A CLINICAL FIELD TRIAL TO DETERMINE:

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

Study Number: FLOR-01-EFF-12

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Abstract

The U. S. 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 according to Good Clinical Practices at the Washoe Park Trout Hatchery (TH; Montana Fish, Wildlife & Parks, Anaconda, MT) in August, 2002, by staff from the NIO and Washoe Park TH following guidelines described in Study Protocol Number FLOR-01-EFF (2nd revision, revised and signed April 1, 2002; Bowker 2002). The study objective was to compare mortality between westslope cutthroat trout (CTT) fry *Oncorhynchus clarki lewisi* fed florfenicol-medicated feed and westslope CTT fry fed non-medicated feed. Fish used in the study had been diagnosed with CWD by presumptive identification of *F. psychrophilum* from fish sampled from the test population 1 d before the start of the study. A completely randomized design procedure was used to assign a treatment condition of either "treated" or "untreated" to each of 12 test tanks. Test fish in six of the 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 six test tanks were fed non-medicated feed during the same 10-d period. The study lasted 25 d and consisted of a 1-d acclimation period, a 10-d treatment period, and a 14-d post-treatment period. Following the treatment period, test fish in all 12 test tanks were fed non-medicated feed. Blinding techniques were employed to minimize

bias in data collection. Total mortality that occurred during the treatment and post-treatment periods of the study was the primary response variable. The null hypothesis tested was that mean percent total mortality of test fish in treated tanks was equal to or greater than mean percent total mortality in untreated tanks (H_o: $\mu_{treated} \geq \mu_{untreated}$). The alternative hypothesis was H_a: $\mu_{treated} < \mu_{untreated}$. Based on a one-tailed t-test for two independent samples, mean percent total mortality in the treated group (75.0%) was significantly less ($P \leq 0.001$) than mean percent total mortality in the untreated group (94.0%). The higher than expected mortality among treated test fish was considered to have been due to factors such as: (1) the suspected high virulence of the *F. psychrophilum* strain isolated from fish in this study; (2) starting the study 3 - 4 d after the detection of increased mortality and observation of clinical signs indicative of CWD; and (3) because salmonid fry are particularly susceptible to CWD (Holt 1993; Brown et al. 1997; Bader and Starliper 2002). In spite of the high mortality in both treated and untreated groups of test fish, results from this study demonstrated that florfenicol-medicated feed treatment therapy was efficacious in controlling mortality in westslope CTT fry caused by CWD.

Introduction

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 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).

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 (Study Number FLOR-01-EFF-12) 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 westslope cutthroat trout fry (CTT) *Oncorhynchus clarki lewisi* caused by bacterial coldwater disease (CWD), causative agent *Flavobacterium psychrophilum*. The study was conducted under the Pivotal Study Protocol FLOR-01-EFF (2nd revision, revised and signed April 1, 2002; Bowker 2002) according to Good Clinical Practices, and was intended to provide the FDA Center for Veterinary Medicine (CVM) with pivotal field data documenting the efficacy of the florfenicol treatment regimen.

Materials and Methods

Study location and schedule - The study was conducted at the Washoe Park Trout Hatchery (TH), Montana Fish, Wildlife & Parks (MFWP; Anaconda, MT; Appendix A). The 25-d study began on August 26, 2002, and ended on September 19, 2002. The study consisted of a 1-d pre-treatment period (August 26, 2002), a 10-d treatment period that extended from August 27 to September 5, 2002, and a post-treatment period that lasted 14 d (September 6 - 19, 2002; 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; Appendix B), which is a 50% medicated premix in a palatable base for salmon. The Aquaflor® premix was top-coated onto Silver Cup Starter Feed (Nelson & Sons Inc., Murray, UT) at the U. S. Fish and Wildlife Service's (USFWS) Bozeman Fish Technology Center (BFTC) using a Marion Laboratory Mixer (Model SPS-1224; Marion, IA) according to procedures described in SOP No. MISC 218.0 and SOP No. INST 126.0 (Appendix G). The medicated feed contained a calculated dose of 0.25 g active florfenicol per kg of feed (10 mg active florfenicol/kg fish ÷ daily feed rate of 4.0% body weight). Non-medicated Silver Cup Starter Feed was fed to untreated (i.e., positive control) test fish during the treatment period and to all test fish during the post-treatment period.

Test fish - Westslope CTT fry (Lot #M010102H; Appendix A) used in the study were progeny of broodstock kept at Washoe Park TH. Broodstock were spawned at the hatchery by hatchery personnel. Eggs were fertilized, incubated, and hatched at Washoe Park TH. Resultant fish were reared at the hatchery in a single fish culture tank at the hatchery until they were used in the study.

Study design - The study design consisted of two treatment conditions: "treated" and "untreated." Each treatment condition was replicated six times; consequently, 12 test tanks of fish were used at the start of the study. Individual test tanks were

numbered 1 - 12. Fish in six test tanks were fed florfenicol-medicated feed (i.e., treated tanks; Figure 2). Fish in the remaining six test tanks were fed non-medicated feed (i.e., untreated tanks; Figure 2).

On the first day of the study a completely randomized design procedure was used to assign fish to test tanks (Appendix D; SOP No. MISC 205.2). Based on sample counts done on the first day of the study, estimated mean weight of test fish was 0.29 g (Appendix C, Table C1). The total weight of fish in the single rearing tank was then measured (i.e., approximately 1,400 g), and the total number of fish in this tank was estimated. To attempt to achieve a nearly uniform level of disease among fish in all test tanks, fish were transferred from the tank holding the test population to the appropriate test tanks in two aliquots of 58 g of fish each (2 aliquots x 58 g of fish each = 116 g of fish total).

Determining fish numbers by sample-counting is subject to error. To more accurately determine the total number of test fish per test tank the number of test fish remaining in each test tank at the end of the study were counted and summed with the total mortality from each test tank (i.e., actual number of fish). The total actual number of fish used in the study was then divided into the total weight of the test fish population determined by sample counting on the first day of the study. Based on actual counts, a more accurate mean weight of individual fish was determined to have been 0.17 g (Appendix C, Table C2). Based on the more accurate fish weight, the estimated mean

length of the test fish was 2.6 cm, using the appropriate length-weight conversion table for CTT (condition factor 3,500 x 10^{-7}; Piper et al. 1982).

Before the study started, fish were held in one tank (dimensions 4.55 m long x 0.61 m wide x 0.36 m deep = 1.0 m^3 or 35 ft^3). Mean flow index and density index values in this tank before fish were transferred to test tanks were 0.53 and 0.09, respectively (Appendix C). Test tanks were 61-cm diameter circular fiberglass tanks that were 46 cm deep (Figures 1 and 2). Water depth and flow rate in test tanks were adjusted after test fish had been transferred to test tanks (mean number of fish/tank = 667) so that flow index and density index values in the test tanks were similar to those in the tank that had held the entire test population. With a 20-cm standpipe in place and water flow set at 1.9 Lpm (0.5 gpm; Figures 3 and 4), the rearing volume of each test tank was 0.06 m^3 (2.1 ft^3), and mean flow index and density index values in test tanks were 0.50 and 0.12, respectively (note: flow index and density index values were calculated based on actual number of fish/tank; Appendix C). Flow index and density index values in the tank holding the test population and test tanks were within the acceptable range for rearing healthy salmonids (Piper et al. 1982).

A completely randomized design procedure was used to assign treatment conditions to test tanks (Appendix D; SOP No. MISC 206.2). Based on the randomization procedure, test tanks 1, 2, 6, 7, 8, and 10 were assigned a treatment condition of “treated” and test tanks 3, 4, 5, 9, 11, and 12 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 test fish in treated tanks for 10 consecutive days. Actual dosages were determined by feed assay.

Inclusion criteria - Inclusion criteria for the study site and test fish were met before starting the study. The pathogen suspected of causing morbidity and mortality was presumptively identified as *F. psychrophilum* based on a fish health evaluation of moribund fish sampled from the tank holding the test fish population before the start of the treatment period (Appendix E). Fish mortality in the tank holding the test population increased over a 4-d period before the study was started. Based on (1) the level of mortality in the tank holding the test population, (2) detection of *F. psychrophilum* isolated from moribund fish, and (3) that the logistical criteria and personnel were in place, the study was begun.

Exclusion criteria - According to the study protocol, a test unit can be excluded from the study if a “fatal” event occurs, e.g., (1) multiple disease infections among test fish in a test tank that could affect the outcome of the study, (2) interruption of water flow for a period of time that unduly stresses test fish, or (3) a failure to replace a standpipe after cleaning, resulting in dewatering of the test tank for a period of time that unduly stresses test. No such “fatal” event occurred during this study.

Pre-study Mortality - Increased mortality in the tank holding the test population before starting the study indicated either a fish disease or fish culture-related problem.

Pre-study fish mortality in the test population increased over a 4-d period (i.e., August 21 - 24, 2002) from 25 to 321 on August 21 and 24, 2002, respectively (Appendix N). Based on the increased mortality, increased prevalence of fish showing clinical signs of CWD observed by fish health biologists and culturists, and a determination that no obvious fish culture-related problems were evident (i.e., adequate fish loading density, water flow, and dissolved oxygen), pre-study mortality and morbidity were determined to have been caused by CWD.

Fish health

Pre-study fish health evaluation - Diagnosis of the bacterial infection was based on routine bacteriological evaluation of five fish and histological evaluation of an additional four fish sampled from the test population on August 25, 2002 (1-d before to the start of the study). Routine bacteriological evaluation was done by Mr. Ken Staigmiller (Study Monitor; MFWP Fish Health Lab; Appendix E). Mr. Staigmiller was alerted by hatchery staff to the possibility of a CWD infection based on observations of increased daily mortality before the start of the study, and an increased prevalence of moribund fish exhibiting darkening of the skin pigmentation in the caudal peduncle region giving fish the appearance of a “saddleback” condition, which is a typical sign of CWD (Appendix E, Photo E1; Post 1987; Noga 2000; Bader and Starliper 2002). Mr. Staigmiller (1) microscopically examined wet mounts of skin, gills, and hindgut, (2) streaked skin, gill, brain, and “internal” organ tissues from five of five fish individually on trypticase yeast extract (TYE) to evaluate bacteria culture growth, and (3) imprinted tissues from all five fish on microscope slides for microscopic detection of bacteria

resembling *F. psychrophilum* and any other suspected fish pathogens. (Note: fish were too small to dissect and remove separate internal organs for fish health evaluation, so all exposed internal organs were used collectively when streaked on TYE or to make tissue imprints on microscope slides.) Imprinted tissues were stained with a gram stain. Cultures grown from one of the five fish were sent to the Washington Animal Disease Diagnostic Lab (WADDL; Pullman, WA) for confirmation using polymerase chain reaction (PCR). Fish sampled for histological evaluation were fixed in Davidson's solution and processed. Histological evaluation of pre-study fish, and all subsequent fish sampled for histological evaluation, were done by Ms. Beth MacConnell and Ms. Linda Staton of the USFWS Bozeman Fish Health Center (Bozeman, MT).

Fish health evaluation during treatment - One to five moribund fish were collected from each of 12 test tanks (n = 36 fish total; Appendix E, Table E1) on day 9 of the treatment period (September 4, 2002; Deviation 1). No other moribund fish were observed during this sampling event. Skin, gill, brain, and "internal" organ tissues from at least one fish per test tank (n = 17 total fish) were streaked on TYE to evaluate bacteria culture growth. Cultures grown from six fish were sent to the WADDL for confirmation that the pathogen was *F. psychrophilum*. Skin, gills, internal, or brain samples were taken in some combination from at least one fish from each tank (n = 20 fish total), imprinted on microscope slides, stained using a gram stain and evaluated under a light microscope (at 200 or 400 times magnification) to detect presence of bacteria resembling *F. psychrophilum*. In all but two cases, at least one fish from each test tank (n = 17 total fish) was fixed in Davidson's solution and processed for

histopathological evaluation. (Note: Only one fish was collected from each of tanks 2 and 8 (treated tanks), and selected tissues from these fish were streaked on TYE and imprinted on microscope slides only.) Embedded tissues were sectioned, mounted on glass microscope slides, stained with Hematoxylin and Eosin (H&E) stain or Giemsa stain, and covered with a cover slip. Tissues were evaluated for presence *F. psychrophilum* or evidence of CWD infection.

Fish health evaluation after treatment - On day 7 of the post-treatment period, three to seven moribund fish were collected from each of 12 test tanks (n = 37 fish total), fixed in Davidson's solution, and processed for histological evaluation. On day 14 of the post-treatment period, one to five moribund fish were collected from all 12 test tanks (n = 29 fish total; Deviation 2), fixed in Davidson's solution, and processed for histological evaluation. Embedded tissues were sectioned, mounted on glass microscope slides, stained with H&E stain or Giemsa stain, and covered with a cover slip. Tissues were evaluated for presence *F. psychrophilum* or of evidence of CWD infection.

Mortality - Mortality was recorded daily throughout the study. Test tanks were checked each morning for dead fish, which were netted from each tank, counted, and disposed of according to routine Washoe Park TH procedures. The number of dead fish removed from each tank was recorded on an appropriate data-collection form.

Fish behavior and appetite - General fish behavior and appetite were observed daily (Deviation 3), usually when tanks were cleaned and fish fed. General behavior was characterized and recorded as “normal” or “abnormal.” Normal behavior was assigned a value of “1," and abnormal behavior was assigned a value of “0.” If abnormal behavior was observed, then a description of that behavior was documented. Appetite was characterized and recorded as “good,” “fair,” or “poor,” depending on the amount of feed remaining on the bottom of each tank after feeding. “Good” meant that little or no feed remained in the tank after feeding; such an observation was assigned a value of “2.” “Fair” meant that approximately one-half of the feed remained in the tank after feeding; such an observation was assigned a value of “1.” “Poor” meant that nearly all of the feed remained in the tank after feeding; such an observation was assigned a value of “0.” Evaluation of the amount of feed remaining at the bottom of each test tank, which was determined to have been the total amount of uneaten feed (i.e., no feed was flushed from test tanks at times other than when tanks were cleaned), was usually made before feed was administered each day and indicated how much feed had been eaten during the previous day.

Feed and feeding rate - Test fish were fed Silver Cup Starter Feed (Nelson & Sons Inc., Murray, UT), which is a standard, commercial salmonid feed. The test fish were fed daily at a rate of 4.0% of their estimated body weight (i.e., 116 g fish/test tank). A copy of the feed label listing percent protein, fat, fiber, moisture, ash, and selected nutrients is in Appendix F. Amount of feed administered to each test tank was calculated on the first day of the study (i.e., 116 g x 4.0% = 4.6 g feed/tank/d). On the

first day of the study, daily feed rations for the entire treatment period were weighed out by a non-blinded study participant into containers labeled so that the individual(s) administering feed to the test tanks did not know which tanks received medicated feed and which tanks received non-medicated feed. Because of a staff shortage at the Washoe Park TH (i.e., all staff were blinded study participants) feed rations were weighed out for the 10-d treatment period in this manner. Feed amounts were adjusted from 4.6 g/d/tank to 1.0 g/d/tank on day 6 of the post-treatment period.

Feed samples and feed assay - Feed samples were collected to be assayed for florfenicol and oxytetracycline on August 26, 2002, using the following procedure: Three 200-g samples of medicated feed and one 600-g sample of non-medicated feed were collected on the first day of the treatment period by a non-blinded study participant. Samples were placed in zip-lock plastic bags. Zip-lock plastic bags containing the feed samples were labeled with name of the collector, date, and time of collection and stored at the Washoe Park TH in a freezer at approximately -20^{o}C until they were shipped to the Upper Midwest Environmental Sciences Center (UMESC; United States Geological Service (USGS), Biological Resources Division, La Crosse, WI). Feed samples were shipped overnight in an ice-packed cooler to the UMESC. Feed samples were received at the UMESC frozen and in good condition and placed in a Revco freezer at -80^{o}C until analyzed. Appropriate chain-of-custody forms were completed and shipped along with feed samples to ensure sample integrity.

Feed samples were assayed for florfenicol on September 19, 2002, and for oxytetracycline on September 24, 2002 (Appendix G). All feed samples were analyzed for florfenicol using UMESC SOP No. CAP 423.2 (entitled "Determination of florfenicol in fish feed") and for oxytetracycline using UMESC SOP No. CAP 416.3 (entitled "Determination of oxytetracycline in fish feed"). (Note: SOP No. CAP 416.3 is in Appendix G; SOP No. CAP 423.2 is proprietary and is available to CVM only with authorization from Schering-Plough Animal Health, Division of Schering Canada, Inc.)

Water quality - Water temperature and dissolved oxygen (DO) concentration were measured at least once daily throughout the study with a YSI Model 95 DO meter (YSI Inc, Yellowsprings OH) according to SOP No. INST 120.0. Data were recorded on appropriate data collection forms. Water hardness, alkalinity, and pH were measured two times during the study according to procedures described in SOPs No. INST 105.0, 104.0, and 125.0, respectively (Appendix K).

Data analysis - The null hypothesis tested in this study was that mean percent total mortality of test fish in treated tanks was equal to or greater than mean percent total mortality of test fish in untreated tanks (H_o : $u_{\text{treated}} \geq u_{\text{untreated}}$). The alternative hypothesis tested was that mean percent total mortality of test fish in treated tanks was less than the mean percent total mortality of test fish in the untreated tanks (H_a : $u_{\text{treated}} < u_{\text{untreated}}$). For each test tank, total mortality was converted to percent total mortality by dividing the total mortality by the actual number of fish in the tank at the start of the study. Mean percent total mortality in treated test tanks was calculated by determining

the percent total mortality in each treated test tank, summing the percent total mortality for treated test tanks, and dividing by the number of treated test tanks used in the study. Mean percent total mortality in untreated test tanks was calculated by determining the percent total mortality in each untreated test tank, summing the percent total mortality for untreated test tanks, and dividing by the number of untreated test tanks used in the study. Percent total mortality data were arcsine-transformed to radians (Zar 1984) with the transformation $P = arcsin\sqrt{P}$. Transformed percent data were analyzed with a one-tailed t-test for two independent samples ($\alpha = 0.05$; Deviations 4 and 5). The statistical software packages used were SigmaStat 2.03 (SPSS 1997) and SYSTAT Version 10.2.01 (SPSS 2002).

Quality assurance and personnel - Quality assurance procedures followed in this study were in compliance with CVM's Guidance for Industry Good Laboratory Practice (Final Guidance VICH GL9; USFDA, 2001). Names of the Study Director, Study Monitor, Investigator, and all other personnel involved in the study, as well as curriculum vitaes documenting qualifications, are listed in Appendix H.

Results

Mortality - Mean percent total mortality of westslope CTT fry in treated test tanks (75.0%) was significantly lower ($P \leq 0.001$) than that of westslope CTT fry in untreated test tanks (94.0%; Tables 2a and 2b; Appendix Table I2). During the treatment period,

mean mortality in treated and untreated tanks ranged from 11.5 to 69.0 and 24.2 to 55.8 fish/d, respectively. During the post-treatment period, mean mortality in treated and untreated tanks ranged from 9.5 to 30.2 and 4.3 to 42.5 fish/d, respectively (Appendix Table I3). Mean percent cumulative mortality in the treated and untreated test tanks were nearly identical during the first 7 - 8 d of the treatment period. However, from day 9 of the treatment period through the end of the study, mean percent cumulative mortality in treated test tanks was consistently less than that in untreated tanks (Appendix Table I3; Figure 5).

Fish health

Pre-study fish health evaluation -Presumptive diagnosis of CWD was made based on gram-negative bacteria exhibiting typical *F. psychrophilum* morphology which were found extensively on the skin, gills, and/or internally in all five fish samples. *F. psychrophilum* were detected on gill or skin of all five fish. A systemic infection of *F. psychrophilum* was detected in three of the five fish based on presumptive identification of cultures that grew on TYE streaked with brain and collective internal organ tissue (Appendix E, pgs E3 - E6). Cultures from the fish sent to the WADDL and analyzed using PCR were positively confirmed as *F. psychrophilum*. No other bacteria were observed from four of the fish samples. However, an *Acinetobacter sp.* was isolated from one of the five fish sampled for bacteriology. *Acinetobacter sp.* was most likely a secondary infection because it was not observed on stained slides. Of the fish collected for histological evaluation, all showed clinical signs of CWD, and *F. psychrophilum*

bacteria were observed in three of the four fish evaluated. No parasites were observed on wet mounts of skin, gill, and hindgut. Pre-study fish health evaluation results indicated that fish morbidity and mortality were caused by CWD.

Fish health evaluation during treatment - Cultures of *F. psychrophilum* grew on TYE streaked with skin, gill, brain, and collective internal organ tissue from 14 of 17 moribund fish collected for bacteriological evaluation on treatment day 9. Bacteria cultures from four of six fish sent to the WADDL were positively identified as *F. psychrophilum*. In addition, low levels of *Acinetobacter sp* were detected in cultures from two of six fish sent to the WADDL. Bacteria presumptively identified as *F. psychrophilum* were observed on 19 of 20 gram-stained smears of skin samples and 11 of 18 gram-stained internal- or spleen-tissue smear samples. Mr. Staigmiller's fish health evaluation summary during this sampling period is as follows:

> Externally, fish showed a very high rate (90%) of infection with *F. psychrophilum* as determined by bacterial culture and by visual observation of gram-stained slides. Internally, about 63% of fish examined were determined to have been infected with the bacteria. Bacterial coldwater disease caused by *F. psychrophilum* appears to have been the primary cause of mortality in this group of fish. *Acinetobacter sp.* was also observed and/or cultured in about 30% of the fish examined. This bacteria was predominantly an external infection and appears to have been a secondary factor. Other pathogens that were screened for but not

observed and have been ruled out as factors include: viruses, *Aeromonas salmonicida*, *Yersinia ruckeri*, and parasites.

Of the fish collected for histological evaluation during the treatment period, all showed clinical signs of CWD, and *F. psychrophilum* bacteria were observed in 9 of the 17 fish evaluated. Results indicated that morbidity and mortality were caused by CWD.

Fish health evaluation after treatment - All moribund fish collected and evaluated on day 7 of the post-treatment period showed clinical signs of CWD. *F. psychrophilum* bacteria were observed in 20 of the 34 fish evaluated histologically. Of the fish collected and evaluated on day 14 of the post-treatment period, 24 of 27 moribund fish showed clinical signs of CWD, and *F. psychrophilum* bacteria were observed in 18 of the 27 fish evaluated histologically.

Fish behavior and appetite - General fish behavior almost always appeared normal throughout the study (Appendix Table J1). The overall mean fish behavior value for all tanks throughout the study, using the scale described in the methods, was 1.0 (Appendix J1) indicating that overall observed general fish behavior was normal. The mean appetite value for all tanks throughout the study, based on the ordinal scale used, was 1.1 (Appendix Table J2). This result indicated that approximately just more than half of the feed remained in the tanks after feeding. One reason that may explain why fish were apparently not consuming the entire ration of feed may have been that salmonid fry are typically fed to satiation at feeding rates of 4 - 5% body weight to

encourage fish to accept hand administered feed. As fish grow and become accustom to hand-feeding, feed rates are typically reduced to 1 - 2% body weight. In addition, hatchery-reared CTT are known to be relatively selective about consuming hand-delivered feed, and typically do not take food immediately from the surface as is common with other species of salmonids (M. Sweeney, MFWP, Washoe Park TH, personal communication). Another reason that may explain why fish were apparently not consuming the entire ration of feed was that feed amounts were not adjusted to compensate for mortality during the first 16 d of the study. It's likely that both were factors resulting in so much uneaten feed observed at the bottom of each test tank.

Feed samples and feed assay - Three medicated feed samples and one non-medicated feed sample were analyzed for detectable concentrations of florfenicol and oxytetracycline. The overall mean florfenicol concentration of the medicated feed samples analyzed by HPLC was 0.244 g florfenicol/kg of feed (Appendix G, Table G1, pg G3). Thus, the mean treatment dosage was calculated to have been 9.7 mg florfenicol/kg of fish/d for 10 consecutive days (Appendix G, Table G1). This assayed dosage was 3% less than the calculated target dosage, but is likely to be within the limits acceptable to FDA. Florfenicol was not detected in the non-medicated feed. Oxytetracycline was not detected in either medicated or non-medicated feed samples (Appendix G, Table G1, pg G3).

Water quality - Mean water hardness, alkalinity, and pH were 201 mg/L (as $CaCO_3$), 142 mg/L (as $CaCO_3$), and 7.78 respectively (Appendix K). Overall, mean

water temperature in the test tanks was 9.3°C (±1SD = 0.280, n = 564; Appendix Table L1). Daily mean water temperatures were relatively consistent from day-to-day and from test tank to test tank (Figure 6). Overall, mean DO concentration in the test tanks was 9.2 mg/L (±1SD = 0.331, n = 564; Appendix Table M1). Daily mean DO concentrations were relatively consistent from day-to-day and from test tank to test tank (Figure 7). All water quality variables measured were suitable for rearing healthy salmonids (Piper et al. 1982).

Discussion

Bacterial coldwater disease causes significant mortality among cultured salmonid fishes (Bader and Starliper 2002) and is present among wild fish populations when conditions are conducive for the invasion of *F. psychrophilum* (Post 1987). Generally, the disease occurs in cold-water fishes at temperatures below 12 - 13°C and is most prevalent below 10°C (Bader and Starliper 2002). Bacterial coldwater disease affects salmonids ranging in age from yolk-sac fry to yearlings; usually the younger the fish, the more severe the disease (Leek 1987). Mortality in yolk-sac fry often ranges from 30 to 50%, and as fish begin feeding, mortality is usually in the 20% range (Plumb 1999). Mortality can be even higher than 20%, as shown in a study conducted by Schmidtke and Carson (1995) in which mortality in Atlantic salmon *Salmo salar* diagnosed with CWD reached as high as 80%.

Mortality of hatchery-reared fish infected with CWD can result from a number of factors, including malnutrition, presence of toxic substances in the water, and physiological imbalances (Post 1987). However, water temperature and bacterial strain virulence are the most important factors determining disease severity (Noga 2000). Experimentally, Holt (1993) showed that *F. psychrophilum* virulence varies with bacterial strain. In Holt's study, mortality of coho salmon *O. kisutch* infected with different strains of *F. psychrophilum* varied from 0 to 100%. Although tests to evaluate the virulence of the *F. psychrophilum* strain isolated in our study were not conducted, it was suspected that the bacterial strain was particularly virulent based on total mortality and the prevalence of moribund fish during the 24-d treatment and post-treatment periods.

Post (1983) noted for CWD that not only does significant mortality occur when temperature of the rearing water is below 10^{o}C, but also that there is low physiological resistance of young fish to diseases in general. This is typically the case with a salmonid fry because its immune system has not yet fully developed (J. Evered, DVM, USFWS, Olympia Fish Health Center, Olympia WA, personal communication). As a salmonid fry grows to the fingerling stage, its immune system becomes more developed, resulting in lower cumulative mortality among older fish infected with bacterial agents.

Salmonid fry may also be particularly susceptible to high CWD-induced mortality because of the development of the many skin micro-lesions characteristic of CWD (V. Ostland, Ph. D., Kent SeaTech Corp., Mecca, CA, personal communication).

Apparently, the micro-lesions allow an increased passive osmotic exchange of internal body fluids and the external water medium. Vital body fluids may either “leak” out, or water may “leak” in. Either scenario results in impaired fish health.

Studies that have been conducted under Protocol FLOR-01-EFF on fingerling salmonids (which have more fully developed immune systems; i.e., studies FLOR-01-EFF-03, FLOR-01-EFF-04, and FLOR-01-EFF-06), resulted in lower mortality, indicating that the test fish, particularly those in untreated tanks, were better able to withstand the effects of CWD than were fry. Hence, the daily cumulative mortality from these studies showed a chronic, low-level mortality pattern that is more typical of CWD.

In the current study, another factor that may have contributed to the higher-than-expected mortality was that the start of therapeutic treatment was delayed for several days after CWD was initially diagnosed. Based on the sudden onset of mortality of pre-study test fish and the prevalence of moribund fish observed with clinical signs characteristic of CWD, it would have been better to start the study several days before we did.

In spite of these factors (i.e., bacterial virulence, susceptibility of salmonid fry to bacterial diseases, delay in starting the study), florfenicol-medicated feed therapy controlled mortality caused by CWD in westslope CTT more effectively than withholding medicated feed. As in other studies conducted under Protocol FLOR-01-EFF (i.e., studies FLOR-01-EFF-03 and FLOR-01-EFF-06) mean percent cumulative mortality

seen in the current study was nearly identical in treated and untreated test tanks during the first few days of the treatment period. Such mortality in the treated tanks began to decrease on day 7 of the treatment period relative to the mean percent cumulative mortality in the untreated tanks. It has been the experience of trained fish health biologists and fish culturists that evidence of efficacy becomes apparent after 4 - 5 d of oral antibiotic treatment (J. Evered, DVM, USFWS, Olympia Fish Health Center, personal communication). Based on the mean percent cumulative mortality pattern of fish in the treated and untreated test tanks, treatment efficacy became noticeable on day 7 of the treatment period, and this pattern continued throughout the remainder of the treatment period and the post-treatment period. As a result, mean percent total mortality of fish fed florfenicol-medicated feed was lower than that of fish fed non-medicated feed.

Conclusions

Results from this study showed that florfenicol-medicated feed, administered at a dosage of 10 mg/kg of fish/d for 10 consecutive days, was effective in controlling mortality of CCT caused by CWD. Evidence to support this conclusion is as follows: (1) mean percent total mortality was significantly less in treated tanks than in untreated tanks; (2) fish health evaluations indicated that morbidity and mortality were caused by *F. psychrophilum*; (3) fish behavior and appetite were mostly normal; and (4) water quality measurements were suitable for rearing healthy salmonids. Test fish mortality

was primarily attributed to CWD, and control of mortality was attributed to treatment with florfenicol-medicated feed.

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Deviations from the Study Protocol

Deviation 1. **Study Protocol Section 5.12: Provisions for necropsy and disposal of expired test subjects**

The study protocol states that no more than five moribund fish will be sampled from each test tank at some point during days 6 - 10 of the treatment period. It was anticipated that during this period, moribund fish would not be observed in treated tanks and that five moribund fish would be readily observed and sampled from each untreated tank. However, because of the nature of the disease diagnosed in this study (i.e., coldwater disease), fewer moribund fish were detected and sampled during this period than anticipated. On day 9 of the treatment period, a total of 16 moribund fish were collected from the treated tanks and 20 moribund fish were collected from the untreated tanks. Although fewer moribund fish were collected during this fish health sampling event than anticipated, we believe a sufficient number of fish were collected and evaluated to make an accurate fish health assessment . As a result, this deviation did not adversely affect the outcome of the study.

Deviation 2. **Study Protocol Section 5.12: Provisions for necropsy and disposal of expired test subjects**

The study protocol states that no more than five moribund fish will be sampled from each test tank at some point during the post-treatment period. It was anticipated that during this period, moribund fish would not be observed in treated tanks and that five moribund fish would be readily observed and sampled from each untreated tank. However, because of the nature of the disease diagnosed in this study (i.e., coldwater disease), fewer moribund fish were detected and sampled during this period than anticipated. On days 7 and 14 of the post-treatment period, at least 5 fish were sampled from each treated tank (n = 36 total fish) but at least 5 fish were sampled from only 3 of 6 untreated tanks (n = 30 total fish). Although fewer moribund fish were collected during the two post-treatment fish health sampling events than anticipated, we believe a sufficient number of fish were collected and evaluated to make an accurate fish health assessment . As a result, this deviation did not adversely affect the outcome of the study.

Deviation 3. **Study Protocol Section 6.3: Adverse reactions:**

The study protocol states that general fish behavioral data such as fish activity and appetite or fish feeding behavior will be collected on appropriate data collection forms. Although not explicitly stated, it was assumed that general fish behavior and appetite would be collected daily. During the study, general fish behavior and appetite data were not observed and recorded for any tank on day 24 of the study. Failure to collect such data on this day did not adversely affect the outcome of the study.

Deviation 4. **Study Protocol Section 7.3.1 and 7.3.2: Null hypothesis and Alternative (research) hypothesis:**

The study protocol states that the null and alternative hypotheses would be H_o: $\mu_1 = \mu_2$ and H_a: $\mu_1 \leq \mu_2$, respectively (μ_1 = treated; μ_2 = untreated). However, the null and alternative hypotheses were based on using a two-sample, two-tailed t-test. Before the study started we anticipated that mean total mortality in the treated tanks would be lower than mean total mortality in the untreated tanks. Because of this *a priori* comparison reasoning, we believed it was more appropriate to analyzed data using a one-tailed t-test for independent samples. Therefore, the null and alternate hypothesis were amended to H_o: $\mu_1 \geq \mu_2$ and H_a: $\mu_1 < \mu_2$, respectively. Using a one-tailed t-test at the $\propto = 0.05$ level instead of a two-tailed t-test at the same level resulted in detecting a significant difference in the mean percent total mortality (arc-sine transformed) between treated and untreated tanks. Differences would not have been significant using a two-tailed t-test. We believe the one-tailed t-test is an appropriate test to use, and as a result, the statistical test used in this study did not adversely affect the outcome of the study.

Deviation 5. **Study Protocol Section 7.3.4: Biostatistical procedures used:**

The study protocol states that mortality data will be analyzed by logistic regression or general linear mixed model using fish nested within tank with treatment as a fixed effect and tank (treatment) as a random effect. In this study, data were analyzed using a two-sample, one-tailed t-test. We would like to analyze data as described in the study protocol and are in the process of learning these procedures. However, we are not yet at a point where we feel we can analyze such data correctly. Consequently, a test (two-sample, one-tailed t-test) was used that we are comfortable with and confident using. The test used to analyze data in this study did not adversely affect the outcome of the study.

Table 1. Schedule and description of significant events for study FLOR-01-EFF-12.

Study day and description of significant event	
Pre-treatment acclimation phase (study day 1; August 26, 2002)	
First day of pre-treatment period (study day 1)	Set water inflow to each test tank (gpm). Calculated initial flow and density indices in test tanks. Randomly assigned treatment condition to each test tank. Began collecting daily water temperature and dissolved oxygen data. Calculated feed amounts to be fed to fish in each test tank (fish were fed at a rate of 4.0% body weight per day). Collected water samples for hardness, alkalinity, and pH. Collected medicated feed samples and non-medicated feed samples. Weighed out feed for entire treatment period.
Treatment phase (study days 2 - 11; August 27 - September 5, 2002)	
First day of treatment period (study day 2)	Began florfenicol-medicated feed treatment.
Ninth day of treatment period (study day 10)	Collected fish for fish necropsy and histology samples. Collected water samples for hardness, alkalinity, and pH.
Post-treatment phase (study days 12 - 25; September 6 - 19, 2002)	
Seventh day of post-treatment period (study day 18)	Collected fish for histology samples.
Fourteenth day of post-treatment period (study day 25)	Collected fish for histology samples. Terminated study.

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

Number of mortalities recorded in florfenicol-medicated feed treated test tanks[a]						
	Test tank 1	**Test tank 2**	**Test tank 6**	**Test tank 7**	**Test tank 8**	**Test tank 10**
Total mortality	459	528	529	523	515	536
Number of fish in each test tank at start of study	664	693	692	699	697	682
Percent total mortality	69.0	76.0	76.0	75.0	74.0	79.0
Total number of fish counted into test tanks = 4,127; mean number of fish per tank = 688						
Total mortality = 3,090; mean mortality = 515; ±1SD = 28.305						
Mean total (%) mortality = 75.0						

[a] Florfenicol-medicated feed treated group: Mean total mortality (75.0) = (((459/664) + (528/693) + (529/692) + (523/699) + (515/697) + (536/682))/6) x 100

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

Number of mortalities recorded in untreated test tanks[b]						
	Test tank 3	**Test tank 4**	**Test tank 5**	**Test tank 9**	**Test tank 11**	**Test tank 12**
Total mortality	633	598	655	588	550	608
Number of fish in each test tank at start of study	660	645	705	631	600	640
Percent total mortality	96.0	93.0	93.0	93.0	92.0	95.0
Total number of fish counted into test tanks = 3,881; mean number of fish per tank = 647						
Total mortality = 3,632; mean mortality = 605; ±1SD = 36.484						
Mean total (%) mortality = 94.0						

[b] Untreated group: Mean total mortality (94.0) = (((633/660) + (598/645) + (655/705) + (588/631) + (550/600) + (608/640))/6) x 100

Figure 1. Photograph of the study tanks (tanks numbered 1 - 12) that were used in study FLOR-01-EFF-12.

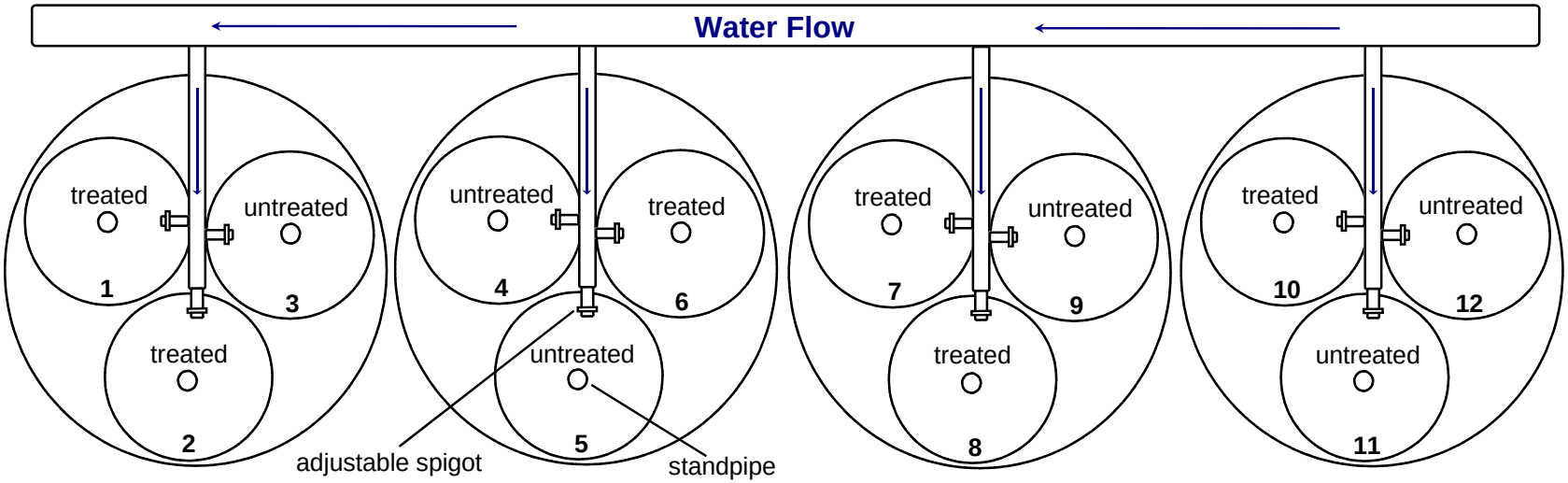
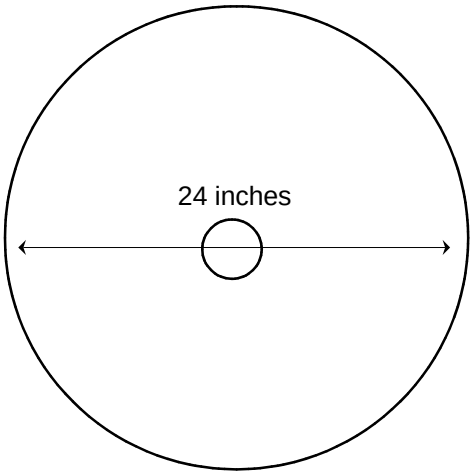


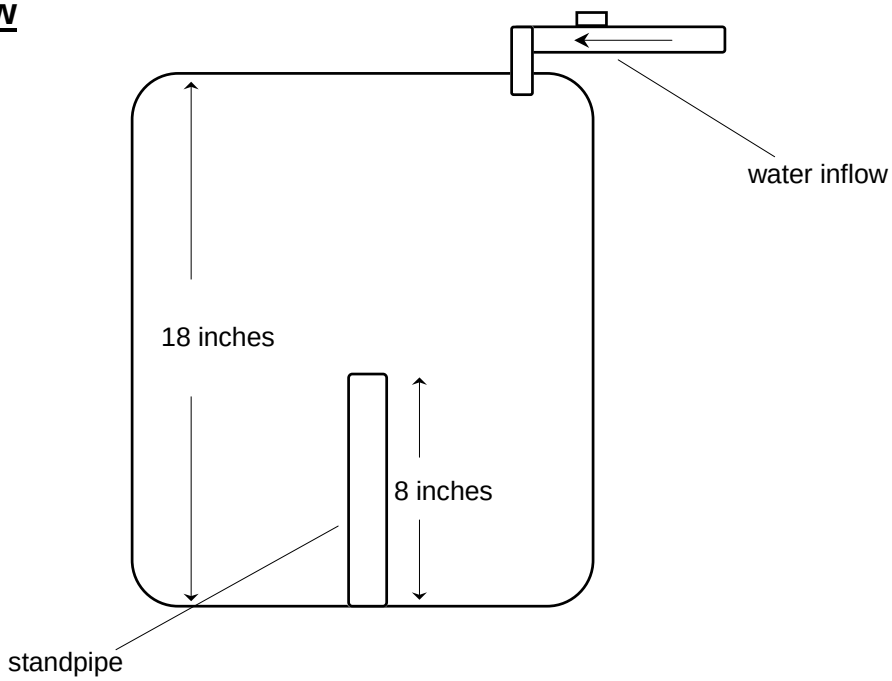
Figure 2. Study tanks 1 - 12 showing water inflow, outflow standpipes, adjustable spigots, tank numbers, and treatment conditions.

Figure 3. Photograph of an individual study tank used at Washoe Park TH showing standpipe, adjustable spigot, and westslope cutthroat trout fry.

Top View



Side View



Rearing Volume = 2.09 ft^3

Figure 4. Diagram of test tank used in study (FLOR-01-EFF-12) showing dimensions.

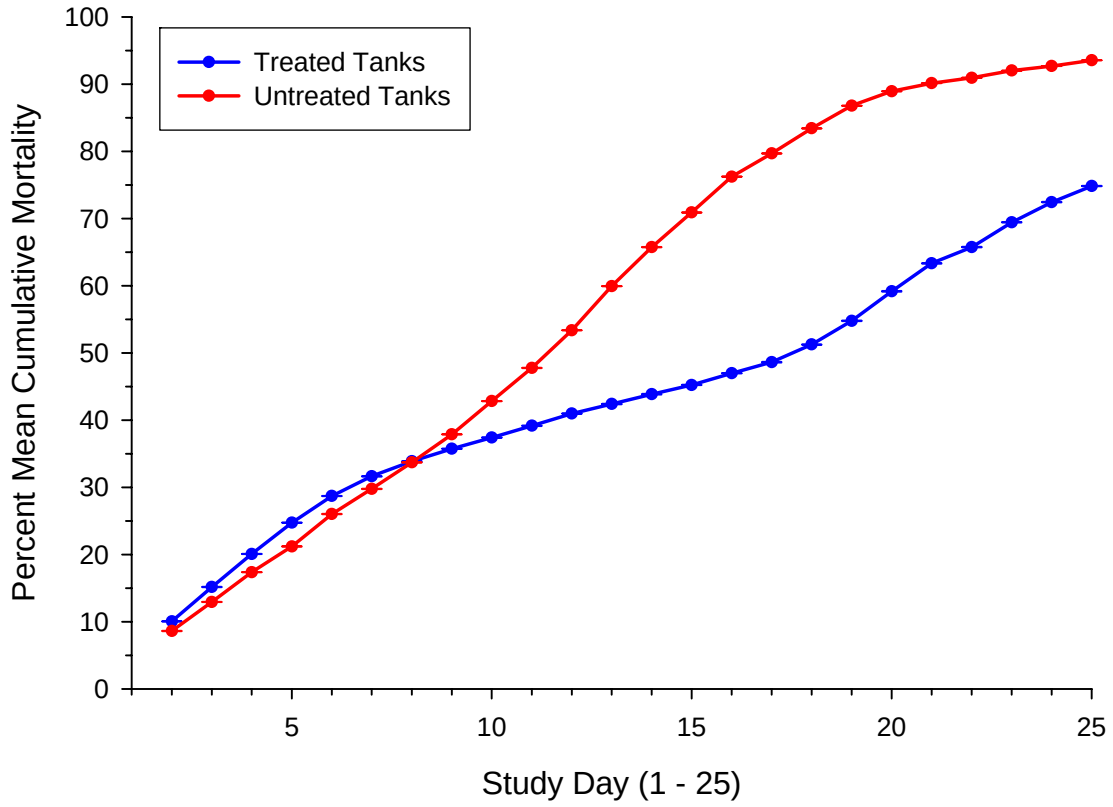


Figure 5. Percent mean cumulative mortality of six treated and six untreated tanks of westslope cutthroat trout at Washoe Park TH (FLOR-01-EFF-12).
Error bars = ±1SD

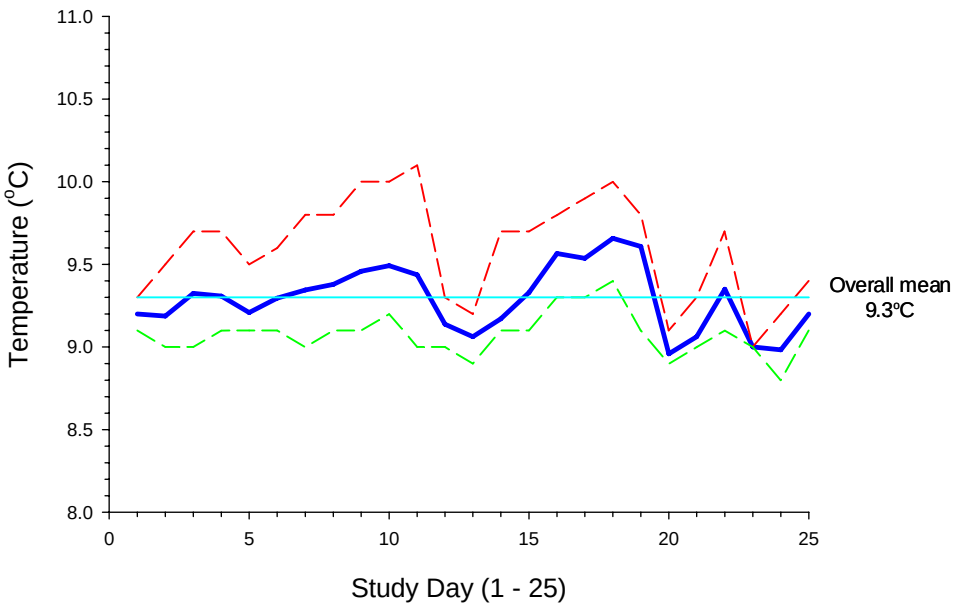


Figure 6. Daily mean, maximum, and minimum water temperatures recorded during the experiment.

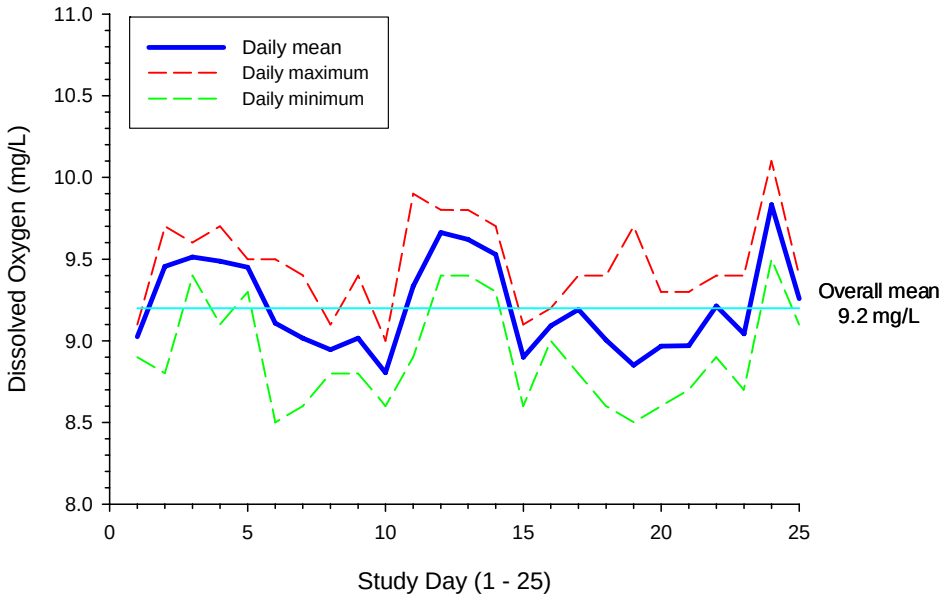


Figure 7. Daily mean, maximum, and minimum dissolved oxygen concentrations recorded during the experiment.