

A CLINICAL FIELD TRIAL TO DETERMINE:

The Efficacy of Florfenicol-Medicated Feed to Control Mortality of Fingerling Westslope Cutthroat Trout *Oncorhynchus clarki* Caused by Bacterial Coldwater Disease, Causative Agent *Flavobacterium psychrophilum*.

Study Number: FLOR-01-EFF-04

Study Director

James D. Bowker
U.S. Fish and Wildlife Service
Bozeman Fish Technology Center - National INAD Office
4050 Bridger Canyon Road
Bozeman, MT 59715
Phone: 406-587-9265 ext. 126
FAX: 406-582-0242

Investigator

Jim Schreiber
Murray Springs Trout Hatchery
Montana Fish, Wildlife, and Parks
5475 Sophie Lake Road
Eureka, MT 59917
(406) 889-3489

Testing Site: Murray Springs State Fish Hatchery
Montana Fish, Wildlife, and Parks
5475 Sophie Lake Road
Eureka, MT 59917
(406) 889-3489

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James D. Bowker _____

Daniel Carty _____

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Abstract

The United States Fish and Wildlife Service's (USFWS) National Investigational New Animal Drug Office (NIO) designed and conducted an efficacy study to generate data needed to obtain U.S. Food and Drug Administration approval for the use of florfenicol-medicated feed to control mortality in hatchery-reared salmonids diagnosed with bacterial coldwater disease (CWD), causative agent *Flavobacterium psychrophilum*. The study was conducted at Murray Springs Trout Hatchery (TH; Eureka, MT) by staff from the NIO and Murray Springs TH following guidelines described in Study Protocol Number FLOR-01-EFF. The objective of the study was to compare mortality between fingerling westslope cutthroat *Oncorhynchus clarki* fed florfenicol-medicated feed and fingerling westslope cutthroat (CTT) fed non-medicated feed. Fish used in the study had been diagnosed with bacterial CWD by identification of *F. psychrophilum* cultures grown on Tryptone-Yeast Extract agar (TYE) that had been streaked with spleen tissue from fish sampled at the start of the study and confirmed by polymerase chain reaction (PCR). On day one of the study a completely randomized design procedure was used to assign a treatment condition of either "treated" or "untreated" to each test tank. Test fish in 4 of the 8 test tanks were fed florfenicol-medicated feed at a target dosage of 10 mg florfenicol/kg of fish/d for 10 consecutive days. Test fish in the other 4 test tanks were fed non-medicated feed during the same 10-d period. Following the treatment period, test fish in all 8 test tanks were fed non-medicated feed. Blinding techniques were employed to ensure that study participants

involved in day-to-day data collection did not know which test tanks of fish were fed medicated feed and which test tanks of fish were fed non-medicated feed. The study lasted 41 d and consisted of a 1-d acclimation period, a 10-d treatment period, and a 30-d post-treatment period. Total mortality that occurred during the treatment and post-treatment periods of the study was the primary response variable. Percent total mortality for each test tank was calculated by dividing the number of dead fish removed from each test tank during the treatment and post-treatment periods by the number of live fish transferred to each test tank at the beginning of the study. At the end of the study, mean percent total mortality in the group treated with florfenicol-medicated feed was lower (1.9%) than the mean percent total mortality in the group not treated with florfenicol-medicated feed (3.2%), although differences were not significant ($P = 0.332$). Mean mortality among treated tanks was controlled within 4 - 5 d whereas mean mortality in untreated tanks was not controlled until day 29 of the post-treatment period. Although mortality in two of the untreated tanks was relatively low throughout the entire study period, cumulative mortality in the other two untreated tanks continued to increase throughout the study. The disparity in the disease level among untreated test tanks may have resulted in lower than expected mean mortality at the end of the study. It was suspected that had there been a more uniform level of disease in all test tanks, differences in total mortality between treated and untreated groups would have been more dramatic.

Introduction

Florfenicol is a potent, broad-spectrum antimicrobial agent with bacteriostatic properties (Horsberg et al. 1996). It is a fluorinated analogue of thiamphenicol and is also similar in structure to chloramphenicol, both of which have been used as broad-spectrum, veterinary antibiotics (Nagata and Oka 1996). Bacterial diseases are a major problem in aquaculture and account for significant losses of fish (Clarke and Scott 1989; Frerichs and Roberts 1989; Bjorndal 1990). While the importance of environmental conditions (McCarthy and Roberts 1980; Haastein 1988; Munro and Roberts 1989) and the value of effective vaccines, where available (Ellis 1989), are acknowledged, antimicrobial therapy presently has an important role to play in aquaculture (Klontz 1987; Alderman 1988).

Florfenicol has great potential for treatment of infectious diseases, and because of its high potency and safety to humans, it could become an important drug in veterinary medicine, especially with respect to animals used by humans for food (Powers et al. 1990). Additionally, because florfenicol is not currently used in human medicine, it has become a strong candidate for use in aquaculture, and there is considerable interest to obtain U.S. Food and Drug Administration (FDA) approval for its use in fish culture.

The proposed treatment strategy (i.e., dosage and duration) for the use of florfenicol-medicated feed in fish is designed to meet the needs of individual fish species, individual fish lots, and a variety of environmental conditions. In all cases,

treatment goals are to (1) minimize the negative effects of disease on fish health, quality, and survival, and (2) help meet fishery management objectives. Because many factors can affect the success or failure of florfenicol-medicated feed therapy, efficacy data from controlled, replicated studies that are scientifically valid and statistically defensible (i.e., pivotal) are needed to gain approval of florfenicol-medicated feed use in aquaculture.

The objective of this field-based, pivotal study was to evaluate the efficacy of florfenicol-medicated feed treatment (administered orally at a dosage of 10 mg of florfenicol/kg of fish/d for 10 consecutive days) to control mortality in fingerling westslope CTT caused by CWD. The study was conducted under the Pivotal Study Protocol FLOR-01-EFF and is intended to provide FDA/Center for Veterinary Medicine (CVM) with pivotal field data documenting efficacy of the florfenicol treatment regimen.

Materials and Methods

Study location and schedule- The study was conducted at the Murray Springs Trout Hatchery (TH), Montana Fish, Wildlife, and Parks (5475 Sophie Lake Rd, Eureka, MT; Appendix A; Figure 1). The study (41-d) began on October 2, 2001, and ended on November 11, 2001. The study consisted of a 1 d pre-treatment period (October 2, 2001), a 10 d treatment period that extended from October 3 - 12, 2001, and a post-treatment period that lasted 30 d (October 13 - November 11, 2001; see Table 1 for a

schedule and description of significant study events).

Test article - The florfenicol used in this study was Aquaflor[®] (Lot Number UK-1-BGCA-01; Schering-Plough Animal Health, Division of Schering Canada, Inc., Pointe Claire, Quebec), which is a 50% medicated premix in a palatable base for salmon. The Aquaflor[®] premix was mixed with Rangen (Buhl, ID) Custom Trout Starter #2 (Lot #4762) using a Marion Mixer (Model Number SPS-1224) according to SOP No. INST 126.0 and SOP No. MISC 218.0. The medicated feed contained a target dose of 0.6 g active florfenicol per kg of feed. The non-medicated Rangen Custom Trout Starter #2 was fed to the untreated (i.e., control) test fish groups during the treatment phase of the study and was fed to all test fish during the post-treatment phase of the study.

Test fish - Fingerling westslope CTT (Lot # M015101E; Appendix A) used in the study were progeny of broodstock held at Washoe Park State Fish Hatchery (SFH; Anaconda, MT), and were spawned at the Washoe Park SFH by hatchery personnel. Eggs were transferred to Murray Springs TH where they were raised until used in this study.

Estimated mean length and weight of test fish were 5.6 cm and 1.7 g, respectively. Mean fish length and weight estimates were based on sample counts conducted on October 2, 2001, in which all fish in each tank were weighed to more

accurately estimate the number of fish in each tank. Following this procedure, it was estimated that there was an average of 3,695 fish/tank (± 1 SD = 976) among the eight test tanks. At the start of the study there was no significant difference ($P = 214$) between the mean number of fish in treated tanks (3,244 fish/tank) and untreated tanks (4,144 fish/tank). Sample-count data were converted to mean fish length and weight using the appropriate length-weight conversion table for westslope CTT (condition factor $3,500 \times 10^{-7}$; Piper et al. 1982). Based on the estimated number of fish/tank and the estimated fish length and weight, the mean flow index and density index values were calculated to have been 0.21 and 0.6, respectively (Appendix L). These values are will within the acceptable range for rearing healthy salmonids (Piper et al. 1982).

Study design - The study design consisted of two treatment conditions: "treated" or "untreated." Each treatment condition was replicated 4 times; consequently, 8 test tanks of fish were used in the study. Individual test tanks were numbered 3, 4, 5, 6, 9, 13, 15, and 16. Fish in four test tanks were fed florfenicol-medicated feed (i.e., treated tanks; Figure 2). Fish in the remaining four test tanks were fed non-medicated feed (i.e., untreated tanks; Figure 2). A completely randomized design procedure was used to assign treatment conditions to test tanks (Appendix B; SOP No. MISC 206.2). Test tanks 4, 5, 6, and 9 were assigned a treatment condition of "treated", and test tanks 3, 13, 15, and 16 were assigned a treatment condition of "untreated." The intended treatment regimen was to administer florfenicol-medicated feed at a dosage of 10 mg active drug/kg of fish/d to treated test fish for 10 consecutive days. Actual dosage rates

were determined by feed assay.

Pre-study mortality - Prior to the start of the study, mortality had been increasing daily in test tanks (Appendix C). Based on the level of mortality, clinical signs, and culture findings following a preliminary fish health evaluation, pre-study mortality and morbidity was suspected to have been caused by bacterial CWD (causative agent *Flavobacterium psychrophilum*). Mean total mortality during the 7 d period prior to the start of the treatment period in treated and untreated tanks was 61.0 and 28.3, respectively. Although results from a Mann - Whitney Rank Sum test detected no significant difference ($P = 0.114$) in mortality between the treated and untreated groups there was a substantial difference in pre-study mortality between the two groups.

Fish health evaluation - Diagnosis of the bacterial infection was based on evaluation of 3 - 7 fish sampled per tank by Jim Peterson, Study Monitor, Montana Fish, Wildlife, and Parks (MT FWP; Appendix D). Jim Peterson's preliminary assessment was based on observation of increased daily mortality prior to the start of the study and moribund fish with external lesions on the dorsal surface. The observed lesions resembled lesions seen previously at this hatchery in fish diagnosed with bacterial CWD. Fish were diagnosed with bacterial CWD by identification of *F. psychrophilum* cultures grown on Tryptone-Yeast Extract agar (TYE) that had been streaked with spleen tissue and confirmed by polymerase chain reaction (PCR; Appendix D).

Infection of westslope CTT with CWD has been a recurring problem at Murray Springs TH and based on past history, it was suspected that CWD was the cause of the elevated morbidity and mortality. Fish behavior and feeding were observed periodically throughout the study, usually when tanks cleaned and fish were fed. Although this data was not recorded, healthy-appearing fish behaved and fed normally during the treatment and post-treatment periods.

Feed - Test fish were fed a standard commercial salmonid diet. A copy of the feed label, listing percent protein, fat, fiber, moisture, ash, and selected nutrients can be found in Appendix E. Amount of feed administered to each test tank was calculated on the first day of the study. Daily feed rations for the entire study were weighed out and placed in plastic containers on the first day of the study.

Feed samples - Feed samples were collected and an actual concentration of florfenicol in medicated feed was assayed using the following procedure: three 150-g samples of medicated feed and one 600-g sample of non-medicated feed were collected on the first day of the study (Deviation 1). Samples were placed in zip-lock plastic bags and stored at the Murray Springs TH in a freezer at approximately -20°C until they were shipped to the Upper Midwest Environmental Sciences Center (UMESC; United States Geological Service (USGS), Biological Resources Division). Zip-lock plastic sample bags were labeled with name of the collector, date, and time of collection. After the final feed samples were collected, all samples were shipped

overnight in an ice-packed cooler to the UMESC. Feed samples were received at the UMESC frozen and in good condition. When feed samples were received at the UMESC, they were transferred and kept in a Revco freezer at -80°C until analyzed. Appropriate chain-of-custody forms were completed and shipped along with feed samples to ensure sample integrity.

Water quality - Water temperature and dissolved oxygen concentration were measured daily throughout the study using a YSI Model 95 DO meter according to SOP No. INST. 120.0. Water hardness and alkalinity were measured three times during the study according to procedures described in SOP No. INST 105.0 and 104.0. Water pH was not measured (Deviation 2).

Data analysis - The null hypothesis ($H_0 : u_{\text{treated}} = u_{\text{untreated}}$) tested in this study was that there was no difference in percent mean total mortality between the groups of test fish that were treated with florfenicol-medicated feed (target dosage of 10 mg florfenicol/kg of fish/d for 10 consecutive days) and the groups of test fish that were fed non-medicated feed. Mean total mortality in treated and untreated test tanks was calculated by averaging the total mortality of the four treated tanks and the four untreated tanks, respectively, during the treatment and post-treatment periods. For each test tank, total mortality was converted to percent total mortality by dividing the sum of the mortality during the treatment and post-treatment periods by the number of live fish in the tank at the start of the study. Mean percent total mortality was calculated

in the same manner as that used to determine mean total mortality. Percent total mortality data were arcsine-transformed to degrees (Zar 1984), with the transformation $P = \arcsin \sqrt{P}$. Degrees were transformed to radians by dividing resultant transformed degrees by 57.296 (Zar 1984). Transformed data were then analyzed with a two-sided t-test for independent samples ($\alpha = 0.05$). The statistical software packages used were SigmaStat 2.03 (SPSS 1997) and SYSTAT Version 8.0 (SPSS 1998).

Quality assurance and personnel - Quality assurance procedures that were followed in this study are described in the study protocol. Names of the Study Director, Study Monitor, Investigator, and all other personnel involved in the study, as well as curriculum vitae documenting qualifications, are listed in Appendix F.

Results

Mortality - Mean percent total mortality of fingerling westslope CTT in treated test tanks (1.9%) was lower than that of fingerling westslope CTT in untreated test tanks (3.2%; Tables 2a and 2b; Appendix Table G2). However, differences were not significant ($P = 0.332$). Total mortality of test fish in treated and untreated tanks ranged from 21 to 90 and from 33 to 220, respectively. Mean daily mortality in treated tanks was consistently ≤ 1 fish/d by the first day of the post-treatment period and continued to decrease to the 0.0 - 1.0 fish/d level of mortality for the duration of the post-treatment period. Mean daily mortality in untreated tanks ranged from 2.3 - 6.5 fish/d during the

first 14 d of the post-treatment period and did not reach the ≤ 1 fish/d level of mortality until day 29 of the post-treatment period (Figures 3 and 4; Appendix Table G3).

Feed assay - Feed samples were analyzed on October 18, 2001, by high pressure liquid chromatography (HPLC) according to UMESC SOP No. CAP 423.2 entitled "Determination of Florfenicol in Fish Feed." Five replicates of each of the medicated and non-medicated feed samples were analyzed. The mean florfenicol concentration of the medicated feed samples analyzed by HPLC was 0.55 g florfenicol/kg of feed (Appendix H). The mean treatment dosage was calculated to have been 9.6 mg florfenicol/kg of fish/d. Results of non-medicated feed analysis verified that no quantifiable drug concentrations were detected in feed fed to untreated test fish during the treatment period or to any fish during the post-treatment period.

Water quality - Water hardness and alkalinity were 139 mg/L CaCO₃ and 140 mg/L CaCO₃, respectively (Appendix I). Overall, water temperature in the test tanks averaged 11.1°C (± 1 SD = 0.012, n = 400; Appendix Table J1), and daily mean water temperature was constant throughout the study (Figure 5). Overall, DO concentration in the test tanks averaged 9.4 mg/L (± 1 SD = 0.229, n = 392; Appendix Table K1), and daily mean DO concentration was relatively consistent throughout the study (Figure 6).

Discussion and Conclusions

Although there was no significant difference in mortality between the two treatment groups, there is strong evidence that florfenicol-medicated feed therapy was effective in controlling mortality in test fish caused by CWD. Mean total mortality in the treated tanks over a 7-d period prior to initiating the treatment period (61 dead fish) was more than two times greater than the mean total mortality in untreated tanks (28.3 dead fish) over the same period. Not only was mortality lower among untreated tanks, but total mortality in untreated Tank 13 (12 dead fish) over this period was less than half of the mean total mortality for all untreated tanks. In hindsight, this tank should not have been included in the study because mortality data suggested that the disease level in this tank was substantially lower than the disease level in the other seven test tanks. Total mortality in two of the treated tanks (93 dead fish in Tank 4 and 90 dead fish in Tank 5) during this 7-d pre-study period was two times greater than the total mortality in test tank 3 (46 dead fish), which was one of the untreated tanks that had the highest mortality during this period. Mortality during the 7 d period prior to the onset of the treatment period indicates that the disease level was higher among the treated tanks than among the untreated tanks. In addition, mean mortality observed during the above-mentioned 7-d period prior to the start of the treatment period was similar to the mean mortality observed during the first few days of the treatment period. Mean total mortality during the first four days of the treatment period in treated tanks (0.25%/day) was nearly two times higher than the mean total mortality in the untreated tanks (0.14%/day) during the same period. However, on day 5 of the treatment period daily mortality among treated tanks began to decrease while daily mortality among untreated tanks remained consistent. Mean mortality among treated tanks during the last two

days of the treatment period (0.05%/day) was five times lower than the mean mortality in these same tanks during the first four days of the treatment period. Mean mortality in untreated tanks over the last 2 d of the treatment period (0.16%/day) was similar to the mean mortality in the untreated tanks during the first four days of the treatment period (0.14% /day) indicating no change in mortality over this period. The observed decrease in mortality in treated tanks was consistent with what has typically been observed in other trials in which fish with coldwater disease or columnaris (causative agent *F. columnare*) when treated with oral antibiotics such as oxytetracycline or florfenicol (personal communication, Dr. Joy Evered, USFWS, Olympia Fish Health Lab). Among untreated tanks there appeared to be a greater disparity in daily mortality over the course of the study than that observed in treated tanks. At the end of the study, mortality in tanks Tank 3 and Tank 15 had not been controlled while mortality in untreated tanks 13 and 16 had returned to near-zero. From this data it was apparent that florfenicol-medicated feed therapy was effective in controlling mortality caused by CWD while mortality in two of the untreated tanks had not controlled. It was suspected that had the decision regarding which test tanks to include in the study been more conservative with respect to daily mortality prior to the start of the study, differences in mortality between the treatment groups would have probably been more dramatic.

Acknowledgments

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Springs TH for assisting in this study; Jim Peterson of MT FWP for conducting fish health evaluations; and Chue Vue of the USGS UMESC for assaying feed to determine florfenicol concentrations.

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Table 1. Schedule and description of significant events for study FLOR-01-EFF-04.

Study day and description of significant event	
Pre-treatment acclimation phase (study day 1; October 2, 2001)	
First day of pre-treatment period (study day 1)	<p>Sample counted all fish in each tank to estimate number of fish per study tank.</p> <p>Calculated initial flow and density indices in test tanks.</p> <p>Randomly assigned treatment condition to each test tank.</p> <p>Began collecting daily water temperature and dissolved oxygen data.</p> <p>Collected and measured water samples for water hardness, alkalinity, and pH.</p> <p>Calculated feed amounts to be fed fish in each test tank (fish were fed at a rate of 1.75% body weight).</p> <p>Collected all samples of medicated and non-medicated feed.</p> <p>Collected fish samples for fish health and histology.</p>
Treatment phase (study days 2 - 11; October 3 - October 12, 2001)	
First day of treatment period (study day 2)	<p>Began florfenicol-medicated feed treatment.</p> <p>Collected and measured water samples for water hardness and alkalinity.</p>
Post-treatment phase (study days 12 - 25; October 13 - November 11, 2001)	
Thirtieth day of post-treatment period (study day 41)	Terminated study.

Table 2a. Mortality of fingerling westslope cutthroat recorded during the treatment and post-treatment phases of the study in treated tanks.

Number of mortalities in florfenicol-medicated feed treated test tanks ^a				
	Test tank 4	Test tank 5	Test tank 6	Test tank 9
Total mortality	90	79	57	21
Number of fish in tanks at start of study	3,276	3,144	3,197	3,360
Total number of fish in test tanks = 12,977; mean number of fish per tank = 3,244				
Total mortality = 247; mean mortality = 61.75; ±1SD = 30.434				
Mean total (%) mortality = 1.9				

^a Florfenicol-medicated feed treated group: Mean total mortality (1.9%) = $((90/3,276) + (79/3,144) + (57/3,197) + (21/3,360))/4 \times 100$

Table 2b. Mortality of fingerling westslope cutthroat recorded during the treatment and post-treatment phases of the study in untreated tanks.

Number of mortalities in untreated test tanks ^a				
	Test tank 3	Test tank 13	Test tank 15	Test tank 16
Total mortality	215	33	199	89
Number of fish in tanks at start of study	3,555	2,776	5,789	4,459
Total number of fish in test tanks = 16,579; mean number of fish per tank = 4,145				
Total mortality = 536; mean mortality = 134.0; ±1SD = 87.582				
Mean total (%) mortality = 3.2				

^b Untreated group: Mean total mortality (3.2%) = (((215/3,555) + (33/2,776) + (199/5,789) + (89/4,459))/4) x 100



Figure 1. Tank room at Murray Springs Trout Hatchery.

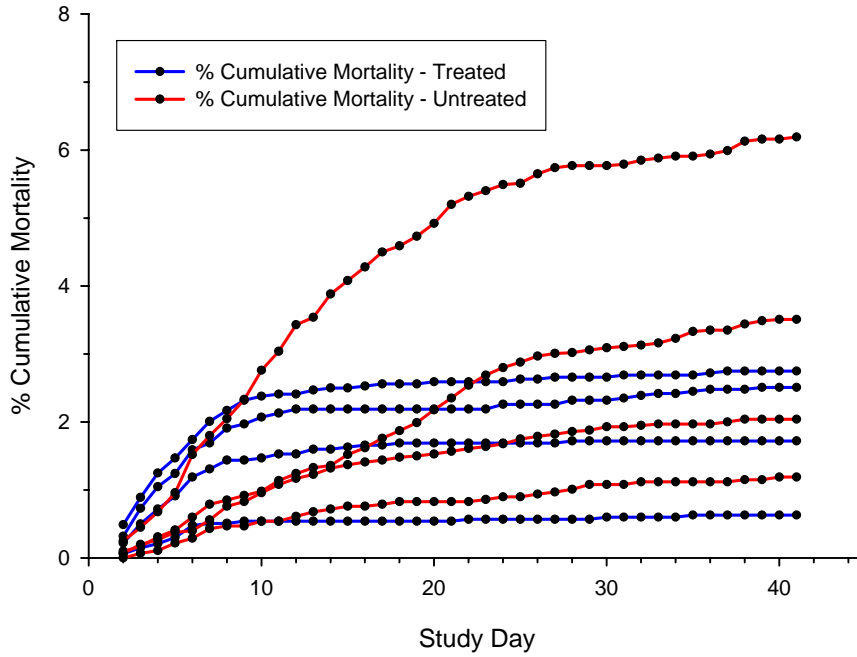


Figure 3. Percent cumulative mortality of each test tank.

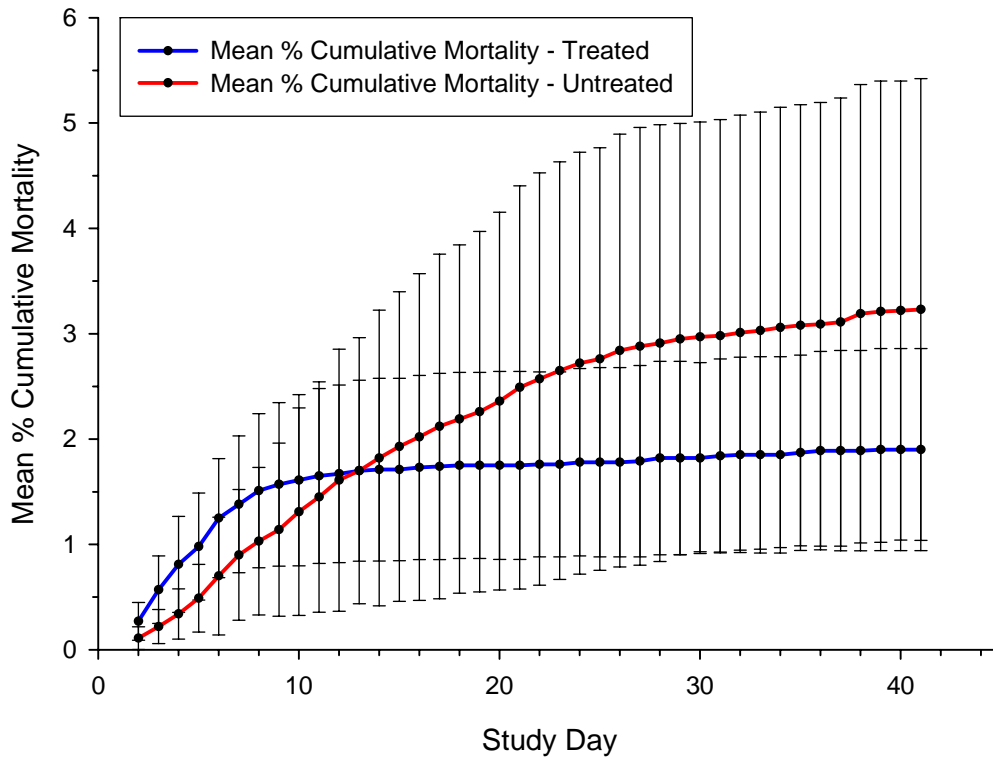


Figure 4. Mean percent cumulative mortality of 4 treated and 4 untreated test tanks in study FLOR-01-EFF-04.

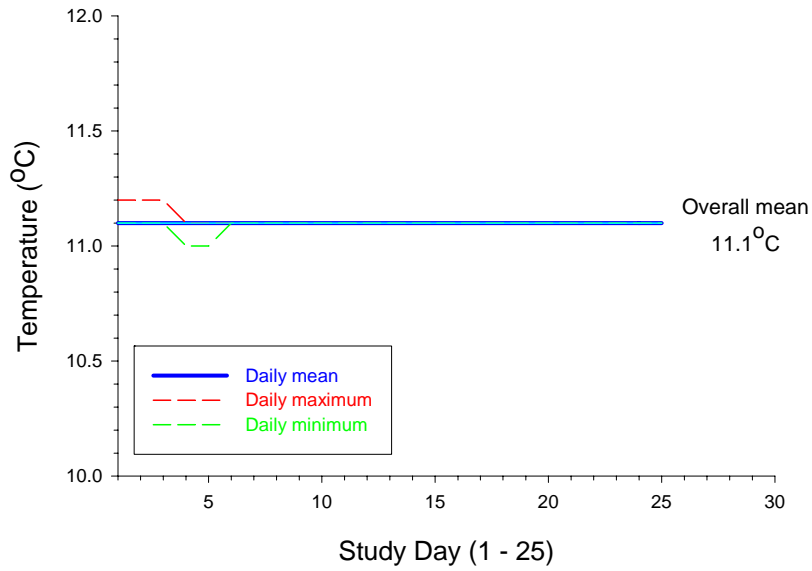


Figure 5. Daily mean, maximum, and minimum water temperatures recorded during the study.

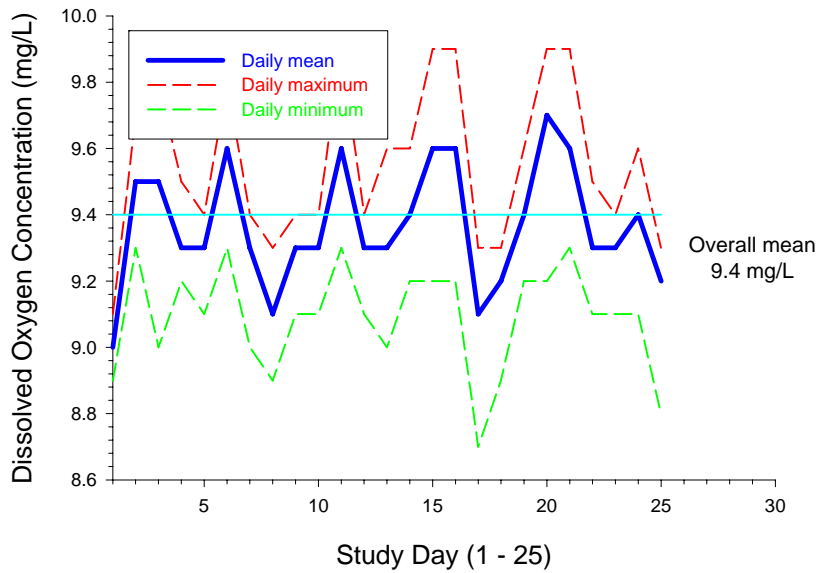


Figure 6. Daily mean, maximum, and minimum dissolved oxygen concentrations recorded during the study.

Deviations from the Study Protocol

Deviation 1. Study Protocol Section 6.5.1.1.5: Number of feed assay replicates

The study protocol states that three samples will be collected from each lot of medicated feed during the study. One medicated feed sample will be collected at the beginning of the treatment period, one during the middle of the treatment period, and one at the end of the treatment period. Also, one non-medicated feed sample will be collected at the beginning of the treatment period. However, during this study, all three medicated-feed samples and one non-medicated feed sample were collected on the first day of the study. The feed for the entire treatment period of the study was weighed out in individually labeled containers on day one of the study. Although, it was not possible for the Investigator to take feed samples at times described in the study protocol feed was sampled in a manner consistent with this procedure because all feed was weighed out on the first day (i.e., one sample was collected from the top of the bag, one sample was collected from the middle of the bag, and one sample was collected from the bottom of the bag). This deviation did not adversely affect the outcome of the study.

Deviation 2. Study Protocol Section 5.9.3 Frequency of monitoring water chemistry parameters

The study protocol states that the pH will be measured and recorded once during the study. However, pH was not measured during this study.