°C. From the study of effect of temperature on growth of these *F. columnare* strains in TYI culture medium described later in this report, the growth temperature of *F. columnare* strains is most rapid at about 27°C (25-30°C). More rapid growth at 27°C resulted in no more increased loss of fish at this temperature.

It appears other factors besides or in combination with the strain of pathogen and elevated water temperatures that are involved in the production of the fish losses.

Table 6: Day of fish loss and effect of water temperature on *F. columnare* infection in juvenile Lost River suckers. This shows day of fish loss and culture results. Only those aquaria in which loss occurred are noted.

March 3, 2000 Began Test.

<u>Date</u>	<u>22°C</u>		<u>25°C</u>		<u>27°C</u>	
	1)	2)	1)TK10	2)TK2	1)Tk12	2)
3/3 day						
3/4 1			1 (-)*			
3/6 3					1 (+)	
3/8 5					1 (+)	
3/10 7				1 (+)		
3/11 8				1 (+)		
3/16 13				1 (+)		

April 6, 2000 Test Terminated.

*= A negative sign indicates no *F. columnare* isolated; while += those fish from which *F. columnare* detected.

Table 7: Fish loss and survivor data for the experiment on temperature effect on *F. columnare* infections in Lost River suckers.

<u>Treatment</u>	<u>Tank #</u>	<u># Died</u>	<u>#Survivors</u>	<u>% loss</u>	
22°C	Control 1	7	-	20	
	Control 2	1	-	19	
	Exposed 1	3	-	20	
	Exposed 2	4	-	20	
25°C	Control 1	6	-	21	
	Control 2	9	-	20	
	Exposed 1	10	1	20	5
	Exposed 2	2	3	17	15
27°C	Control 1	5	-	20	
	Control 2	8	-	20	
	Exposed 1	12	2	18	10
	Exposed 2	11	-	20	
Combine	25°C	10% loss			
	27°C	5% loss			

Table 8: Recovery of *F. columnare* from the bacteriological examination of tissues of dead fish in the effect of water temperature on infections in Lost River suckers..

Recovery of *F. columnare*

	<u>Skin</u>	<u>Gill</u>	<u>Kidney</u>
3/4 Tk 10	-	-	-
3/6 Tk12	+	+	+
3/8 Tk12	+	+	+
3/10 Tk2	+	+	+
3/11 Tk2	+	+	+
3/16 Tk2	+	+	+

Effect of Temperature on Growth of *F. columnare* in TYI Broth.

Purpose- Compare the growth of the three selected *F. columnare* isolates from the 1996 Upper Klamath Lake fish kill when incubated in TYI broth at 20, 22, 25, 27 and 30°C. Determine at which temperature growth occurs most rapidly.

Method-The bacterial isolates KlsuM17-96, Klsu65-96, and KlsuG1-96 were removed from lyophilization storage by inoculation in 200 ml bottles of TYI broth and incubated at room temperature (~21°C). Also, a *F. columnare* isolate obtained from a juvenile spring chinook salmon that had died with a columnaris gill lesion in September 1996 at Dexter Ponds (Oregon Department of Fish and Wildlife facility) on the middle fork Willamette River was included for comparison. Bacterial incubators at the appropriate temperature were available in the Department of Microbiology, Oregon State University. Temperature data recorders (Hobo XT, Onset Computer Corp.) were placed in each incubator. Temperatures were maintained within +/- 1° except for the 25°C incubator room which was 24°C for the first 30 hours, and dropped to 23°C for the remainder of the test. Growth of isolates KlsuM17-96 and KlsuG5-96 were tested at all five temperatures of 20, 22, 24, 25, 27 and 30°C, but KlsuG1-96 and DexChS1-96 were tested at four temperatures of 22, 24, 27 and 30°C.

Duplicate side-arm flasks (300ml) containing 50 ml of TYI broth were inoculated for each *F. columnare* strain at each temperature. Transmission and absorbance readings to

track growth were taken every 4 to 6 hours for the first 44 hours, and then 12 hours for the last reading at 56 hours. A Spectronic 21 (Milton Roy Company) was used to measure the percent of transmission and absorbance at a 525 nm wave length. The side-arm flasks containing the sterile media were placed in the incubators at the appropriate temperature for at least two hours then inoculated with 0.5 ml of an actively growing 14 hour culture which had been incubating at 23°C on a New Brunswick rotary shaker at 120 rev/min. A purity check was conducted on the inoculum of each strain by preparing wet mounts and smears for Gram stain and observing for appropriate cell morphology microscopically. Also, TYES plates were inoculated and incubated at 21°C to observe for typical colony formation.

At the end of the test (56 hr), samples of growth were inoculated onto TYES agar to demonstrate presence of *F. columnare* and purity. Growth of the isolates at various temperatures was also examined by inoculation of TYES agar plates from the same TYI broth cultures and placing them in the incubators to observe for visible growth. Plates were examined for growth after 11 hours and 20 hours.

Results and Discussion

Figures 2-5 show the change in absorbance in TYI broth over time for each of the *F. columnare* isolates. Growth was greatest at 27 and 30°C in the first 24-30 hours, but after 30 hours, growth at the lower temperatures approached or exceeded that of the higher temperatures. The optimal most rapid growth temperatures for *F. columnare* is reported to be 25-30°C (Reichenbach 1989). The strains from the Upper Klamath Lake suckers appear to be very typical and grow most rapidly nearest 27°C. At the lower incubation temperatures growth was slower but began to reach similar levels after 40-60 hr.

Growth on TYES agar plates at 11 hours after inoculation at the 6 temperatures tested demonstrated rapid growth at 27, 30 and 35°C. Limited growth had developed at 22 and 24°C and almost no evidence of growth was found at 20°C. After 20 hours, extensive growth over the plate was observed at 27-30°C, also growth was very evident at 22 and 24°C and some growth had developed at 25°C. The appearance of growth on TYES agar plates was definitely faster at 27-35°C.

In the laboratory experiment challenge of Lost River suckers held at 22, 25, and 27°C losses with associates *F. columnare* infection were observed at 25 and 27°C which correlates somewhat with most rapid growth of the bacterium. But if 27°C is more optimal for growth, we did not see a corresponding greater loss in this group of fish over the fish held at 25°C.

第 11 章 数据库系统

Comparison of Challenge Methods on Infection of *F. columnare* in Juvenile Lost River Suckers Held at 25°C Water Temperature.

Purpose- Compare infection of *F. columnare* in juvenile Lost River suckers challenged by net stress handling method versus the aquaria draw-down method.

Methods- In this test, eight 20 L aquaria containing 8L of water received an inflow of 0.5L at 25°C water. Eighteen Lost River suckers at ~3.3 g each were distributed into each aquarium on September 2, 2000. These fish had been held in a stock tank at 20°C and were exposed to 22.5°C water in the aquaria. On September 5, 2000 the inflow water temperature was adjusted to 25°C \pm 1°C and maintained at that temperature for the remainder of the test. The fish were fed the same diet of brine shrimp and gel as described previously in the preliminary challenge tests. The following treatments were included in this test:

1. Lowering the water level in the aquarium and exposure of the fish to sterile broth.
2. Lowering the water level in the aquarium and exposure of the fish *F. columnare* culture.
3. Netting of fish prior and after exposure to sterile broth.
4. Netting of fish prior and after exposure to *F. columnare* culture.

Each treatment was replicated and the aquarium randomized.

Handling by netting challenge method-This method was similar to that used in the Preliminary Challenge Test #1 where losses of 44-76% occurred. The differences include using 25°C water in this test versus 22°C in the preliminary challenge, aquaria were 20 L instead of 100L and the fish in this test were adjusted to the aquaria for several days prior to challenge while in the Preliminary test the fish were removed from a common 100L stock tank, challenged and then distributed to a different 100L tank.

The fish were netted out of the aquarium into a beaker containing 1 L. Then, the fish were poured into a net and placed in a plastic basin container with 1900 ml of 25°C water and 100 ml of sterile broth or *F. columnare* culture. After 10 min. the fish were netted out of the challenge solution and placed directly into the aquarium. The aquarium water level (8 L) had been lowered by 2 L and the 2 L challenge solution was added and immediately began diluting out at 0.5 L/min.

Aquarium draw-down method- This method was nearly the same as the main test challenge method used at the University of Wyoming. The inflow water supply was stopped, the tank water level siphoned off down to 1900 ml, then 100 ml of either bacterial culture or sterile broth was added. A net was used to swirl and mix the solution in a figure eight pattern three times. After 10 min. exposure, the influent line was added and the tank filled and gradual dilution occurred at 0.5 L/min.

The bacterial challenge was prepared as described previously. As with other experiments *F. columnare* strains KlsuM17-96, KlsuG5-96 and KlsuG1-96 were removed from lyophilized culture storage and grown in TYI broth. Equal amounts of each isolate were

adjusted to 0.1 absorbance at 525 nm. The *F. columnare* challenge mixture was sampled for bacterial numbers by the spread plate method by inoculation onto TYES agar. The bacterial challenge solution to which the suckers were exposed was 4.65×10^6 CFU/ml. The aquaria were observed twice daily for dead fish. Samples of gill and kidney tissue were inoculated on SCA and TYES agar media, incubated at 18°C for seven days and observed for typical *F. columnare* growth.

At termination on October 7, 2000, five fish from each aquarium were sampled from the gills and kidney for *F. columnare* by inoculating bacterial agar media.

Results and Discussions

The experiment started on September 9, 2000 continued to October 7, 2000 (28 days). Only 3 fish died during this test.

Sept 12 - Aquarium 6 (bacterial challenge by aquarium draw-down) – 1 fish positive for *F. columnare*

Sept 24 - Aquarium 8 (control challenge by aquarium draw down) - 1 fish opportunistic bacteria aeromonad pseudomonad, no *F. columnare*

Sept 25 - Aquarium 1 (Control challenge by aquarium draw down) - 1 fish opportunistic bacteria aeromonad pseudomonad, no *F. columnare*

In the examination of gills and kidney of five fish from each aquarium at termination, no *F. columnare* was detected.

From these results, once again it appears under the conditions of these challenge methods that the strains of *F. columnare* from the 1996 Upper Klamath Lake fish kill are not highly virulent. They are capable of involvement in loss and but in this test only 1 of 36 fish (combined draw-down method again) or 2.8% fish had *F. columnare* infection. In this test the fish were larger 3.3 g versus 0.6-0.8g each in the Preliminary Challenge Test #1 in which high losses occurred. Also, in this test the fish were adjusted to the aquaria several days prior to challenge, while in the first test they were challenge when removed from a stock tank and distributed into their aquaria. It may be that smaller Lost River suckers are more susceptible to *F. columnare*. In this test, a higher water temperature of 25°C was used compared to 22°C in the first preliminary test but this higher temperature did not cause a greater loss as expected.

The *F. columnare* strains used in the test and in previous challenges were stored in lyophilized culture at freezer temperatures and had received only a very limited number of passes in culture media before storage. This makes it unlikely they had lost any virulence since primary isolation from the diseased fish. Obviously, it would be good to conduct challenges with other *F. columnare* isolates from fish kills in other years from the Upper Klamath Lake to determine if this low virulence is a common characteristic in the strains involved in most fish losses in the lake. In the examination of adult Lost River and Shortnose suckers dying in Upper Klamath Lake, obvious lesions of *F. columnare* were observed on the gills and mouth cavity and body. Columnaris disease is a factor in the fish kills but other parameters of the host and environment must also play a large role

in development of disease outbreaks in this lake. Often parasites such as anchor worms and leeches were also involved in infestations of the fish. Pacha and Ordal (1970 or 1967) indicate low virulent strains of *F. columnare* tend to cause more overt lesions on fish while highly virulent strains may kill the fish quickly without gross lesion development.

Acknowledgements

Thanks to Larry Dunsmoor, Klamath Tribes Native Fish Hatchery, for providing the Lost River suckers for the challenge experiments in this study. Thanks to the personnel of the University of Wyoming Red Buttes Environmental Biology Laboratory especially Dr. Joe Meyer, Jeffrey Morris and Mike Suedkamp for their assistance and information during the experiments. Thanks to Dr. Elaine Snyder-Conn for the opportunity to examine the *F. columnare* strains from the Upper Klamath Lake.

Craig Banner, Tony Amandi, and John Kaufman of ODFW Fish Pathology assisted in the necropsy of the adult Lost River suckers during the 1996 fish kill and helped in the isolation of *F. columnare* strains.

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Presentations

Morris, J.M., M.J.Suedkamp, E. Snyder-Conn, R.A. Holt, H.M. Lease, S.J. Clearwater, and J.S. Meyer. Survival and Growth of Juvenile Lost River Suckers (*Deltistes luxatus*) challenged with a bacterial pathogen (*Flavobacterium columnare*) during exposure to sublethal ammonia concentrations at pH 9.5. Presentation at the Annual Meeting of the Rocky Mountain Chapter of the Society of Environmental Toxicology and Chemistry, Laramie, Wyoming, 7 April 2000.

Abstract

The purpose of this study was to investigate the effects of a 12-week training program on the physical fitness and health-related quality of life of sedentary middle-aged men. The study was a randomized controlled trial. The participants were divided into two groups: a training group and a control group. The training group performed a supervised exercise program consisting of aerobic and resistance training. The control group remained sedentary. The primary outcome was the change in maximum oxygen consumption (VO₂max). Secondary outcomes included changes in body mass index (BMI), blood pressure, and health-related quality of life. The results showed that the training group had a significant increase in VO₂max compared to the control group. There were also significant improvements in BMI, blood pressure, and health-related quality of life in the training group. The control group showed no significant changes in any of the outcomes. The findings suggest that a 12-week supervised exercise program can improve physical fitness and health-related quality of life in sedentary middle-aged men.

Appendix

A-1

Description of bacterial growth observed on initial isolation plates. These were inoculated from tissue of dead or morbid Lost River suckers in the columnaris challenge experiment involving prior elevated ammonia and pH exposure at the University of Wyoming Red Buttes Environmental Biology Laboratory.

Treatment

Ammonia
(0.006 mg NH₃-N/L)

<u>Tank/Fish No.</u>	<u>Tissue</u>	<u>Medium</u>	<u>*Results</u>
TK 13 FO 2	Skin	SCA	>30 F. col colonies
	Gill	C	~12 F. col colonies, moderate number APS
	Gill	T	mix of APS no F. col
	Kidney	C	~6 F.col colonies moderate number APS
TK 13 FO4	Kidney	T	mix of APS colonies, no F. col
	Skin	SCA	No growth
	Gill	SCA	~20 F. col also small white APS
	Gill	C	much APS mixture of colonies
	Gill	T	Much APS type colonies one type
	Kidney	C	2 F.col colonies and 4 APS colonies
	Kidney	SCA	2 F. col colonies and few small APS
kidney	T	moderate number APS	

Abbreviations

F. col = typical *Flavobacterium columnare* colonies

APS = Aeromonads and pseudomonads type opportunistic bacteria which are cytochrome oxidase positive. These are commonly found associated with F. col infections or found in fish that have died especially at warm water temperatures.

*On primary isolation plates, F. col was identified by typical colony morphology, color, adherence to agar.

Appendix

A-2

Treatment

Ammonia

(0.006 mg NH₃-N/L) Cont.

<u>Tank/Fish No.</u>	<u>Tissue</u>	<u>Medium</u>	<u>*Results</u>
TK 14 FO1	Skin	SCA	No growth
	Gill	SCA	1 F. col colony\
	Gill	C	Several F. col and much APS mix types
	Gill	T	3 F. col, much APS mix
	Kidney	SCA	7-8 F. col colonies
	Kidney	C	>30 F. col colonies and 2 pink colored colonies
	Kidney	T	6-8 F. col colonies
TK 14 F04	Gill	SCA	Much F. col colonies, few Mixtures APS
	Gill	C	Much mixture APS
	Kidney	SCA	3 F. col colonies
	Kidney	C	Much mix APS colonies
TK 14 FO5	Gill	SCA	Much F. col colonies
	Gill	C	Much APS colonies
	Kidney	SCA	5 F. col colonies and 4 APS mix colonies
	Kidney	C	Much APS mix colonies
TK 14 FO6	Gill	SCA	Much F. col colonies, also Few yellow pigment F. col
	Gill	C	1 F. col colony and much mix APS
	Kidney	SCA	3 mix APS and 1 yellow pigment not F. col
	Kidney	C	Much mix APS colonies

Appendix

A-3

Treatment

Ammonia
(0.006 mg NH₃-N/L) Cont.

<u>Tank/Fish No.</u>	<u>Tissue</u>	<u>Medium</u>	<u>*Results</u>
TK 19 FO1	Skin	SCA	I pink colony
	Gill	SCA	Much F. col and few yellow pigmented not F. col
	Gill	C	~10 F.col and 19 APS mix colonies
	Kidney	SCA	No growth
	Kidney	C	Few misc. yellow and pink opportunistic
TK 19 FO2	Skin	SCA	Much F. col colonies and few white colonies
	Gill	SCA	4 F. col colonies
	Gill	C	4 F. col colonies and much APS colonies
	Kidney	SCA	Much F. col colonies and few small APS colonies
	Kidney	C	Much APS mix

#1 Ammonia
(0.117 mg NH₃-N/2)

TK 1 FO1	Skin	SCA	No growth
	Gill	SCA	Much F. col colonies
	Gill	C	10 F. col Colonies and 5 APS
	Gill	T	Much F. col and 3 APS Colonies
	Kidney	C	Much F. col, few APS Colonies
	Kidney	SCA	Much F. col colonies
	Kidney	T	Much F. col colonies

Appendix

A-4

#1 Ammonia
(0.117 mg NH₃-N/2)

<u>Tank/Fish No.</u>	<u>Tissue</u>	<u>Medium</u>	<u>*Results</u>
TK 1 FO2	Gill	SCA	No growth
	Gill	C	Much APS colonies
	Kidney	SCA	No growth
	Kidney	C	Much APS mix colonies
TK 1 FO3	Gill	SCA	10 F. col colonies, 1 mold
	Gill	C	11 F. col colonies, 4 APS colonies
	Kidney	SCA	12 F. col colonies
	Kidney	C	Few F. col colonies, much APS mix
TK 5 FO1	Skin	SCA	No growth
	Gill	SCA	2 fungi
	Gill	C	Few F. col and moderate mix of APS
	Gill	T	Moderate APS mix
	Kidney	C	~10 F. col colonies and much mix APS
	Kidney	SCA	Much F. col colonies and few APS colonies
TK 20 FO1	Kidney	T	Mix APS colonies
	Skin	SCA	5 F. col colonies
	Gill	SCA	Many F. col colonies
	Gill	C	20-25 F. col colonies and mix ~20 APS colonies
	Gill	T	7-8 F. col and 20 APS colonies
	Kidney	SCA	Many F. col colonies
	Kidney	C	18 F. col colonies and mix APS colonies
Kidney	T	3 F. col colonies and 10	

Appendix

A-5

Treatment

2 Ammonia
(0.220 mg NH₃-N/L)

<u>Tank/Fish No.</u>	<u>Tissue</u>	<u>Medium</u>	<u>*Results</u>
TK 7 FO1	Skin	SCA	No Growth
	Gill	SCA	Much F. col colonies, 1 fungal colony and few APS
	Gill	C	Much mix APS colonies
	Gill	T	Much mix APS colonies
	Kidney	SCA	Much F. col, and few APS colonies
	Kidney	C	Much mix APS colonies
	Kidney	T	Much mix APS colonies
	TK 16 FO1	Gill	SCA
Gill		C	3 F. col colonies and 7 APS colonies
Kidney		SCA	No growth
Kidney		C	Mix of APS colonies
TK 16 FO2	Skin	SCA	Much F. col colonies and few yellow pigmental color
	Gill	SCA	20 F. col colonies and many small APS
	Gill	C	Much APS mix colonies
	Kidney	SCA	~20 F. col colonies and few yellow pigment colony not F. col
	Kidney	C	Much APS white colonies
TK 16 FO3	Gill	SCA	Much F. col and moderate APS colonies
	Gill	C	5 F. col colonies and one yellow pigmented

Kidney

SCA

Much F. col and few
APS colonies

Kidney

C

15 F. col colonies and
moderate mix APS
colonies