A CLINICAL FIELD TRIAL TO DETERMINE:

The Efficacy of Chloramine-T to Control Mortality of Walleye *Sander vitreus* Caused by Columnaris, Causative Agent *Flavobacterium columnare*

Study Number: CHLT-96-EFF-07

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Study start date: June 6, 2006

Study end date: June 22, 2006

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Abstract

The U.S. Fish and Wildlife Service's (USFWS) Aquatic Animal Drug Approval Partnership (AADAP) program designed and conducted a pivotal efficacy study to generate data needed to help obtain U.S. Food and Drug Administration approval for the use of chloramine-T (CL-T) to control mortality in hatchery-reared freshwater fish diagnosed with external columnaris (causative agent, *Flavobacterium columnare*). The study was conducted under a CVM-accepted research study protocol in accordance with Good Clinical Practices at the Iowa Department of Natural Resource's Rathbun Fish Culture Research Facility (RFCRF; Moravia, IA) in June, 2006, by Alan Johnson and RFCRF staff. The test fish used in the study were walleye (WAE) *Sander vitreus* fingerlings drawn from a reference population that had been presumptively diagnosed with external columnaris before the study started. Test fish used in the study were considered "very sick" as evidenced by the high level of pre-study mortality and by the number of fish in the reference population showing clinical signs of columnaris disease (i.e., saddleback lesions). To compound matters, the start of the treatment phase of the study was delayed for several days due to the long holiday weekend. The study's null hypothesis was H_o: $\mu_{treated} \geq \mu_{untreated}$ (i.e., mean percent total mortality of WAE treated with CL-T at a target dosage of 20 mg/L for 60 min daily in a static bath on three alternate days (i.e., "treated" fish) was equal to or greater than that of WAE not treated with CL-T (i.e., "untreated" fish). The study's alternative hypothesis was H_a: $\mu_{treated} < \mu_{untreated}$. To test the null hypothesis, a completely randomized block design (CRBD) was used to allocate four replicates of each of the two treatment conditions (treated vs.

untreated). During the study, blinding techniques were employed to minimize data-collection bias. The study lasted 17 d and comprised a 1-d pre-treatment (i.e., acclimation) period, a 5-d treatment period during which CL-T was administered three times on alternate days, and an 11-d post-treatment period. Fish were fed a commercial walleye diet daily except on treatment days. Results from a t-test performed at the end of the 17-d study revealed that there was not a significant difference (P = 0.154) between mean percent total mortality of fish in the treated test tanks ($\bar{x}$ = 79%) and in the untreated test tanks ($\bar{x}$ = 83%). However, results from a t-test performed on day 10 (4-d post-treatment) revealed mean percent mortality of fish in treated test tanks ($\bar{x}$ = 75.5%) was significantly lower (P = 0.035) than that in the untreated test tanks ($\bar{x}$ = 82.0%). Based on analysis of mortality data, chloramine-T treatment was efficacious, but several factors, including delaying start of treatment, confounded results so that differences in mortality between treated and untreated test tanks at the end of the study were not significant. Although this factor would have also affected test fish in untreated tanks, the relative mortality was likely lower in untreated tanks due to the fact that there were fewer fish (remaining during the latter part of the study) in these test tanks than in treated tanks and resultant culture conditions in untreated test tanks were “better” than in treated test tanks. Thus, our interpretation of the mortality data indicates that the CL-T treatment was effective in controlling mortality in treated test fish in spite of the relatively high degree of incidence and severity of the disease and the delay in administering treatments, and that the post-treatment mortality was likely a reinfection caused by the continual presence of *F. columnare* in RFCRF water supply (and possibly in remaining fish with systemic infections?). Therefore,

mortality results from this study—combined with (a) fish health data collected during the study, and (b) the fact that overall mean dose-verification showed that CL-T administered to treated tanks was within 9% of the target dose—support the claim that CL-T is effective in controlling mortality in WAE caused by external columnaris.

Introduction

Bacterial gill disease (BGD) is one of the most common diseases of hatchery-reared salmonids (Bullock et al. 1991) and causes more hatchery fish losses than any other bacterial disease (Bills et al. 1988). It was once thought that no single organism was responsible for BGD; however, it is now generally accepted that filamentous, yellow-pigmented bacteria are probably the cause of the disease (Wakabayashi et al. 1980; Farkas 1985). Although bacterial isolates causing BGD in fish in Japan, Hungary, and the USA (Oregon) have been compared and shown to constitute a new species, *Flavobacterium branchiophilum* (Wakabayashi et al. 1989), many fish health professionals still regard the disease to be of mixed etiology (Post 1987). As such, external columnaris (causative agent, *F. columnare*) of the gills is often considered as a form of BGD.

Columnaris disease is an acute-to-chronic bacterial infection that has been reported to cause mortality in several species of cultured salmonids, catfish, bait minnows, goldfish, basses, and sunfish (Post 1987). Anderson and Conroy (1969) listed 36 species of fish for which columnaris disease has been described. However, all

freshwater fishes are probably susceptible to columnaris under environmental conditions favorable to the bacterium and stressful to the fish. Columnaris outbreaks and epizootics can and do occur at water temperatures $\geq 15^{\circ}C$ (Post 1987), although the optimum temperature for columnaris disease is near 28 - 30°C (Post 1987). There is usually no indication of columnaris disease at water temperatures < 10°C.

During a columnaris outbreak, bacterial growth first invades the skin of the head region, including the mouth, lips, cheeks, and gills (Post 1987). Subsequently, bacterial growth on the skin, fins, and gills results in the "shedding" of many organisms into the water, thus creating a self-perpetuating infection in the affected population (Post 1987). Infected fish release large numbers of bacteria which infect other hosts. These hosts then begin to release more pathogens, which if left untreated, will increase the severity of infections on the present hosts or attack new hosts until every member of the population has the disease (Post 1987). Morbidity for columnaris disease in overcrowded or unsanitary conditions may reach 100% of the fishes. Mortalities under the same unsatisfactory conditions may reach 70% or higher among young and most susceptible fishes (Post 1987). Columnaris disease can be presumptively diagnosed from disease signs of the skin and gills of the host and from squash preparations made from scrapings of the affected areas or the gills. Presence of long, slender, possibly filamentous, rod-shaped, gram-negative bacteria assists in presumptive diagnosis (Post 1987). The bacteria are not usually found systemically until a relatively large amount of external skin or gill damage has taken place. In virtually all instances, an outbreak of

columnaris will require intervention, e.g., improving fish culture conditions and/or using chemotherapeutants to reduce the external bacterial load on fish.

Historically, several chemotherapeutants, including benzalkonium chloride, (available as Hyamine 16, 22, and 3500), diquat, chloramine-T (CL-T; Bullock et al. 1991) and hydrogen peroxide (Jeff Rach, USGS, UMESC, personal communication) have been used to control mortality caused by BGD and external columnaris. However, with the exception of hydrogen peroxide, none of these chemicals are approved by the U. S. Food and Drug Administration (FDA) Center for Veterinary Medicine (CVM) for use to control mortality in freshwater fish caused by these diseases. Because CL-T appears to be one of the most effective therapeutant for salmonids diagnosed with BGD (From 1980; Bowker 1997a, 1997b, 1998; Bullock et al. 1991), it has become a prime candidate for FDA/CVM approval as a bath treatment for all freshwater finfish. Chloramine-T has been characterized as a non-selective sanitizing agent, and it has been shown to improve the condition of gills infected with bacteria and coated with mucous.

Salmonids and representative coolwater finfish are relatively tolerant of CL-T. Bills et al. (1988) established the 1-h LC50 value for CL-T in rainbow trout *Oncorhynchus mykiss* at greater than 60 mg/L, except in soft acidic waters (pH 6.5) where the LC50 was 55.8. Bowker and Carty (2001) demonstrated that the margin of safety associated with exposing juvenile rainbow trout (identified as the most sensitive life-stage likely to be treated with CL-T) for 3 h on three alternate or consecutive days at

14^{o}C was between 50 and 60 mg/L. Gaikowski et al. (In Press) found that the margin of safety associated with exposing a variety of small (i.e., fry) coolwater fish species (i.e., northern pike *Esox lucius*, walleye *Sander vitreus* , and lake sturgeon *Acipenser fulvescens*) to CL-T for 60 min on four consecutive days was $\geq$ 100 mg/L.

As per FDA/CVM guidelines, drug efficacy must be demonstrated at the lowest proposed concentration on a variety of representative fish species within specified groups of fish (e.g., salmonids, coolwater, or warmwater). For a variety of reasons, 20 mg/L CL-T was selected as the lowest proposed efficacious concentration for use on coolwater fish. Although the treatment concentration administered in the current study (20 mg/L) is higher than concentrations identified by others as effective in controlling mortality in salmonids caused by BGD (Bullock et al. 1991; Ostland et al. 1995; Bowker and Erdahl 1998, Bowker et al. In Press), it was presumed that 20 mg/L was more likely to consistently control mortality in a variety of coolwater finfish under different environmental and culture conditions than lower concentrations. In addition, the treatment regimen administered in the current study is described in the U.S. Fish and Wildlife Services (USFWS) Compassionate Chloramine-T INAD #9321 (Erdahl 2003) and has been shown to consistently control mortality in a variety of finfish under various environmental conditions (Bonnie Johnson, USFWS, AADAP, personal communication).

The CL-T treatment regimen currently proposed for use on salmonids (12 - 20 mg/L for 60 min on three alternate or consecutive days) and coolwater finfish (20 mg/L for 60 min on three alternate or consecutive days) 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 fish culture and fishery management objectives. Because many factors can affect the success or failure of CL-T therapy, efficacy data from controlled, replicated studies that are scientifically valid and statistically defensible (i.e., pivotal or supportive) are needed to gain approval of this drug for use in aquaculture.

To generate data needed to help obtain FDA approval for the use of CL-T to control mortality in hatchery-reared walleye diagnosed with external columnaris, a pivotal efficacy study was designed and conducted in June, 2006, at the Iowa Department of Natural Resource's Rathbun Fish Culture Research Facility (RFCRF; Moravia, IA; Deviation 1). The study was conducted under Study Protocol #4000-1 (Bowker 1996) in accordance with Good Clinical Practices (Final Guidance VICH GL9; USDHHS, 2001) by Alan Johnson (Investigator; Deviation 2) and RFCRF staff. The study objective was to evaluate the effectiveness of 20 mg/L CL-T (Deviation 3) administered as a 60-min bath treatment on three alternate days to control mortality in walleye (WAE) fingerlings caused by external columnaris.

Materials and Methods

Test Article and Test Fish

The test article was chloramine-T (Lot Number 0299510520572; Deviation 4; Axcentive SARL/International Specialty Chemicals, Inc., Tarrytown, NY; Appendix B; Deviation 5), a pure, water soluble compound not formulated in any way. Test fish were WAE fingerlings (Lot RRFC06-0to; Appendix A), which were progeny of a WAE brood population maintained at the RFCRF. At the RFCRF, hatchery personnel spawned mature WAE broodfish, incubated and hatched the fertilized eggs, and reared resultant fish until the study started.

Study Location and Schedule

The study was conducted at the RFCRF on June 6 - 22, 2006. The 17-d study comprised a 1-d (June 6) pre-treatment period for setting up the experiment and acclimating test fish; a 5-d period (June 7 - 11) during which three 20-mg/L CL-T treatments were administered as static baths for 60 min/d on alternate days (June 7, 9, and 11); and an 11-d post-treatment observation period (June 12 - 22; Appendix A; see Table 1 for a schedule and description of significant study events; Deviation 6). The study was conducted in accordance with FDA/CVM Guidance for Industry Good Clinical Practice (Final Guidance VICH GL9; USFDA, 2001; Deviation 7). The names of the Study Director, Study Monitor, Investigator, and other personnel involved in the study,

as well as curriculum vitae documenting study participants' qualifications, are listed in Appendix H.

Study Hypothesis and Experimental Design

The study's null hypothesis was H_o: $\mu_{treated} \geq \mu_{untreated}$, i.e., that mean percent total mortality in test tanks of WAE fingerlings treated with 20 mg/L CL-T was equal to or greater than that in test tanks of WAE fingerlings not treated with CL-T. The alternative hypothesis was H_a: $\mu_{treated} < \mu_{untreated}$. To test the null hypothesis, a completely randomized block design (CRBD; SOP No. MISC 232) was used to allocate four replicates of each of two treatment conditions (treated vs. non-treated control) across two blocks of test tanks (2 replicates of each treatment condition/block X 4 test tanks/block = 8 test tanks used in the study; Deviation 8; Deviation 9). The experimental unit was the "test tank." Block 1 comprised test tanks 1 - 4, and Block 2 comprised test tanks 5 - 8 (Figure 1; Appendix D). Treated test tanks were #s 1, 2, 5, and 7, and control (non-treated) test tanks were #s 3, 4, 6, and 8.

Experimental Procedures

Pre-treatment mortality and presumptive diagnosis of columnaris - The reference population of WAE fingerlings (mean length, 4.6 cm; mean weight, 0.83 g) comprised approximately 4,758 fish held in tanks #20 and #25 (rearing volume = 1,554

L; water inflow = 32 L/min; water turnover rate = 1.23 turnovers/h; loading rate, tank #20 = 1.99 g/L, tank #25 = 0.66 g/L; Appendix C). Based on the experience of Mr. Alan Johnson, the on-site Investigator, all of these environmental factors were suitable for holding and rearing healthy WAE. Mortality of fish in holding tank #20 and #25 increased during the 10-d period before the study started. For example, in tank #20, mortality ranged from 21 to 28 fish per day on May 27 - 29, and increased to 135 and 231 fish per day on June 4 and 5, respectively (Appendix N). In addition, mortality of fish in holding tank #25 (fish that were from the same lot as those in tank #20) showed a similar increase in mortality (Appendix N). Although there were no obvious fish-culture problems in either holding tank #20 or #25, many of the moribund and dead fish collected from both tanks showed clinical signs of columnaris. Thus, the on-site investigator concluded that the increased mortality was due to an outbreak of columnaris.

On June 7, five moribund fish were collected (three fish were collected from Tank #20 and two fish were collected from Tank #25) for fish health evaluations. All of these fish had external skin lesions characteristic of columnaris disease. Microscopic examination of wet mounts of skin scrapes on glass microscope slides showed bacteria morphologically similar to *F. columnare*. The microscopic detection of columnaris-like bacteria, the clinical signs observed, and a history of columnaris disease at the study site, led the on-site Investigator to make a presumptive diagnosis of a columnaris outbreak in the reference population.

Inclusion and exclusion criteria - Inclusion criteria for the study site and test fish were met. For example, by June 5, a sufficient number of fish in the reference population were showing clinical signs of columnaris disease, and enough test tanks were available for replication. In addition, the June 7 evaluation of moribund fish sampled from tanks #20 and #25 confirmed that *F. columnare* was likely the causative agent of the increased mortality that had been previously observed (Appendix E). Therefore, the study was started on June 6. During the study, no adverse events occurred that excluded test tanks or caused the study to be terminated early.

Transfer of fish from the reference population to the test tanks - On June 6, a completely randomized design procedure was used to allocate 1,736 WAE fingerlings from holding tanks #20 and #25 to the eight test tanks (n = 217 fish per test tank; 50 fish/test tank from tank #25 and 167 fish/test tank from tank #20; Appendix D; SOP No. MISC 232). To attempt to achieve a nearly uniform level of disease among the test tanks, collection and transfer of fish from the holding tank to the test tanks was done in four “rounds” (as follows):

(3 rounds of 50 fish per round) + (1 round of 67 fish) = 217 fish per test tank.

First round of 50 came from holding tank #25

Subsequent rounds came from holding tank #20

Test-tanks were made of fiberglass and were 1.66 ft long x 1.79 ft wide. Each test tank had one 1.12-ft high standpipe; thus, the water volume in each test tank was 3.33 ft^3.

Water inflow to each test tank was set at 2 L/min, and thus the water turnover rate for each test tank was approximately 1.27 water exchanges/h. Initial loading rates (1.9 g/L) in each of the test tanks (Appendix C) were approximately the same as those in holding tank #20 (1.9 g/L) and were suitable for rearing healthy WAE (personal communication, Alan Johnson).

Care and feeding of test fish - On June 6, fish were not fed because feed is routinely withheld from fish on days when they are moved, sample-counted, or treated with a water-bourne therapeutic. During June 7 - 11, fish were not fed on treatment days (June 7, 9, and 11) but were fed on non-treatment days (June 8 and 10). During the 11-d post-treatment period (June 12 - 22), fish were fed daily. Feed administered was 1.0-mm Walleye Grower (Nelson and Sons, Inc., SilverCup Feeds, Murray, UT), which is a standard, commercial walleye feed (Appendix F). Fish were fed at a daily rate of 8% of their estimated body weight. Feed was administered via automatic, Louden-style, trough-type feeders modified such that there were individual feed compartments for each test tank. As per routine RFCRC procedures, the feeders dispensed feed at 5-min intervals over a 24-h period.

Administration of chloramine-T and control treatments- Chloramine-T and control static-bath treatments were administered on June 7, 9, and 11. Four aliquots of CL-T (1.96 g per aliquot) were weighed out each treatment day (Sartorius Balance Model 1702 MP8). Eight 200-mL Pyrex beakers labeled 1 - 8 were filled with approximately 100 mL hatchery water. Chloramine-T aliquots were added to the

appropriate beakers (i.e., four beakers received CL-T) by the non-blinded study participant (Alan Johnson). Water flow was turned off to all tanks, and the contents of each beaker was poured into its corresponding test tank. Each beaker was rinsed three times to make sure all of its contents were transferred to each test tank. Tank contents were stirred to ensure thorough mixing, and the beakers placed on the side of their respective tanks. A 60-min treatment period began once the contents of the last container was added to its test tank. During the study, all tanks were dosed within 3 min. Water samples were collected from each test tank approximately 30 min into a 60-min treatment for CL-T dose-verification purposes. At the end of the 60-min treatment period, water flow to each tank was resumed.

Post-treatment fish health evaluations - On June 12 (first post-treatment day), one moribund or recently dead fish was sampled from each of four tanks for fish health evaluations (Deviation 10). Fish were collected from test tanks 8 (n = 1; control), 5 (n = 1; treated), 1 (n = 1; treated), and 7 (n = 1; treated). All fish so sampled had external skin lesions characteristic of columnaris disease. Microscopic examination of wet mounts of skin scrapes showed “haystack colonies of long, slender rods indicative of a columnaris infection”;however, microscopic examination of gill tissue wet mounts revealed no such bacteria. In addition, no parasites were found on skin or gill.

Data Collection (Including Blinding Procedures)

The study was designed to be single-blinded to minimize data collection bias. The non-blinded participant was the on-site Investigator (Alan Johnson) who was aware of which tanks received CL-T treatments and which tanks were controls. However, Mr. Johnson was occasionally involved in the day-to-day data collection (e.g., mortality, behavior, water temperature, and dissolved oxygen concentration; Deviation 11), as well as being involved in fish health evaluations, water hardness, alkalinity, pH measurements, and collection of water samples for dose verification. Blinded study personnel (Brad Bond and Nick Roberts) were responsible for dose verification of water samples collected during treatment.

Mortality - Mortality was recorded daily. Test tanks were checked once or twice daily for dead fish, which were dipnetted from each tank, counted, and disposed of according to routine RFCRC procedures. The number of dead fish removed from each tank, plus the number of moribund/dead fish sampled for fish health examinations, were recorded on appropriate data-collection forms.

Behavior - General, feeding, and appetite behaviors were observed intermittently (Deviation 12) due to use of Louden type raceway feeders to deliver feed over a 24-h period and the low-light environment preferred by WAE (personal communication, Alan Johnson, Study Investigator, IA DNR). General behavior was characterized and recorded as “normal” or “abnormal”; if abnormal behavior was

observed, study personnel had the option to note behavior characteristics such as lethargic, hyperactive, or flashing. If “other” behavior was observed, study personnel were to check the “other” box and describe behavior. Feeding behavior was determined by evaluating the relative amount of fecal material in each tank observed during collection of mortalities and tank cleaning. Appetite behavior was characterized and recorded as “aggressive,” “semi-aggressive,” or “non-aggressive.” “Aggressive” appetite behavior meant that fish appeared to be actively feeding and that nearly all feed offered was consumed (or that there was lots of fecal material). “Semi-aggressive” appetite behavior meant that some fish appeared to be feeding actively and that some fish appeared to not be feeding (no as much fecal matter). “Non-aggressive” appetite behavior meant that the fish appeared to not be feeding (very little fecal matter).

Chloramine-T dose verification - Water samples (100 mL) for CL-T dose-verification were collected from each test tank 30 min into each 60-min treatment. Water samples were analyzed for free and total chlorine with a HACH (Loveland, CO) Pocket Colorimeter (SOP No. INST 101.2). The colorimeter was used in the high range and “zeroed” with a water blank before each sample was measured. Two additional samples were collected from treated tanks, and these samples were analyzed for free and total chlorine and used for quality control purposes.

Water quality - Water temperature (measured to the nearest 0.1 $^{\circ}$C) and dissolved oxygen (DO) concentration (measured to the nearest 0.1 mg/L) were measured at least once daily during the study with a YSI Model 556 DO meter (YSI Inc,

Yellowsprings OH). Water hardness (mg/L $CaCO_3$), and alkalinity (mg/L $CaCO_3$) were measured twice during the study using HACH reagents and a Digital titrator, and pH (measured to the nearest 0.1 unit) was measured twice during the study using YSI Model 556 DO meter. Water quality data were recorded on appropriate data-collection forms.

Data Analysis

For each test tank (i.e., each experimental unit), percent total mortality was calculated by dividing the total mortality at the end of the study by the number of fish placed into the test 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 mortalities for treated test tanks, and dividing by the number of treated test tanks (n = 4) used in the study. Mean percent total mortality in untreated test tanks at any point in the study was calculated by determining the percent total mortality up to that point in each untreated test tank, summing the percent total mortalities for untreated test tanks, and dividing by the number of untreated test tanks (n = 4) used in the study. Mean percent total mortality did not include mortalities that occurred during the 1-d pre-treatment period.

Before being statistically analyzed, percent total mortality data for all test tanks were transformed to radians with the arc sine-square root transformation, where *P'* =

arcsin $\sqrt{P}$ (Zar 1984). Transformed percent mortality data were analyzed with a CRBD analysis of variance (ANOVA; α = 0.05; Deviation 13) to determine whether mean percent total mortality differed significantly between treated and untreated test tanks (a) at the end of the treatment period (after mortalities were collected in the afternoon of June 11; Deviation 6), (b) during the post-treatment period (June 15 - day 4 of the post-treatment period), and (c) at the end of the study (June 22 - day 11 of the post-treatment period), to see whether or not there was a treatment effect or block effect. The statistical software packages used were SigmaStat 3.5 (SYSTAT 2006a) and SYSTAT 11 (SYSTAT 2006b; Deviation 11).

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

Results

Mortality

At the end of the treatment period (Study day 6; June 11), mean percent mortality in treated tanks ($\bar{x}$ = 72% (156 of 217); ± 1 SD = 2.217) was significantly different (*P* =

0.022) from that in control tanks ($\bar{x}$ = 80% (173 of 217); ± 1 SD = 0.577) At 4 days post-treatment (Study day 10, June 15), mean percent mortality in treated test tanks ($\bar{x}$ = 75% (164 of 217); ± 1 SD = 1.414) was also significantly different (*P* = 0.035) from that in control tanks ($\bar{x}$ = 82% (178 of 217); ± 1 SD = 0.577). However, at the end of the study (Study day 17, June 22), mean percent total mortality in treated tanks ($\bar{x}$ = 79% (172 of 217); ± 1 SD = 2.160) was not significantly different (*P* = 0.154) from that in control tanks ($\bar{x}$ = 83% (180 of 217); ± 1 SD = 0.500; Tables 2a and 2b; Appendix Table I2). At the three points in time when the RCBD was run, blocking was not significant during the first two study days (*P* = 0.176 at day 6, *P* = 0.182 at day 10, *P* = 0.042 at day 17).

On day one of the treatment period (before treatment was administered), mean cumulative mortality in treated tanks (29.3 fish/tank; ± 1 SD = 4.787) was similar to that in control tanks (28.3 fish/tank; ± 1 SD = 4.573). Daily mortality was “high” in both treated and control tanks during the first 3 d of the 5-d treatment period, ranging from 24 to 62 fish/tank in treated tanks and from 23 to 78 fish/tank in control tanks (Appendix Table I1 and I3). Daily mortality was “low” in both treated and control tanks during the last 2 d of the 5-d treatment period, ranging from 3 to 12 fish/tank in treated tanks and from 4 to 12 fish/tank in control tanks. We speculate that the decrease in mortality observed in <u>treated tanks</u> was a result of effective therapy and improved culture conditions (especially in those treated tanks in which fish loading was reduced due to the overall high mortality) and that the decrease in mortality in <u>control tanks</u> was due to improved culture conditions due to the high overall mortality in control tanks.

During the 11-d post-treatment period, mortality in untreated test tanks decreased substantially, likely because fewer fish in the tanks resulted in a lower pathogen load in the water column. Mean total mortality during the 11-d post-treatment period in untreated test tanks was 7.3 dead fish/tank. Mean total mortality during the same period in treated test tanks was 15.8 dead fish/tank. At the end of the 16-d combined treatment and post-treatment periods, mean cumulative mortality in treated tanks was 172 (± 1 SD = 2.160) and in untreated tanks was 180 (± 1 SD = 0.500).

Behavior

General behavior appeared normal throughout the study (Appendix Table J1). The general impression of appetite behavior was that fish fed aggressively.

Chloramine-T Dose Verification

The overall mean daily CL-T concentration (n = 12) administered over the course of the study was 19.6 mg/L (Appendix G, Table G1), and mean daily concentrations ranged from 19.1 to 20.1 mg/L. Daily measurements from each treated tank ranged from 18.3 to 21.4 mg/L (all within 9% of the calculated target dosage). Chloramine-T concentrations measured in samples collected for quality control ranged from 19.1 to 19.9 (Appendix G, Table G2), indicating that, for the purposes of this study, the dose verification methodology was relatively precise and accurate. Chloramine-T was not detected in the control tanks.

Water Quality

Mean water hardness, alkalinity, and pH were 154 mg/L (as $CaCO_3$), 110 mg/L (as $CaCO_3$), and 7.7 respectively (Appendix K). Overall mean water temperature in the test tanks was 24.1°C (n = 239; Appendix Table L1; Figure 3) and mean DO concentration was 8.2 mg/L (n = 240; Appendix Table M1). Daily mean DO concentrations were relatively consistent (Figure 4) throughout the study. In summary, water quality variables measured during the study were well with guidelines considered suitable for rearing healthy walleye.

Discussion and Conclusions

Although there was not a significant difference in mean mortality at the end of the study between treated tanks and control tanks, mortality results from this study indicated that CL-T treatment was effective in controlling mortality caused by external columnaris through the first few days of the post-treatment period. We speculate that, had there not been a delay in starting treatment, overall mortality in all test units would have been less, but more importantly, mortality in treated tanks would have been less than mortality in control tanks. Under routine RFCRC procedures, chemotherapeutic treatment is started early in a disease outbreak to prevent excessive mortality. However, in this case, the columnaris infection was detected during a time (holiday weekend) when the on-site Investigator was not available. During this weekend, the disease apparently progressed to the point where many, if not all, fish in the reference

population showed clinical signs of columnaris, and daily mortality in the reference population reached nearly 5%. As a result of the delay, the infection was allowed to spread to more fish in the reference population, and quite possibly, to become systemic in some of the fish. Once an infection becomes systemic, topical therapeutics such as CL-T have virtually no effect in controlling mortality caused by the infection. We suspect that the delayed intervention (i.e., CL-T treatment) exacerbated the unsatisfactory conditions in the test tanks, resulting in mortality levels similar to that described by Post (1987) if such conditions are not improved. As previously stated, we suspect that had the study started as soon as it was evident that the fish were sick that the number of pathogens shed among fish in treated tanks would have been reduced at an earlier stage, and that resultant mortality would have been more readily controlled. Therefore, in this study, we considered CL-T to have been effective in controlling mortality in WAE fingerlings caused by external columnaris for the following reasons (and support the claim that CL-T is effective in controlling mortality in WAE caused by external columnaris):

(1) At the end of the Study, mean percent total mortality in the treated test tanks was less than that in the untreated test tanks;

(2) Differences in mean cumulative mortality in treated and untreated tanks were significantly different through day 4 of the post-treatment period;

(3) Fish health evaluations conducted during the pre-study and post-treatment periods showed that external columnaris was the primary factor causing morbidity and mortality of fish in the reference population and in the test tanks;

(4) Dose verification of water dosed with CL-T showed that the concentrations were within the ± 25% limits acceptable to FDA.;

(5) Measured water quality variables and culture conditions were suitable for rearing healthy walleye; and

(6) No other external bacteria or parasites were detected on fish examined.

Acknowledgments

The authors thank James B. Rudacille, Brad J. Bond, Jodi Ahrnkiel, and Nick A. Roberts of the Iowa DNR Rathbun Fish Culture and Research Center for assisting in this study, and Dan Carty and Molly P. Bowman of the USFWS's AADAP Program for helping prepare this report.

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Table 1. Schedule and description of significant events for study CHLT-96-EFF-07.

Study day and description of significant event	
June 5, 2006	1. Order fish transferred to test tanks randomly assigned 2. Treatment conditions randomly assigned to test tanks
Pre-treatment acclimation phase (study day 1; June 6, 2006)	
First day of pre-treatment period (June 6; study day 1)	1. Set water inflow to each test tank 2. Transferred fish to test tanks 3. Calculated initial flow and density index values in test tanks 4. Calculated feed amounts to be fed to fish in each test tank (fish fed at a rate of 8% body weight daily) 5. Began collecting study data
Treatment phase (study days 2 - 6; June 7 - 11, 2006)	
First day of treatment period (June 7; study day 2)	1. Fish sampled from reference population and examined for external columnaris and parasites
First, third, and fifth day of treatment period (June 7, 9, and 11; study days 2, 4, and 6)	1. Administered chloramine-T treatments on alternate days (starting with study day 2) 2. Analyzed water sampled collected from each tank during each treatment event for dose verification
Post-treatment phase (study days 7 - 17; June 12 - 22, 2006)	
First day of post-treatment period (June 12; study day 7)	1. Fish sampled from four test tanks for fish health evaluations
First day of post-treatment period (June 12; study day 7)	1. Water samples analyzed for hardness, alkalinity and pH
Fourth day of post-treatment period (June 15, study day 10)	1. Last day during study that mean percent cumulative mortality was significantly lower in treated tanks than in untreated tanks
Tenth day of post-treatment period (June 21; study day 16)	1. Water samples analyzed for hardness, alkalinity and pH
Eleventh day of post-treatment period (June 22; study day 17)	1. Study ended

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

Number of mortalities recorded in chloramine-T treated test tanks[a]				
	Test tank 1	**Test tank 2**	**Test tank 5**	**Test tank 7**
Total mortality	183	182	156	165
Number of fish in each test tank at start of study	217	217	217	217
Percent total mortality	84.3	83.9	71.9	76.0
Total number of fish counted into test tanks = 868; mean number of fish per tank = 217				
Total mortality = 686; mean mortality = 171; ±1SD = 2.160				
Mean total (%) mortality = 78.8				

[a] chloramine-T treated group: Mean total mortality (171) = ((183/217) + (182/217) + (156/217) + (165/217))/4) x 100

Table 2b. Mortality of walleye 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 6**	**Test tank 8**
Total mortality	175	194	177	175
Number of fish in each test tank at start of study	217	217	217	217
Percent total mortality	80.6	89.4	81.6	80.6
Total number of fish counted into test tanks = 868; mean number of fish per tank = 217				
Total mortality = 721; mean mortality = 180; ±1SD = 0.500				
Mean total (%) mortality = 83.0				

[b] Untreated group: Mean total mortality (180) = ((175/217) + (194/217) + (177/217) + (175/217))/4) x 100

Figure 1. Tank set-up, a randomized block design, at RFCRF, showing all eight study tanks and their standpipes, water inflow, and cover screens for study CHLT-96-EFF-07.

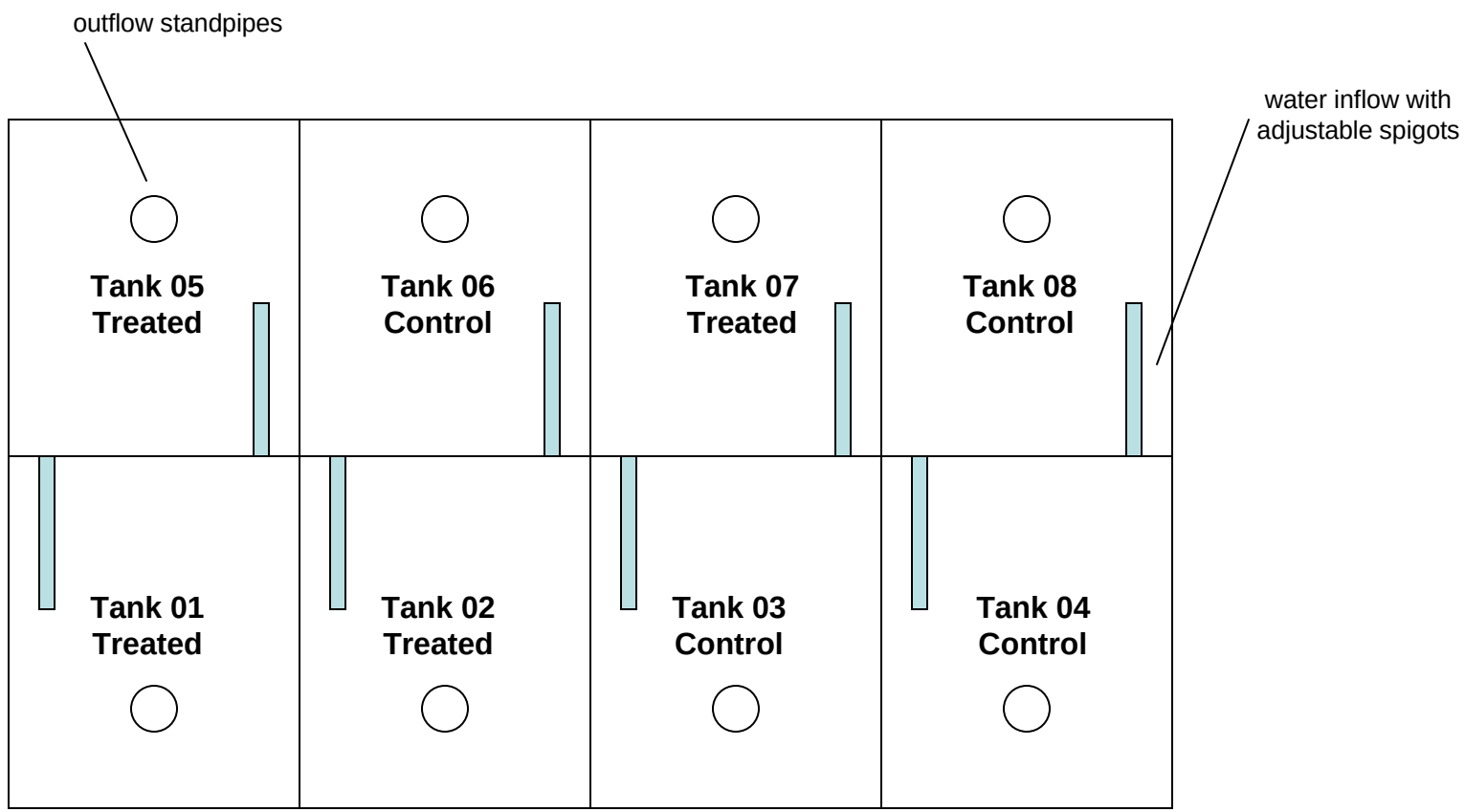
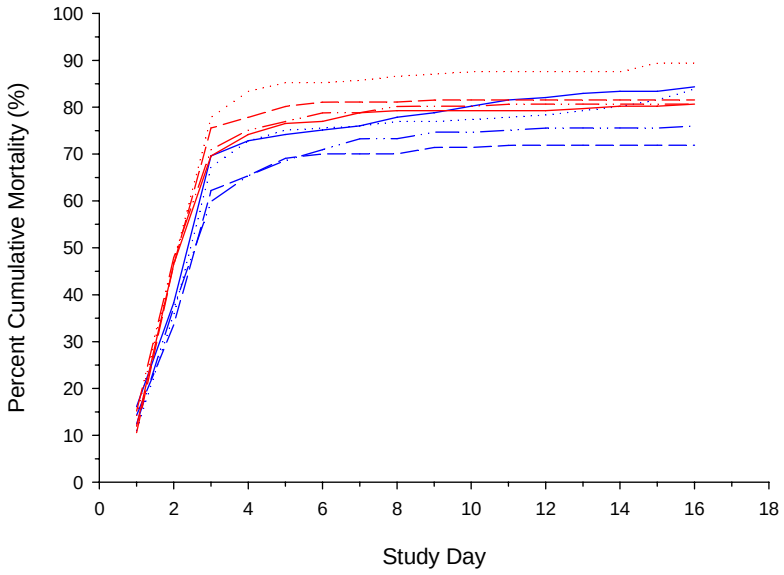


Figure 2. Study tanks 1 – 8 showing water inflow, outflow standpipes, adjustable spigots, and treatment conditions.



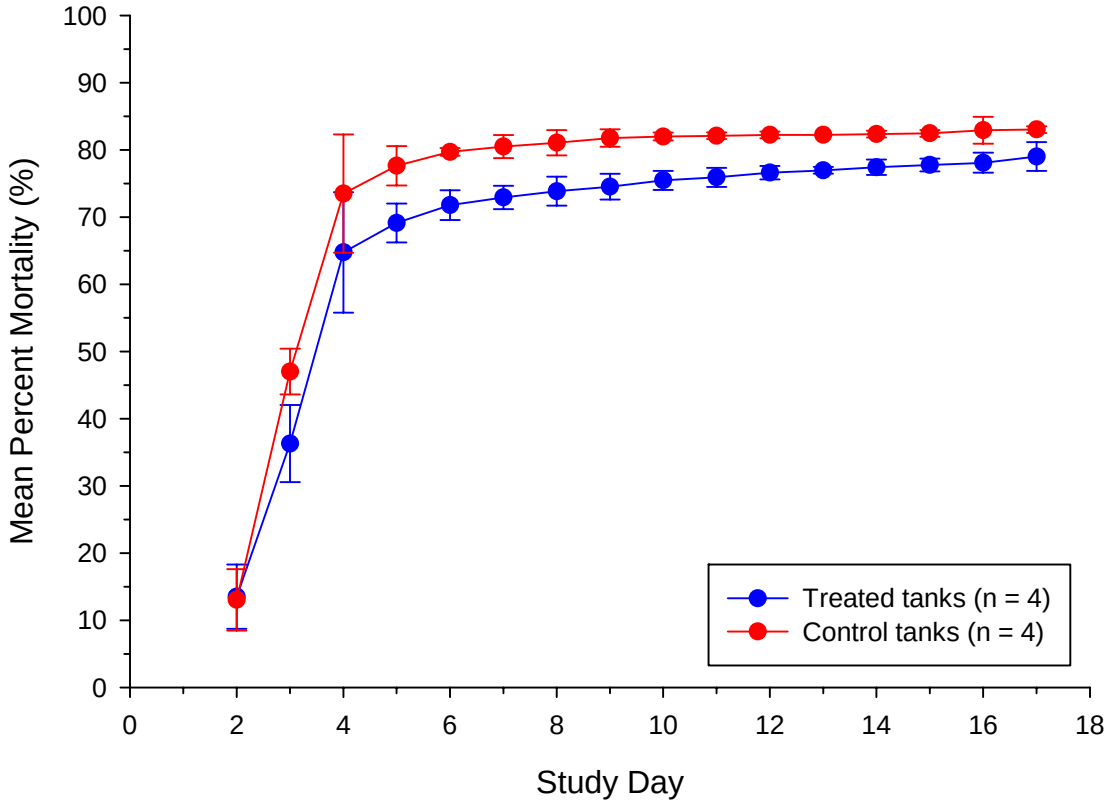


Figure 3. Mean percent mortality of four treated and four control tanks of walleye treated with chloramine-T.
(error bars ± 1SD)

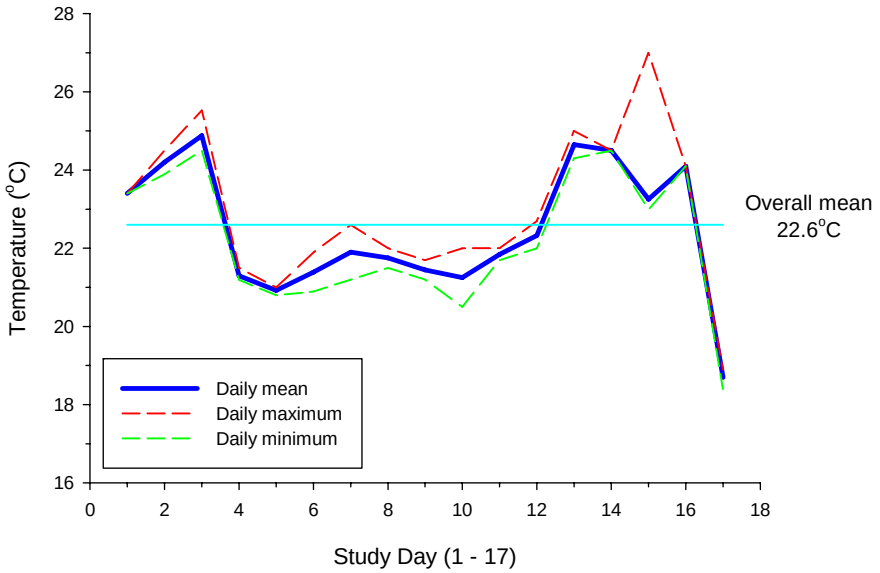


Figure 4. Daily mean, maximum, and minimum water temperature recorded during the study.

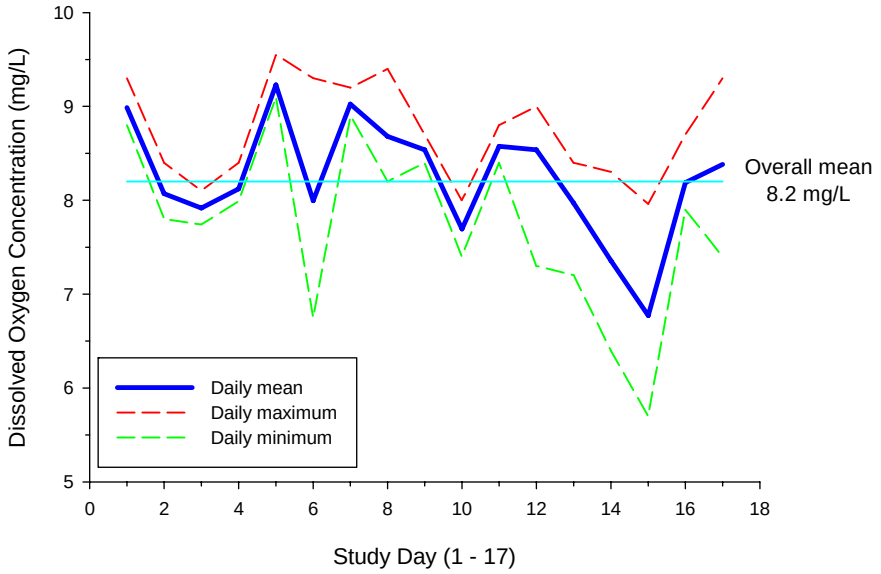


Figure 5. Daily mean, maximum, and minimum dissolved oxygen concentration recorded during the study.

Deviations from the Study Protocol

Deviation 1. **Study Protocol Section 2.1.9 Lot number:**

The study protocol lists study sites that potentially may conduct a chloramine-T efficacy study under this protocol at the time this protocol was drafted. Rathbun Fish Culture and Research Facility (RFCRF) was not included in this list. The current study was conducted at RFCRF. This deviation did not negatively affect the outcome of the study.

Deviation 2. **Study Protocol Section 10.1 Investigators, study monitors, and fish health biologists involved in proposed field based efficacy trials:**

The study protocol lists facilities, Investigators, Monitors, and fish health biologists that may be involved in proposed field based clinical efficacy trials. Rathbun Fish Culture and Research Facility was not included in this list, nor was Mr. Alan Johnson included as an Investigator or Dr. Tom Bell included as a Study Monitor. Such deviations did not negatively impact the outcome of the study.

Deviation 3. **Study Protocol Section 2.1.7 Dose(s) to be tested:**

The study protocol states that 10 mg/L chloramine-T is the only dose to be tested under this protocol. In the current study, a dose of 20 mg/L chloramine-T was tested. Although it was presumed that treatment with 20 mg/L would be more efficacious that treatment with 10 mg/L, the outcome of the study was not negatively affected by this deviation.

Deviation 4. **Study Protocol Section 2.1.8 Manufacturing site:**

The study protocol states that chloramine-T used under this protocol will be manufactured by Akzo Chemical. Between the time the protocol was developed (1996) and the time when the current study was conducted (2006), the manufacture of this brand of chloramine-T changed from Akzo Chemical to Axcentive SARL. This deviation did not negatively affect the outcome of the study.

Deviations from the Study Protocol (continued)

Deviation 5. **Study Protocol Section 2.1.9 Lot number:**

The study protocol lists study sites that potentially may conduct a chloramine-T efficacy study under this protocol and the chloramine-T lot number on hand at the time this protocol was drafted. The chloramine-T lot number used in the current study was not included in this list. This deviation did not negatively affect the outcome of the study.

Deviation 6. **Study Protocol Section 3.2.4 Post-treatment period duration:**

The study protocol states that the post-treatment period duration will be 14 d. In this study, the post-treatment lasted only 11 d. Due to the fact that only 11 d of daily mortality was collected during post-treatment period, we assumed that total cumulative mortality would have been higher in all test tanks had the post-treatment period been extended to 14 d. However, the overall outcome of the study would, in all likelihood, not have changed. Therefore, we contend that this deviation did not negatively impact the outcome of the study.

Deviation 7. **Study Protocol Section 1.1 Objective:**

The study protocol states that Investigators and Monitors will be familiar with a document entitled: Conduct of Clinical Investigations: Responsibilities, of Clinical Investigators and Monitors for Investigational new Animal Drug Studies. This document has been replaced by Guidance for Industry Good Clinical Practice (Guidance Document #85). The Investigator and Monitor in this study were familiar with the contents of this document. This deviation did not negatively impact the outcome of the study.

Deviation 8. **Study Protocol Section 4.2 Experimental design:**

The study protocol states that the experimental design used will be completely randomized. In this study, we used a completely randomized block design to assign treatment conditions to test tanks. A completely randomized block design was used due to test tank configuration and our suspicion that blocking factors may exist. This deviation did not negatively impact the outcome of the study.

Deviations from the Study Protocol (continued)

Deviation 9. **Study Protocol Section 4.4.1 Allocation of animals to test units:**

The study protocol states that animals will be randomly placed in one of six test units. In this study, a total of eight test units were used. This deviation did not negatively impact the outcome of the study.

Deviation 10. **Study Protocol Section 6.2.2 Procedures for assessing other variables:**

The study protocol states three to five fish will be randomly selected and removed from each tank to evaluate the level of bacterial infection. In this study, one fish was selected from four of eight test tanks (three treated tanks and one control tank) for fish health evaluation. Collection of more fish/tank would have provided more evidence that fish death or morbidity was caused by external columnaris. However, all fish sampled showed clinical signs of external columnaris which was confirmed by microscopic examination of skin scrapes. Failure to sample three to five fish/tank did not negatively impact the outcome of the study.

Deviation 11. **Study Protocol Section 5.5.1 Extent of blinding and 5.5.3 List of personnel with access to treatment codes and rationale:**

The study protocol describes blinding procedures and the role of the non-blinded study participant(s). In this study, Mr. Alan Johnson was the non-blinded study participant and should not have been involved in day-to-day data collection. However, due to intermittent work conflicts, blinded study personnel were not always available to collect some data. Therefore, on occasion, Mr. Alan Johnson collected mortality, behavior, and water temperature/DO concentration. With the exception of behavior, it is difficult to bias data collection (a fish is either dead/moribund or it isn't; record digital readout of DO meter). We acknowledge that documenting behavior may be bias by non-blinded study personnel. However, no behavior observations were made that would affect the outcome of the study. Therefore, we believe that this deviation did not negatively impact the outcome of the study.

Deviations from the Study Protocol (continued)

Deviation 12. **Study Protocol Section 6.2.2 Procedures for assessing other variables:**

Although not explicitly stated in the study protocol, fish behavior should have been observed and documented daily. In this study, such observations were made on 4 of 17 study days. We acknowledge that daily observations would have been preferred and would have provided more information relative to test article induced (negative) behavior. However, all observations were denoted as "normal" and it is presumed that this behavior was indicative of the behavior during the rest of the study. In addition, fish were observed every day that automatic feeders were loaded with the daily ration of feed, and no abnormal behavior was noted during these periods. Therefore, we believe that this deviation did not negatively impact the outcome of the study.

Deviation 13. **Study Protocol Section 7.3.4 Biostatistical procedures used:**

The study protocol states the an independent t-test will be used to detect differences between treated and untreated fish with regards to total fish mortality/test unit. In this study, percent total mortality data for all test tanks were transformed to radians with the arc sine-square root transformation, where $P' = arcsin\sqrt{P}$ (Zar 1984), and then transformed percent mortality data were analyzed with a completely randomized block design analysis of variance. This deviation did not negatively impact the outcome of the study.