U.S. Fish & Wildlife Service

California Nevada Fish Health Center
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Efficacy of florfenicol (Aquaflor®) medicated feed to control columnaris disease in juvenile Steelhead trout (*Oncorhynchus mykiss*). Trial FLOR-01-EFF-32

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Summary: Two florfenicol (Aquaflor® premix, Schering Plough Animal Health, Union, NJ) feed trials were conducted with juvenile Steelhead trout *Oncorhynchus mykiss* challenged by cohabitation with fish infected with *Flavobacterium columnare*. The standard treatment regimen of 10 mg active florfenicol / kg fish / day administered in feed for 10 consecutive days was employed in both trials at a feed rate of 2% bodyweight per day (% bw / d) in trial 1 (0.100% Aquaflor® in feed) and 3.5% bw / d in trial 2 (0.057% Aquaflor® in feed). Control groups received the same diet without medication administered at the same feed rate. In each study, treatment groups consisted of four replicate tanks. During the post-treatment period, it was determined that the 10 d treatments were unsuccessful in controlling mortality caused by columnaris. There was no significant difference in mortality between treated groups over the spontaneous recovery background levels shown by the controls. Resistant *F. columnare* isolates were not detected during the trials. Success in Trial 1 was negatively impacted by feeding fish in treated tanks at doses below the standard treatment regimen. This was a result of administering medicated feed at inconsistent feed rates due to calculation errors in the initial loading as well as the use of mortality weights to re-adjust feed amounts. Feed rates in trial 2 were estimated to be within 6-16% of the target level. No adverse reaction to the medicated diet was detected in either feeding behavior observations or florfenicol-induced tissue changes in the liver, kidney, and intestine. Antibiotic concentration in liver and kidney tissue was assayed by a microbiological method with a sensitivity threshold of 0.25 ng. No inhibition of indicator bacteria in the microbiological assay was detected in the samples suggesting low antibiotic levels in the target tissue. Additional validation testing for this assay is warranted. Analytically verified florfenicol concentration in the medicated diet was only 53% of the target dosage (265.2 mg / kg feed instead of 500 mg / kg) in trial 1, but was 87% (247.5 / 285.5) in trial 2. The poor performance of the medicated diet in trial 1 can be explained by inadequate consumption of the antibiotic by test fish. However, similar results in trial 2 suggest that the standard dosage of 10 mg active florfenicol / kg fish may be insufficient to rapidly control columnaris outbreaks.

Background:

Florfenicol is a potent, broad-spectrum, antibacterial agent with bacteriostatic properties. It is currently labeled to control mortality caused by *Edwardseilla ictaluri* in catfish, and *Flavobacterium psychrophilum* and *Aeromonas salmonicida* in salmonid fishes in the United States (http://www.spah.com/usa). The feed additive Aquaflor® (50% florfenicol) is produced by Schering Plough Animal Health (Union NJ). At the request of and in cooperation with the USFWS Aquatic Animal Drug Approval Partnership Program (AADAP), the California Nevada Fish Health Center (FHC) conducted two trials with Coleman National Fish Hatchery (CNFH) juvenile Steelhead trout at the FHC wet laboratory in May and June 2007. The objectives were:

1. Determine if the standard treatment regimen (10 mg active florfenicol / kg fish / day for 10 consecutive days) is effective in controlling columnaris disease in cultured Steelhead trout.
2. Determine adverse responses from the treatment such as anorexia or cellular damage to liver, kidney, and intestinal tract (histology).
3. Determine if resistant *F. columnare* strains develop during treatment.
4. Determine drug content in plasma and tissue (liver and kidney pool) of treated fish throughout the treatment.

Major limitations to such a trial being conducted with a full CNFH production group(s) is the irregular occurrence of columnaris in specific raceway populations and the variability in disease severity. In addition, the risk of substantial disease loss to CNFH production fish is deemed unacceptable. Therefore, an experimental challenge approach was used for the two trials using smaller test tanks in a laboratory setting.

Methods:

FHC wet laboratory – All experimental work with trout was conducted within the FHC wet laboratory. Its water supply is ozone disinfected and effluent is treated with chlorine (45 min retention time system with free chlorine residual of 0.3 – 0.5 mg / L). Other biosecurity measures include limited access to the locked building, iodophor footbaths, and individual nets with iodophor containers for each treatment tank. Eight circular tanks (800L volume) supplied with 5.7 L / min flow of 18° – 20°C water and aeration were used for the four replicate groups per treatment. Treatment group designation for a given tank was obtained by a random number generator.

Medicated feed - Two 7-kg allotments of Silvercup salmon starter #2 (Trial 1) and 1.5mm (Trial 2) commercial diet fed to the general Steelhead population at CNFH were sent to Mr. Jim Bowker (AADAP) for production of the medicated diet. Medicated feed was prepared using a Marion Mixer to obtain a target concentration of 10 mg active florfenicol / kg fish. For Trial 1, test fish were fed at a rate of 2% body weight (bw)/d. Hence, the feed allotment was mixed with 0.100% Aquaflor® premix and topcoated with 0.5% fish oil. For Trial 2, test fish were fed at a rate of 3.5% bw/d, and the feed allotment was mixed with 0.057% Aquaflor® premix and topcoated with 0.5% fish oil.

The control groups received the same Silvercup diet without medication. Diets were kept at 10°C before and during use. To maintain blinding, the florfenicol and control diets were placed in ziplock bags marked with an alpha code known only to the lead Investigator and not study personnel responsible for feeding test fish. Subsamples (200 g) of both the control and medicated diets were collected at day 1, 5, and 10 of the treatment, frozen at -20°C until they were shipped to the AADAP office in Bozeman, MT. Day 1 samples were then shipped to Eurofins AvTech (Kalamazoo, MI) for analytical (high performance liquid chromatography) verification of florfenicol content.

Water quality and fish rearing –Fish were fed twice daily by hand. Fish behavior (position in tank) and feeding response was recorded daily. Water quality parameters measured daily include dissolved oxygen concentration (Hach Co., Loveland CO) and temperature (hourly profile recorded by Hobo probes, Onset Co., Pocasset MA). Hardness, alkalinity, and pH of "source" water that supplied test tanks were measured on the first day of each feeding trial (wet chemistry kits LaMotte Co, Chestertown ML, pH measured with Beckman 12 meter).

Daily feed ration was adjusted during each study by subtracting the previous mortality from the estimated initial population to obtain a tank specific population number (N_t). The resultant N_t was then multiplied by the average fish weight to obtain the estimated tank biomass (gram). A decision was made in consultation with AADAP staff prior to Trial 1 to adjust feed rate as follows:

Study day 1 – 3	feed test fish based on initial tank biomass,
Study day 3 - 6	adjust daily feed amount based on the current tank population estimate ({370 – cumulative mortality number} x average weight of mortalities on day 3),
Study day 7 – 10	feed as above but use average weight of mortalities that were measured on day 6.

In Trial 1, the biomass was multiplied by 0.02 to obtain the grams of diet to feed test fish at the 2% bw / d level. In trial 2, the biomass was multiplied by 0.035 to obtain the grams of diet to feed test fish at the 3.5% bw / d level. Note that in Trial 2, the average weight of the fish in the reference population (2.8g) was used as the weight factor to calculate daily biomass during the 10 d treatment period.

Mortality and fish health - Mortalities were removed from each test tank twice daily during the 10 d treatment and once daily during the 6 – 9 d post-treatment period. The presence of clinical signs of columnaris (gill or skin lesion, ulcer, tail rot) was noted for each fish. Gill and skin imprints were taken from mortalities that did not show obvious clinical signs. Such imprints were gram-stained and examined for filamentous gram-negative bacteria (presumptive *F columnare* diagnosis) using standard procedures.

Bacterial Challenge- A 2005 CNFH isolate of *Flavobacterium columnare* was grown using the following procedure:

1. The isolate was grown in tryptone yeast extract with salts (TYES = 0.4% tryptone, 0.04% yeast extract, 0.05% $CaCl_2$ and $MgSO_4$, 1.5% agar {excluded for broth} in a volume of 1L using distilled water) for 18 – 20 h at 25°C.
2. Culture growth was facilitated by moderate agitation.

3. The resultant cultures were centrifuged (2000 x g, 20°C, 10 min) and the pellet re-suspended in TYES broth to an estimated concentration of 10^9 cfu / mL based on TYES plate counts of 100 µL spiral spread preparations of 10x dilutions. Clumping of the inoculum limited the precision of colony counts.

The *F. columnare* suspension was then added to a 2 L static aquarium (20 mL; ~ final challenge concentration estimated at 10^7 cfu / mL) containing 10 juvenile steelhead trout. The water temperature in the aquarium was held at 18° - 20°C and water was supplied with aeration. Trout were transferred to 20° flow-through aquaria after the 18 h static bath disease challenge. Clinical signs of columnaris on challenged fish were observed within 24 – 36 h post-challenge. Groups of 4 – 5 infected fish (i.e., donors) were placed into 400 cm^3 cages and suspended in the 568 L tank containing the experimental population of test fish.

Trial 1

On May 14, a reference population of Steelhead trout (12.4 kg total weight, average fish wt 2.96 g ± 1.31 SD) was established by transferring fish from a production raceway at CNFH to the 568 L reference population tank (density index (Piper et al. 1982) = 0.51 (D.I. = biomass {27.3lbs} / average length {2.64 in.} x volume {20.1 cf}). Columnaris donors were added to the tank holding the reference population on May 15. Donor mortalities were replaced with moribund donors from the challenge aquaria for the next 3 days. On May 19, the first clinical signs of columnaris were observed in the reference population with mortality of 9.2% occurring on May 20 (386 dead fish from a population of 4,186). Fork length and weight was measured in 16 of the dead fish and the mean of this sub-sample (3.45 g, 1SD = 0.91g) was used to calculate initial loading of 450 fish per tank to achieve a density index of 0.15. Later the same day 1553 g of live fish were impartially selected (by dip-net) and transferred in two rounds (225 fish or approximately 777g / round) to the eight replicate test tanks. The treatment period began the next day (May 21) over which time fish were fed medicated or non-medicated feed for 10 consecutive days. The treatment period ended on May 30.

Trial 2

On June12, a total of 3,344 trout (9 kg total weight, mean wt ± 1 SD = 2.8 g ± 0.8; n = 100) were transferred from a CNFH production raceway to the 568 L tank (DI = 0.474). The columnaris disease challenge was performed as described above. Test fish were moved from the 568 L tank into the circulars when the first clinical sign of columnaris was observed in the reference population on June 17. A total of 370 test fish (target density index of 0.15) were hand counted from the reference into the replicate circular test tanks in three rounds of 120 – 130 fish / round. The treatment period began on June 18; test fish were fed either medicated or non-medicated feed at 3.5 % bw / d for 10 consecutive days; and the treatment period concluded on June 27, 2007.

Disk sensitivity testing of isolates – The diagnostic criteria for *F. columnare* identification (filamentous gram-negative rod, orange-yellow pigmented rhizoid colonies on TYES agar, colony binds 1% aqueous congo red dye) was based on the protocols of Griffin (1992). Isolates of *F. columnare* were obtained from test fish fed florfenicol and control diet sampled on day 1 and 5 of the treatment period. Isolates from the TYES primary cultures were plated onto dilute Mueller-Hinton (M-H) agar and incubated at 20 - 25°C for 24 – 48 h prior to reading of the inhibition zone (CLSI M42-P). Twenty milliliters of dilute Muller-Hinton agar (0.4% M-H broth, 1.7% agar, and 5% calf serum) was poured into 100 mm diameter petri plates and used within 48 h. Two disks containing 30 µg of florfenicol were placed onto each M-H plate and zones of inhibition were measured using a micrometer to obtain the mean zone diameter.

Antibiotic concentration in plasma and tissue – Duplicate 40 L aquaria were loaded with 50 unchallenged healthy trout and fed either medicated or control diets for 10 d in conjunction with challenged groups. Two microbiological methods were used to estimate the concentration of florfenicol in individual fish liver/kidney tissue pools (Kusser and Newman 1990). Plasma was not tested because the volumes obtained from the trout were insufficient to test as they require >20x dilution. Liver / kidney tissue pools were tested using the following procedure:

1. A 2.5 x 10^7 spore suspension of *Bacillus cereus* (Difco) was added to 250 mL of cooled Mueller-Hinton agar containing 50 µg / mL Brilliant Black BN dye.
2. 100 µL of the resultant agar was added to wells of a 96-well plate that was sealed and maintained at 4°C until used for the assay.
3. A 0.5 mg / mL stock solution of florfenicol was prepared by diluting 10 mg of Aquaflor (50% active florfenicol) in 10 mL distilled water.
4. Dilutions (2x and 10x) of this stock were prepare with water.
5. Plates were inoculated with 100 µL of standard or sample and incubated in a moist chamber at 28°C for up to 48 h.
6. Growth of *B cereus* resulted in the dye changing from blue to yellow within 48 h.

Pilot studies with this system, using spiked supernatant of a 5x aqueous dilution of kidney – liver homogenate, demonstrated that inhibition of bacterial growth occurred at concentrations as low as 0.3125 ng florfenicol and was similar to the water-only diluent threshold of 0.25 ng. Kidney-liver homogenates (2x, 3x, and 10x dilutions in water), from five to eight test fish fed florfenicol for 5 and 10 days in trial 1 and for 5 days in trial 2 were assayed by this method.

The second microbiological method used the same *B cereus* agar as described above, without dye, in standard 100mm Petri plates. After blotting the surface of the agar, sterile 4 mm disks were placed on the surface and 25 µL of sample or standard was immediately applied to the disk. Zones of inhibition were measured

after 24h incubation at 28°C. In this system, standards of 1.25 ng and 0.125 ng florfenicol produced 18 and 9 mm diameter zones, respectively. Two fold dilutions of kidney-liver samples from three Trial 1 test fish fed florfenicol for 10d) and three Trial 2 test fish fed florfenicol for 5d were assayed by this method.

Statistics- Kaplan-Meier survival analysis using Log Rank comparison of multiple groups was used to test for differences among treatments (Sigma Stat 3.1, Point Richmond CA). Before analysis, percent cumulative mortality of each treatment group (four replicates) were arcsine transformed and tested by Student's t-test at the $P< 0.05$ significance level.

Results:

Water quality- Mean water temperature in Trial 1 was18.0°C (range, 15.7 – 19.9°C) and in Trial 2 was 19.7°C (range, 18.3 – 22.2°C). Dissolved oxygen concentration ranged from 7.64 to 8.86 mg / L in the test tanks during both trials. Other parameters were considered normal as listed below:

Trial	Date	Hardness mg/L $CaCO_3$	Alkalinity mg/L $CaCO_3$	pH
1	May 19	40	64	6.49
2	June 18	56	68	7.84

Fish behavior and histological observations - Excluding moribund test fish, vigorous feeding behavior was observed in all groups. Initial loading density indices in Trial 1 ranged from 0.156 to 0.176 and from 0.115 to 0.122 in Trial 2. These indices are similar to the loading densities of the production raceways at CNFH. A social hierarchy was apparent in each tank. We observed larger fish positioned nearer the bottom and nipping behavior by the more dominant fish during feeding. This observation was more apparent in Trial 1, where fish were fed at a rate of 2% bw/d and there appeared to more cannibalism of mortalities. This behavior was a likely cause of the mouth and head lesions that were observed in fish necropsied in Trial 1. Unlike linear flow raceways where dead and moribund fish are typically impinged on the tail screen, such fish in circular tanks are lodged along the bottom at the center standpipe and are thereby available for feeding by all trout in the tank.

From a qualitative standpoint, no remarkable difference was observed between the cellular morphology of the posterior kidney, liver, and intestinal tract of the control (n = 3 fish) and treated (n = 6 fish) trout examined from either trial. Hydropic droplets (protein) were seen in a small percentage of the proximal tubule epithelial cells of both control and treated trout in Trial 2. All other tissues were characterized as normal.

Concentration of florfenicol in medicated diets – A sample of medicated diet taken prior to feeding in Trial 1 contained 265.2 mg florfenicol / kg feed (Eurofin, Kalamazoo MI). The target concentration for this diet was 500 mg / kg feed. A similar sample from Trial 2 contained 247.5 mg florfenicol / kg feed with the target dosage being 285.5 mg / kg.

Feed rate error – Actual fish number transferred to each test tank at the beginning of the study was calculated at the end of the experiment by summing all mortalities and survivors. In Trial 1, the use of biomass to load the tanks at the beginning of the study resulted in an average of 466 fish / tank, which was 3.6 % more than the target of 450 fish / tank (Table 1). This error resulted in underfeeding fish in the treated groups (AF1 - 4) by 3 – 19%/ d. This error was further compounded by the decision to adjust tank biomass every three days using mean fish weight of the day 3 and 6 mortalities. We considered the variability in mortality weight data (i.e, mean weight, 3.6 g; std dev 1.0; sample size, n= 145; and coefficient of variation, 27%) from day 3 and 6 of the treatment period (10 fish / tank / sample date) to be unacceptable. It is unlikely that the weight of these fish accurately represented the tank population.

Hand counting fish in Trial 2 to initially load test tanks was much more accurate than in Trial 1 (e.g. average percent error of only 0.5%; Table 2). When the initial mean weight of 2.8 g is multiplied by the actual fish number on day 1 and day 10 in the florfenicol-treated tanks, the estimated feed rate tended to be higher than the target 3.5 % bw /d (Table 2).

Table 1. Trial 1 actual fish number (N) at initial tank loading , percent difference of (N) from 450 fish target, and estimated percent bodyweight / d fed to groups on day 1 to day 10 of treatment for the four Aquaflor (AF) and control (CON) replicates. Target feeding rate was 2% bw / d.

Treatment condition	Actual number of fish/tank at time = 0	% Difference (N)	Estimated % bw fed Day 1 to 10
AF1	461	2.4	1.88 - 1.95
AF2	482	7.1	1.73 – 1.86
AF3	487	8.2	1.73 – 1.84
AF4	479	6.4	1.62 – 1.84
Con1	458	1.8	1.92 – 1.96
Con2	442	-1.8	2.04 – 2.11
Con3	498	10.7	1.56 – 1.79
Con4	495	10.0	1.42 – 1.72

Table 2. Trial 2 actual fish number (N) at initial tank loading , percent difference of (N) from 370 fish target, and estimated percent bodyweight / d (2.8 g average fish weight x actual fish number) fed to group on day 1 to day 10 of treatment for the four Aquaflor (AF) and control (CON) replicates. Target feeding rate was 3.5 % bw / d.

Treatment condition	Actual number of fish/tank at time = 0	% Difference (N)	Estimated % bw fed Day 1 and 10
AF1	363	-2.1	3.57 - 4.26
AF2	370	0	3.50 - 3.97
AF3	378	2.2	3.43 - 3.97
AF4	359	-3.0	3.61 – 4.26
Con1	378	2.2	3.43 – 4.11
Con2	376	1.6	3.44 – 4.58
Con3	377	1.9	3.44 – 4.02
Con4	375	1.4	3.45 – 4.10

We did not attempt to measure weight of the study fish in Trial 2 over the 10-d treatment period and likely underestimated the tank biomass due to growth. If the unchallenged group fed the medicated diet and held in the 40L aquaria were used as surrogates for the test populations held in the circular test tanks, we estimate the use of 2.8 g as the weight factor throughout the treatment period to underestimate the tank biomass by 5.7% – 16.7%:

Day 5 mean weight of unchallenged fish	= 3.11 g (see table 4)
Initial mean weight of reference fish	= 2.80 g
Days between measurements	= 11
(3.1-2.8)/11	= 0.027 g /d estimate weight gain
First day of treatment (6 d after initial wt)	= ave. wt. of 2.96 g
(6 x 0.027) + 2.8 = 2.96 g	
Tenth day of treatment (16 d after initial wt)	= ave. wt of 3.25 g
(5 x 0.027)+ 3.11 (day5 measurement)	= 3.25 g
(2.96 – 2.80)/ 2.8	= 5.7%
(3.25 – 2.80)/ 2.8	= 16.1%

Resistant isolates – No resistant *F. columnare* isolates were detected from treated test fish sampled in either trial. However, there was poor recovery of the primary *F. columnare* isolates on dilute M-H agar. Only 25 – 42 % of the primary *F. columnare* isolates were successfully passed onto the dilute M-H agar (Table 3). All tested isolates had inhibition zones of $\geq$ 30 mm and were considered sensitive to the antibiotic (Table 3). Isolate colony coloration was pale yellow and

morphology was smooth on dilute M-H agar while coloration was orange and morphology was rhizoid on TYES agar. Using the phenotypical characteristics described above, all isolates grown on dilute M-H and passed back onto TYE agar were later confirmed as *F. columnare.* There is no standard inhibition zone reported for the test system (CLSI M42-P). However, an *Aeromonas salmonicida* isolate (ATCC 14174) inoculated onto this media produced 37 – 41 mm zones under these conditions.

Table 3. Florfenicol (30 µg) disk susceptibility results with *F. columnare* isolates collected from trout fed florfenicol in Trial 1 and Trial 2 sampled on day 1 and 5 of the treatment period. Data recorded as number of *F. columnare* isolates that grew on the M-H media / total isolates inoculated (Fc) and the range of inhibition zone diameters observed on the M-H cultures after 48 h.

	Day 1	Day5
Trial1	4 / 12 Fc, 46 - 54 mm	5 / 12, 43 – 50mm
Trial2	not done	1 / 4, 30mm

Antibiotic concentration in kidney and liver – None of the tissue samples tested from either the control or treated unchallenged trout sampled during either trial inhibited the growth of *Bacillus* spores. It appears that the florfenicol concentration in the kidney / liver pool was either: (1) below the 0.25 to 0.125 ng threshold observed in the standard wells or (2) there is a problem in the current assay (e.g.freeze/thaw of tissue, binding of antibiotic to host proteins resulting in poor diffusion into the agar or loss of inhibitory action, etc.) that resulted in lower than expected tissue concentrations. The bioavailability of florfenicol is reported by SPAH to be 66.3% in trout reared at 16°C (i.e., approximately 66% of the ingested drug should be detectable in fish tissue). Therefore, we expected the microbiological assay system used to be able to detect florfenicol in tissue if there was $\geq$ 50% absorption in Steelhead trout. As such, we presumed that the tissue concentrations should have been at least 1 - 5 ng / g (see Example 1).

Example1: 3 g fish consumes 10ng florfenicol / g bw (=10 mg/kg) = 30 ng
30 ng * 0.5 = 15 ng florfenicol in plasma
10 – 30% retained in kidney or liver ~ 1- 5 ng

There was very little difference in growth between unchallenged trout fed medicated or non-medicated diets (Table 4).

Table 4
Mean weight (± 1 SD; measured to the nearest 0.01 g) and fork length (measured to the nearest 1 mm) of the unchallenged trout fed florfenicol or control diet in Trial 1 (AF1) and Trial 2 (AF2). A total of 16 fish were collected at each sampling event prior to dissection of kidney and liver tissue for antibiotic concentration assay.

Sampling Event	Mean wt (± 1 SD) and length (± 1 SD) from treated tanks		Mean wt (± 1 SD) and length (± 1 SD) from control tanks	
AF1-5d	3.68 g ± 0.90	69 mm ± 4	3.43 g ± 0.65	68 mm ± 4
AF1 -10d	4.02 g ± 0.74	71 mm ± 3	4.04 g ± 1.07	70 mm ± 5
AF2- 5d	3.11 g ± 1.08	61 mm ± 6	3.71 g ± 1.12	64 mm ± 7

Bacterial challenge and mortality patterns - As demonstrated by clinical signs, isolation of cultures on TYES, and gram stain imprints, columnaris was the sole cause of mortality in both trials. A total of 27 asymptomatic trout were sampled for gill and / or skin imprints and filamentous gram-negative bacteria were observed in all gram-stained imprints.

No significant difference was detected in the cumulative mortality of treated or control fish in either trial (Trial 1: $t = -0.551$, 6 df, $P = 0.601$; Trial 2: $t = -0.420$, 6 df, $P = 0.689$; trial 2). In Trial 1, mean cumulative mortality in controls (72.3% mortality) was 5.4% higher than that in treated tanks (66.9% mortality) at the end of the 6-d post-treatment period.

In Trial 2, mean cumulative mortality over the entire 19 d study was 22.5 % in both the control and treated tanks. As discussed above, a higher initial mortality was observed in Trial 1 than that in Trial 2. We hypothesize that test fish used in Trial 1 received a stronger *F. columnare* challenge (i.e., were "sicker") and were co-mingled with donor fish for a longer duration within the challenge tank prior to transfer to circular test tanks than test fish used in Trial 2.

Trial 1 - The log rank statistic for Trial 1 survival days demonstrated significant differences between replicate tanks however there was no treatment effect (142.8, df 7, $P<0.001$). The lack of treatment effect is demonstrated by the wide range in mean survival day and cumulative percent mortality data listed for the treated and control replicates (Table 5). Two replicates (AF1 and CON3) showed a bimodal mortality pattern with an increase occurring after the 10d treatment period (Figure1). There was an overall trend for lower mortality with increased mean survival days in all challenged groups with an r-squared value of 0.602 (Figure 2).

Table 5. Mean survival day and percent cumulative mortality in Trial 1 over the 16 day period (10-d treatment + 6-d post-treatment) for the four replicate juvenile Steelhead trout groups undergoing a columnaris epizootic and fed at 2% bw / d rate either Aquaflor (AF = 10 mg drug / kg fish / day) treated feed or the control (CON) diet.

Group	Mean survival day (SE)	16 d Cumulative % Mortality
AF1	7.41 (0.27)	80.0
AF2	9.11 (0.29)	56.4
AF3	9.79 (0.28)	52.4
AF4	7.35 (0.28)	78.9
CON1	9.24 (0.29)	54.6
CON2	6.91 (0.29)	74.9
CON3	7.86 (0.24)	89.4
CON4	7.22 (0.32)	70.5

Figure 1 Daily mortality number for the 4 replicate groups fed florfenicol (AF1-4) or control diet (CON1-4) at the 2% BW/d rate in Trial1.

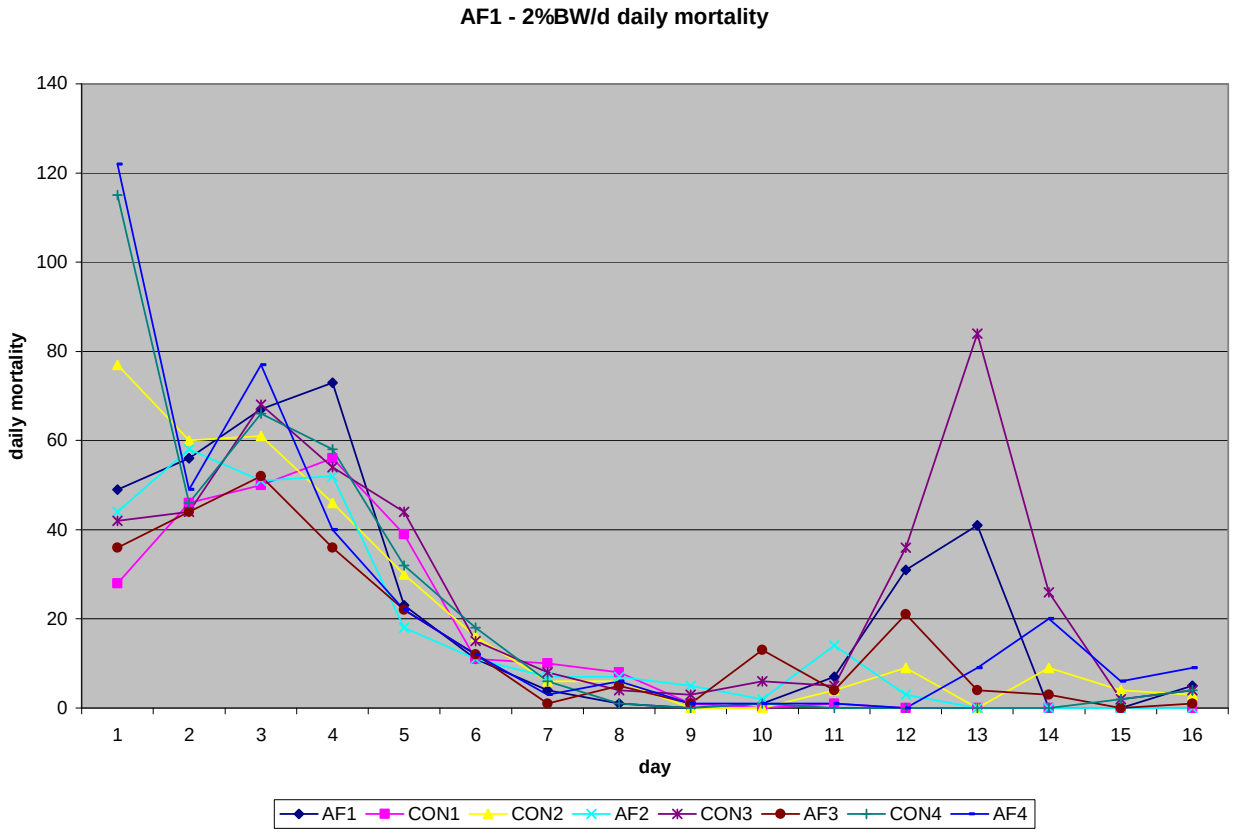
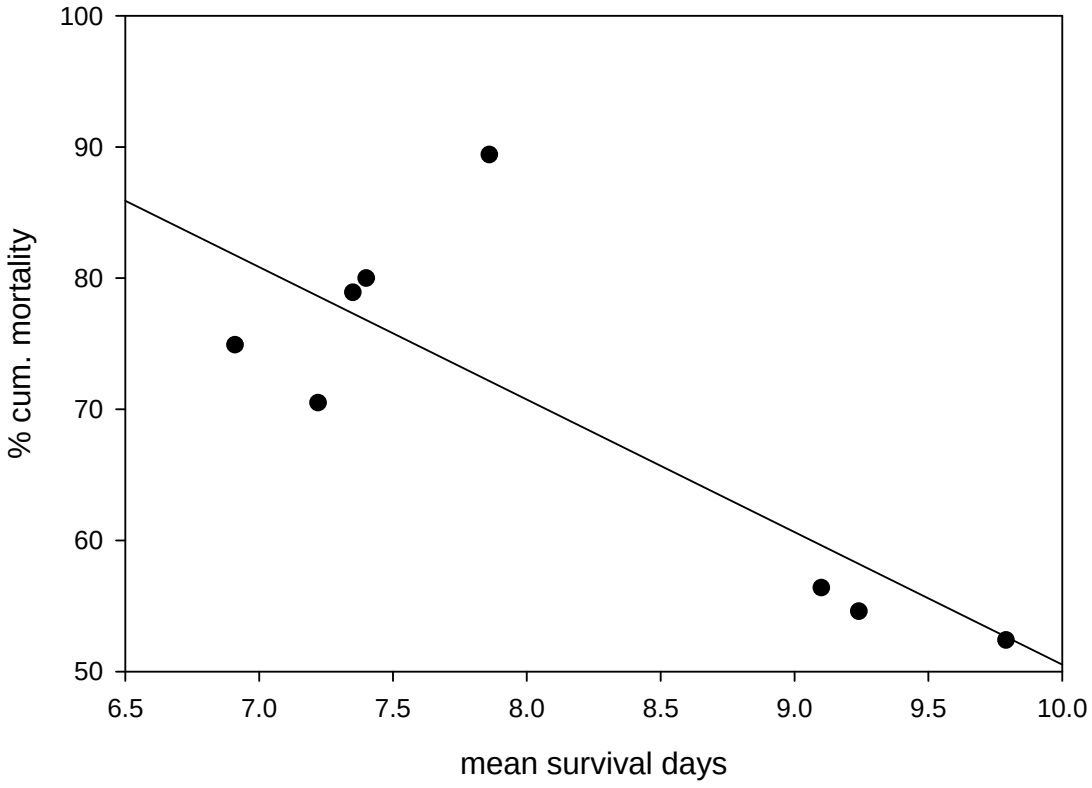


Figure 2. Correlation between mean survival days and cumulative percent mortality for replicate trout groups in Trial 1 ($r^2 = 0.602$). Regression line based on the equation: Survival day = 12.650 – (0.0652*cumulative mortality).



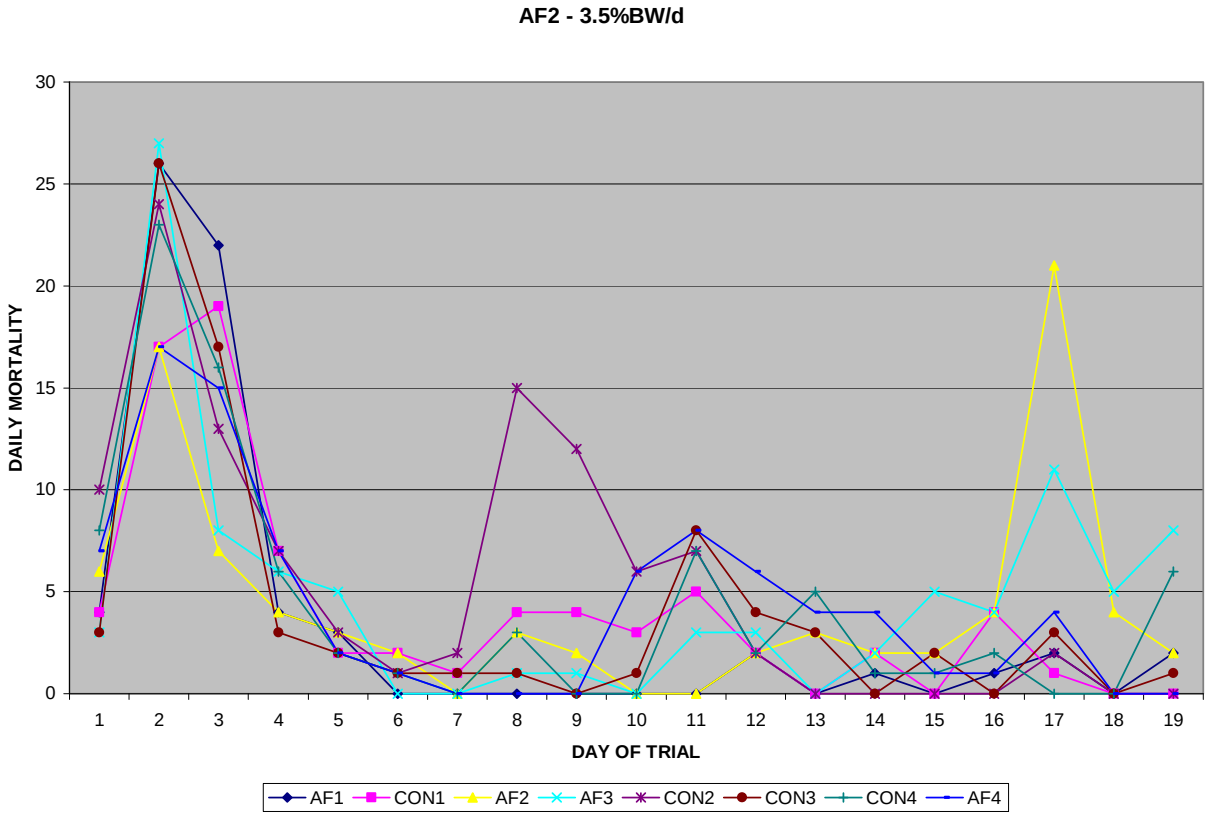
Trial 2 - The log rank statistic for trial 2 survival days demonstrated no significant differences between replicate tanks (12.46, df 7, P= 0.087). The mean survival day and cumulative percent mortality among the treatment replicates were similar (Table 6). The magnitude of daily mortality was lower than that observed in Trial 1 with two florfenicol replicates (AF2 and 3) showing a reoccurrence of columnaris mortality post-treatment (Figure 3).

Daily removal of mortalities appeared to strongly influence the infection rate within replicate tanks. In Trial 1, two of the four control groups showed either no or very low mortality after day 10 (Figure 1). This pattern was repeated in all Trial 2 control tanks (Figure 3). Ogut and Reno (2004) report that the density of recipient (naïve uninfected) salmon had a strong positive relationship to both transmission and disease mortality after exposure of a single donor fish with *Aeromonas salmonicida.* Cessation of columnaris in the untreated populations was likely the result of this density factor and the decrease of infectious challenge due to mortality removal. In order to gain control of the columnaris outbreak, it appears critical that all infected fish be removed from the rearing unit by the end of the 10 d treatment as well as obtaining sufficient antibiotic concentrations in the remaining survivors.

Table 6. Mean survival day and percent cumulative mortality in Trial 2 over the 19 day period (10-d treatment + 9-d post-treatment) for the four replicate juvenile Steelhead trout groups undergoing a columnaris epizootic and fed at 3.5% BW/d rate either Aquaflor (AF = 10 mg drug / kg fish / day) treated feed or the control (CON) diet.

Group	Mean survival day (SE)	19 d Cumulative % Mortality
AF1	16.3 (0.3)	18.5
AF2	16.8 (0.3)	23.5
AF3	16.5 (0.3)	24.9
AF4	16.2 (0.3)	23.1
CON1	16.3 (0.3)	20.4
CON2	15.3 (0.3)	27.7
CON3	16.3 (0.3)	20.2
CON4	16.4 (0.3)	21.9

Figure 3. Daily mortality for the four treated (AF) and control (CON) replicates over the 10 day treatment and 9 day post-treatment period in Trial 2. Fish were fed at the 3.5% BW/ d rate.



Conclusions:

1. Neither 10 d consecutive treatment (at 2 or 3.5% bw /d level of the standard treatment regimen of 10 mg active florfenicol / kg fish / day) was deemed successful in controlling columnaris over the spontaneous recovery background levels shown by the controls.

2. Problems in Trial 1 (underfeeding due to inaccurate population biomass estimates and low antibiotic content of the feed) invalidated the poor results of this trial; however, the same results occurred in Trial 2 that did not have these experimental errors.

3. While the microbiological assay to determine antibiotic content in liver and kidney tissue was not experimentally validated, the data suggested that active florfenicol concentration in the tissue was less than 0.25 ng.

4. We question whether the 10 mg active florfenicol / kg fish / day treatment level is sufficient for the control of columnaris epizootics. Comparative studies should be run with higher antibiotic levels (e.g., 15 and 20 mg active florfenicol / kg fish / day).

5. The 2% bw/d feed rate was deemed adverse for columnaris control in juvenile Steelhead trout as we observed a higher degree of mortality cannibalism in Trial 1 than in Trial 2. This behavior may have exacerbated the columnaris infection as evidenced by infections in the mouth and oral cavity.

6. We did not observe resistant *F. columnare* isolates during the treatment. However, a microdilution technique, in TYE broth with antibiotic samples, may be a better alternative than disk sensitivity in dilute M-H.

7. We hypothesize that a treated population must reach < 1-5% daily mortality rate by day 5 of treatment and be near zero by day 10 in order to reduce the probability of re-infection triggering a continued epizootic in the population.

Future areas of study prior to repeating feed trials:

1 Use a more sensitive assay (0.001 ng / g tissue range) for determination of florfenicol concentration in plasma and tissue. Either contract out analysis by HPLC or develop a more sensitive in-house microbiological assay.

2. Determine the efficacy of oral treatment to prevent initial infection. Intubate precise quantities of medicated diet, based on individual fish weight, to marked trout for 5, 3, 1, and 0 days prior to columnaris challenge. Tissues from cohorts will be assayed for florfenicol concentration by the method described above in #1.

3. Validate a microdilution drug sensitivity assay for *F. columnare* isolates to replace the disk diffusion assay.

4. Conduct a comparative study with challenged fish fed at both 10 and 20 mg florfenicol / kg fish / day treatment levels.

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