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A RESEARCH PROTOCOL TO DETERMINE:

The Efficacy of Chloramine-T to Control Mortality Due to Bacterial Gill Disease or External Columnaris in Cool and Warmwater Finfish

Protocol CHLT-07-EFF.1

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Testing Facilities: Federal, State and Private Facilities throughout the United States

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1. INTRODUCTION:

1.1 Objective:

1. To evaluate the efficacy of chloramine-T (Chl-T) to control mortality caused by bacterial gill disease (BGD; causative agent, *Flavobacterium sp*.) or external columnaris (EC; causative agent, *Flavobacterium columnare*) in all freshwater reared cool and warmwater finfish. Chl-T is to be administered as a bath solution (static bath) for 60 minutes three times on alternate or consecutive days.

2. H_o: $\mu_{\text{treated}} \geq \mu_{\text{non-treated (control)}}$

   Mean percent total mortality in experimental units of test fish treated with Chl-T at a dosage of 20 mg/L for 60 min three times on alternate or consecutive days *is equal to or greater than that* in experimental units of test fish not treated with Chl-T.

3. H_a: $\mu_{\text{treated}} < \mu_{\text{non-treated (control)}}$

   Mean percent total mortality in experimental units of test fish treated with Chl-T at a dosage of 20 mg/L for 60 min three times on alternate or consecutive days *is less than that* in experimental units of test fish not treated with Chl-T.

4. Study Director (SD), On-Site Investigator (OSI), and Study Monitor (SM) will be familiar with this Study Protocol and Guidance Document 85 for Good Clinical Practice (VICH GL9 GCP; Appendix D).

1.2 Background and Justification:

Bacterial gill disease (BGD) is one of the most common diseases of hatchery-reared salmonids (Bullock 1990) and causes more fish losses than any other bacterial disease (Bills et al. 1988). Stressors associated with intensive fish culture may predispose fish to infection (Piper et al. 1982). In Ontario, Canada, BGD accounts for nearly 21% of all diagnostic submissions from fish farms to the Fish Pathology Laboratory of the Ontario Veterinary College (Ferguson et al. 1991). Proliferation of gill epithelial tissue and subsequent loss of gill surface by clubbing and fusing of lamellae are often associated with BGD infections (Bullock 1990). It is generally believed that death is not a direct result of the bacterial infection, but rather a consequence of asphyxiation from lack of adequate oxygen exchange at severely congested gills. The disease is characterized by an acute onset, flared opercula, increased ventilation rate, decreased fright response, equidistant spacing of infected animals, reduced food consumption, and high mortality (Lumsden et al. 1994; Lasee 1995). If BGD, which is horizontally

transmitted, is not diagnosed and treated during the early stages of infection, an epizootic may occur within a 24-h period (Bullock et al. 1991).

Historically, several chemicals, including benzalkonium chloride (available as Hyamine 1622 and 3500), diquat, and chloramine-T (Bullock et al. 1991) have been used to control mortality caused by BGD (causative agent, *Flavobacteria* spp.). However, none of these chemicals are approved by the U.S. Food and Drug Administration (FDA) as medicinal drugs for use in aquaculture. Chloramine-T (Chl-T; N-sodium-N-chloro-p-toluene sulfonamide), manufactured by Akzo Chemical, Inc. (Chicago, Illinois), has become one of the prime candidates for approval because it is recognized as one of the most effective therapeutants for treating salmonids with BGD (From 1980; Speare and Ferguson 1989; Bullock et al. 1991; Thorburn and Moccia 1993; Ostland et al. 1995). Currently, all use of Chl-T in the U. S. is granted by the FDA under a compassionate Investigational New Animal Drug (INAD) exemption. In simple terms, to gain FDA approval of a drug, sufficient data must be generated and accepted by FDA's Center for Veterinary Medicine (CVM) to demonstrate the drug is safe to humans, target animals, and the environment; can be manufactured consistently; and is effective for its proposed use(s). Clinical efficacy must be demonstrated in "pivotal" field trials. Pivotal studies are those done following research study protocols accepted by CVM's Aquaculture Team, that are adequate and well-controlled, and produce scientifically valid, statistically defensible results.

The objective of field based clinical efficacy studies is to evaluate the efficacy of 20 mg/L Chl-T treatment (dose confirmation study) to control mortality in a variety of cool and warmwater finfish species caused by BGD or EC. Efficacy studies will be conducted at fish culture facilities (i.e., hatcheries) after the following have been considered: (1) history of predictable, recurring outbreaks of fish diseases of interest at the hatchery, (2) each hatchery has spaced to dedicate to conducting studies, (3) at each hatchery there are additional resources available to conduct studies (e.g., test units, test fish, staff to monitor study and collect data); and (4) each has demonstrated commitment to adhere to protocols and guidelines. Studies will be conducted under this protocol and are intended to provide CVM with clinical efficacy data to support a New Animal Drug Application.

2. INVESTIGATIONAL DRUG AND CONTROL

2.1 Test Substances

2.1.1 Trade name:

Halamid®

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2.1.2 Chemical name:
Benzene sulfonaminde, N-Chloro-4-methyl, sodium salt

2.1.3 Synonyms:
Sodium *p*-toluenesulfonchloramide ; Chloramine-T

2.1.4 Molecular formula:
$C_7 H_7 Cl N NaO_2 S$ (and hydrates)

2.1.5 Product use:
Oxidizing agent

2.1.6 Appearance and odor:
White crystalline powder with a weak chlorine odor.

2.1.7 Active/inactive ingredients:
Benzenesulfonadmide, N-chloramini-4-methyl-, sodium salt (99.0 – 100.0% pure). This is the final product.

2.1.8 Dosage form:
Chl-T for use in treating BGD and EC in fish is a pure, water soluble compound and is not formulated in any way. During use, dry Chl-T will be dissolved in water and applied as a bath solution to achieve the target dose. At the end of the treatment period, the Chl-T working solution will be flushed from experimental units with hatchery water.

2.1.9 Dose(s) to be tested:
20 mg/L

2.1.10 Manufacturing site:
Axcentive SARL
Chemin de Champouse
13320 Bouc Bel Air, France
Tel. (33) 442 694 090

2.1.11 Distribution site:
Axcentive SARL/International Specialty Chemicals
303 South Broadway
Suite 425
Tarrytown, NY 10591

2.1.12 Lot number:
The lot number of Chl-T used in each study will be documented and reported in each Final Study Report (FSR).

2.1.13 Packaging:
Chl-T is packaged in a variety of quantities (i.e., 5 and 25lbs) and hence different packages (i.e., plastic tub, plastic-lined thick paper bag).

2.1.14 Storage conditions:
Store away from foodstuffs and animal feed. Containers should be kept tightly capped and stored in a cool, dry, well-ventilated area away from flammable, reducing, or oxidizing materials and sources of heat or flame. Maximum storage temperature: 140°F (60°C). Storage at higher temperatures will cause loss of crystalline structure.

2.1.15 Drug storage during study:
Chl-T used in each study will be stored in its original container in a cool, dry place. The container should be labeled with an expiration date of the product.

2.1.16 Drug handling procedures:
Avoid inhalation of dust and prolonged and/or repeated skin and eye contact.

2.1.17 Verification of drug integrity/strength:
A request should be made of a Certificate of Analysis (COA) each time Chl-T is purchased from International Specialty Chemicals. The COA will contain information pertaining to the drug integrity and strength.

2.1.18 Investigational labeling:
A copy of the Investigational label should be affixed to each container of Chl-T. It will be the responsibility of the on-site Investigator to ensure proper labeling of such containers. Investigational labeling can be obtained from the USFWS's AADAP program.

2.1.19 Material safety data sheet (MSDS):
A complete MSDS is included in Appendix A.

2.1.20 Accountability:
The FDA will be notified when Chl-T is ordered from the distributor. A drug use log will document use of the Chl-T.

2.2 Controls:

Control treatment will be a dose of hatchery water (the same hatchery water used at the particular testing site to prepare the chloramine-T solution). The amount of control (mL hatchery water) will be the same as the amount of chloramine-T solution (mL) administered to treated fish.

3. STUDY SCHEDULES:

3.1 Proposed date(s) of initiation:

Proposed date(s) of initiation will be study site specific. Studies may be conducted at (1) FWS hatcheries, (2) state natural resource hatcheries, (3) tribal hatcheries, or (4) private commercial hatcheries. Studies will be initiated when there is a disease outbreak and daily mortality is sufficient to adequately address the study objective. Ideally, the SD or designee will be available to assist initiating the treatment phase of the study. However, if that is not possible, then the on-site Investigator will initiate the study with telephone support from the SD or designee. Dates of study initiation and significant events in each study will be summarized in the FSR.

3.2 Schedule of events:

Study Day	Period	Event
A (≥ 1 d)	Acclimation period	Presumptive diagnosis of pathogen from a subsample of fish from reference population (≥7 of 10 fish) Measure size of reference population tank/raceway and experimental units Measure water inflow to test units Calculate loading rates in reference population and experimental units Sample count study fish Transfer fish into test units Measure water temperature, dissolved oxygen (DO) concentration, hardness, alkalinity, and pH in reference population
B	Treatment Day 01	Administer treatment Perform dose-verification of samples Collect mortalities twice from each test unit Measure water temperature and DO in test units Observe general behavior and appetite behavior
C	Treatment Day 02	Administer treatment Perform dose-verification of samples Collect mortalities twice from each test unit Measure water temperature and DO in test units Observe general behavior and appetite behavior
D	Treatment Day 03	Administer treatment Perform dose-verification of samples Collect mortalities twice from each test unit Measure water temperature and DO in test units Observe general behavior and appetite behavior
E – R	Post-Treatment (14 d)	Collect mortalities twice daily from each test unit Measure water temperature and DO once daily in test units Measure hardness, alkalinity, and pH once from source water Observe general behavior and appetite behavior

3.2.1 Transfer of test animals:

Fish will be transferred into experimental units no less than 1 d prior to initiation of treatment. Allocation of fish will be done in a manner to minimize bias among experimental units with respect to level of disease, and will follow SOPs MISC 237 and MISC 239 (Appendix E). Allocation of fish-to-tanks will be done following a completely randomized design. All information pertaining to transfer of fish to experimental units, including randomization procedures

used to determine allocation of fish to experimental units, date(s) fish were transferred, and date study was started will be described in the FSR.

3.2.2 Initiation of treatment:

Treatment will begin no sooner than 1 d after fish have been transferred to experimental units.

3.2.3 Treatment period duration:

The treatment period will begin on the day treatment is initiated (day 1) and last either 3-d (three treatments administered on consecutive days) or 5-d (three treatments administered on alternate days).

3.2.4 Post-treatment period duration:

The intended duration of the post-treatment period will be at 14 d. However, due to the species of fish to be tested (i.e., cool- and warmwater fish species), that unless fish testing conditions are improved (e.g., decrease fish densities, increase water flow, etc.), it is likely that fish will be re-infected with BGD or EC or colonized/infected (due to warm water) with an ectoparasite If such an event occurs during the post-treatment period, the duration of the post-treatment period will be 10 d.

3.2.5 Data analysis and report writing:

Each study conducted under this protocol is independent and will vary according to design, species, and disease; therefore a separate analysis will be done for each study. Data collection, analysis, and preparation of the FSR will require a maximum of 12 months after the study is complete. By this time, the FSR will be submitted to CVM's Aquaculture Team for review.

3.3 Proposed date(s) of completion:

It is anticipated that (1) one study on a representative coolwater fish species other than walleye (e.g., northern pike, tiger musky, yellow perch, bluegill) is required to complete the effectiveness data requirements for all coolwater fish, and (2) two studies on representative warmwater fish (e.g., channel catfish and largemouth bass) are required to complete the effectiveness data requirements for all warmwater fish. As a result, we are uncertain at this time when all studies will be completed. The proposed date for completion of all studies will be December 31, 2010.

Temperature Range	# Pivotal Studies	# Supportive Studies	Species
Bacterial Gill Disease			
Coolwater	1	1	Two different coolwater species.
Warmwater	1	1	Two different warmwater species.
External Columnaris Disease			
Coolwater	0	1	A coolwater species other than walleye.
Warmwater	1	1	Two different warmwater species.

4. STUDY DESIGN

4.1 Treatment groups:

All fish in a particular study will be drawn from the same fish production lot. Studies will consist of two treatment conditions (treated vs. non-treated control). Treated experimental units (i.e., test tanks) will be treated three times with Chl-T. Control experimental units (i.e., test tanks) will receive a sham water treatment in a manner similar to the treatment that treated fish will receive. There will be at least three replicates per treatment condition in each study.

4.2 Experimental design:

The experimental design used will be a completely randomized design. If another experimental design is used (e.g., randomized block design) it will be described in the FSR.

4.3 Blocking factors:

The experimental design used will be a completely randomized design. But, in situations where such blocking factor-conditions exist and blocking is considered appropriate, a description of the blocking factors will be included in the FSR.

4.4 Randomization procedures:

Completely randomized design procedures will be used to assign fish to experimental units, treatment conditions to experimental units, and to select water samples to be assayed for dose verification. All completely randomized design procedures will follow SOP MISC 327 (Appendix E) and will be detailed in the FSR.

4.5 Configuration of experimental units:

The experimental unit will be a “tank of fish.” Configuration of experimental units will be different at each study site. Experimental units will be set up according to the recommendation of the On-Site Investigator. All experimental units will be plumbed in the same manner with water from the same source. The configuration of experimental units at each study site will be described in the FSR.

5. STUDY PROCEDURES

5.1 Test animals

5.1.1 Description:

5.1.1.1 Test animal (i.e., test fish) species

Species of test fish used will be characterized as representative cool- or warmwater finfish. Species selected for testing will be done in support of an “all-fish” claim and will be described in the FSR.

5.1.1.2 Sex:

Sex of test fish will not be determined or considered. The test chemical is an oxidizing agent that will oxidize organic material on the external surface of test fish. Therefore, sex of the early life-stage test fish used in each study should not be a factor in treatment efficacy. However, it will be assumed that males and females will be present in roughly equal proportions.

5.1.1.3 Breed/class:

Fish species will be confirmed by the hatchery manager or designee.

5.1.1.4 Initial body weight:

Initial body size (length and weight) will be determined at the start of each study. Body weight will be determined by one of the following (1) weighing individual fish separately and generating a mean, (2) weighing a known number of fish and estimating individual weight, or (3) measuring fish length and then estimating weight based on length-weight relationship tables in Piper et al 1982 (Appendix I; Form 11). This information will be documented and described in the FSR.

5.1.1.5 Physiological state:

The physiological state of test fish as related to organ function will not be evaluated; however general fish behavior will be recorded as part of daily observations (Form 5).

5.1.1.6 Age/Size:

Test animals (i.e., test fish) used in the study will be characterized as early life stage (i.e., fry, fingerling, or juvenile). Fish species and size (total length) will be described in the FSR.

5.1.1.7 History of test fish

Test fish will most likely be progeny of on-site wild or hatchery-held broodfish. Eggs will have been collected by hatchery staff, incubated, hatched, and resultant fry will be reared using standard hatchery procedures. A brief description will be included in the FSR detailing information such as egg source, management practices, and environmental conditions under which fish were reared prior to inclusion in the study (e.g., species and size of test fish, stocking densities, water flow, rearing temperature). Prior drug treatments will not be reported provided the withdrawal period for the particular drug has elapsed. In cases where the withdrawal period has not elapsed prior to starting the study, a full description of the treatment and purpose for the treatment will be included in the FSR.

5.1.2 Number of test fish:

A sufficient number of test fish (≥ 100 fish) will be loaded into each experimental unit to approximate near-production loading levels. Ideally, the relative number of fish per volume of water (g fish/L water) in experimental units will be similar (or slightly more crowded) to that of the tank or raceway holding the reference population.

5.1.2.2 Loading rates:

Loading rates will differ for different fish species. In most cases, loading rates of fish in the tank or raceway holding the reference population will be determined by the hatchery owner or manager. For the reference population, the loading rate will be based on the estimated number of fish/tank or raceway, fish weight based on sample counts, and tank or raceway dimensions. In experimental units, we will mathematically calculate (Appendix I) the number of fish from the reference population required to be transferred to each experimental unit to ensure that the loading rates in experimental units match or slightly exceed that of the unit holding the reference population. It is critical to match or slightly exceed the loading rate of the unit holding the reference population to minimize the occurrence of “spontaneous recovery” (Appendix I).

5.1.3 Source of test fish:

Source of test fish will be documented on Form 1 and will be described in the FSR.

5.1.4 Identification method if not client-owned fish:

Test fish will not possess any artificial or man-made mark to identify individual fish. Precautions will be taken to minimize test fish escaping or jumping from one experimental unit to another. This will be accomplished by placing covers over experimental units or by maintaining a low water level in experimental units to prevent fish escapement.

5.2 Inclusion criteria:

The entrance criteria for inclusion in the study will include the following: 1) study fish showing clinical signs will have been presumptively diagnosed with BGD or EC (Appendix B, Sections 1.3 Bacterial Gilll Disease, D. Disease Signs and 1.5 Columnaris Disease, D. Disease Signs), and fish-pathogen related mortality will be adequate to sufficiently address the study objective (i.e., there will be higher than normal (unacceptable) mortality in the reference population); 2) fish are known not to have any secondary disease pathogens that could compromise the outcome of the study; and 3) based on appropriate allocation procedures (allocate fish to experimental units without known bias), a uniform level of disease is present in each experimental unit.

5.2.1 Ability of Investigator to fulfill all the requirements of the protocol:

The SD and OSI will be fully capable of ensuring all requirements of the protocol are fulfilled. The SD (or designee) will work cooperatively with the OSI to ensure that the protocol is followed. Study sites will be selected, in part, because the OSI has experience using Chl-T in accordance with compassionate INAD exemption protocols to treat for BGD or EC. The OSI will be familiar with this research protocol, data collection forms, and how to correctly record data.

5.2.2 Level of disease:

Level of disease-related mortality and the number of fish showing clinical signs of BGD or EC will have increased among fish in the reference population (Appendix B, Sections 1.3 Bacterial Gilll Disease, D. Disease Signs and 1.5 Columnaris Disease, D. Disease Signs). Pre-study daily mortality records will be used to demonstrate the increase in mortality (a copy of the facility records capturing this data will be included in the FSR). Typical disease signs (Appendix B) should be detectable in at least a few fish and the causative agent presumptively identified in most moribund fish. The level of disease (and corresponding morbidity/mortality) should be adequate to warrant treatment. However, the level of disease-related mortality needs to be low enough (i.e., the disease is in early stages of development throughout the population) to obtain control by administering Chl-T. If the level of the disease is too advanced throughout the population and an epizootic is imminent, Chl-T treatment may be ineffective. Therefore, prompt diagnosis and treatment is imperative. Diagnosis will consist of a necropsy performed on 10 fish from the reference population (Appendix B, Noga 1996) by qualified study personnel and presence of BGD or EC confirmed in at least 7 of the 10 fish.

5.3 Exclusion criteria:

An experimental unit will be excluded from the study if a “fatal” event occurs, such as 1) multiple disease infections among test fish; 2) water flow interruption for a period of time that unduly stresses test fish (unduly stressed: position in water column is not normally distributed (e.g., all fish are at the surface or at the tail end of the tank or raceway), fish losing equilibrium, and hyperactivity and jumping); 3) a standpipe is left out resulting in dewatering of the experimental unit for a period of time that unduly stresses test fish; or 4) an unacceptable number of fish jump from an experimental unit or are preyed upon by bird or mammal (≥20% of fish). Fish used in Section 5.2 to determine disease uniformity will be evaluated using a standard fish health diagnostic scheme to determine if other infectious fish pathogens are present at levels to adversely affect the outcome of the study (Appendix B). If it is discovered that mortality/morbidity among test fish may not be attributed to BGD or EC, then steps for exclusion will be taken. If exclusion of any experimental unit is deemed necessary, results from this experimental unit will be excluded from analysis, and it will be documented and described in the FSR.

5.4 Acclimation of test fish:

5.4.1 Duration:

Test conditions will be nearly identical to conditions fish in the reference population were subject to when: 1) fish in the reference population initially became infected with BGD or EC; 2) fish began to show clinical sign(s) of the disease; and 3) BGD or EC – related fish mortality increased. The ≥1-d period between when test fish are transferred from the reference population to experimental units and when the first Chl-T treatment is administered will be termed the acclimation period. If an acclimation period longer or shorter than 1-d is used, the duration and reasons for using such an acclimation period will be described in the FSR.

5.4.2 Medication and/or vaccination during acclimation period:

During the acclimation period, no medication or vaccinations will be administered to test fish.

5.4.3 Baseline data collected prior to initiating study:

(1) Daily mortality in the rearing unit(s) holding the reference population will be collected for at least 3 – 5 d before allocating fish to test units (a copy or transcription of the facility records capturing this data will be included in the FSR), (2) A sample of fish (10) from the reference population will be examined before starting the study to presumptively confirm that mortality is related to BGD or EC (see section 5.2 Inclusion Criteria), (3) Water temperature and dissolved oxygen concentration in the reference population tank will be measured, (4) Size of reference population tank or raceway will be recorded, (5) Loading density of the reference population, (6) Size of the experimental units, (7) A sample count to determine size of test fish, and (8) Water inflow to the experimental units. All the information on Form 1 will be completed (Appendix C).

5.5 Blinding of study:

5.5.1 Extent of blinding:

At least one study participant will be non-blinded and know the treatment condition of each experimental unit. Non-blinded study participant(s) will not be involved in the day-to-day data collection during the course of the study. Blinded study participants will collect data critical to addressing the study objective (e.g., mortality, behavior, appetite, dose verification) as well as measure daily water quality parameters and perform routine fish care procedures (e.g., tank cleaning and fish feeding). Non-blinded study participant(s) will be responsible for (1) dosing treated experimental units with Chl-T and dosing untreated experimental units with hatchery water or (2) coding Chl-T and sham water treatment containers in such a manner that blinded personnel can dose experimental units without knowing which units

receive Chl-T and which units do not (see Form 7). Some tasks, such as allocating fish to experimental units, sample counting, measuring water hardness, alkalinity, and pH may be done by either blinded or non-blinded study personnel.

5.5.2 Blinding method(s) and procedure(s):

A single blinding method will be used.

5.5.3 List of personnel with access to treatment codes and rational:

Study personnel with access to treatment codes is not known at this time but will be determined before the start of each study, The SD and OSI will agree which study personnel will be designated to have access to the treatment code. At least one study participant with access to the treatment code will possess sufficient scientific background to assist in the study, but will not play a role in the day-to-day activities of the study.

5.6 Drug administration:

5.6.1 Dosing regimen:

The dosing regimen for all studies will be 20 mg/L Chl-T in a static bath for 60 min. Fish will be treated three times on alternate or consecutive days.

5.6.2 Route of administration:

All treatments will be administered as static bath treatments. Inflowing water to experimental units will be turned off, Chl-T (or the sham control) will be added to a unit as a stock solution, and contents of the experimental unit will be stirred to ensure a homogeneous solution. At the end of the 60 min treatment period, the water flow to each unit will be turned on and the standpipe removed to facilitate flushing the Chl-T solution from the unit.

5.6.3 Investigational withdrawal period:

The investigational withdrawal period will be 0 days (Appendix G).

5.6.4 Proposed withdrawal period:

The proposed withdrawal period for studies conducted under this research protocol will be equal to the duration of the post-treatment period.

5.7 Removal of test fish from the study:

5.7.1 Criteria for removal of subjects from the study:

Only dead or moribund (i.e., nearly dead or approaching death) fish will be removed from experimental units. Note: To avoid complications relative to data analysis, no healthy-appearing live fish will be removed from test tanks once the study starts (see section 5.12).

5.7.2 Procedures for removal of subjects from the study:

Subjects will be removed from experimental units using dip nets. Nets will be sanitized with disinfectant before and after use.

5.7.3 Fate of removed study animals:

Study animals removed from the study will be disposed of in a manner consistent with disposal procedures at the particular study site (e.g., landfill).

5.8 Concurrent/concominant medication/therapies:

No concurrent/concominant medication/therapy will be administered to test fish during the course of the study. If medication/therapy was administered to fish in the reference population before the start of the study, the withdrawal period for that particular therapeutant will have elapsed before fish are included in the study.

5.9 General management practices:

5.9.1 Site visits:

Ideally, the SD (or designee) will be at the study site at least 1-d prior to initiating the treatment phase of the study and will remain on site during the early stage of the treatment period. However, due to the quick onset of BGD or EC, it may be necessary for the SD (or designee) to visit the site before a disease outbreak and work out details associated tank set up, dose verification, sample collections, collecting data, maintaining blinding scheme, etc with the OSI. The OSI (i.e., the facility manager, asst. manager, or a qualified fishery biologist) or their designee will be on site before and during the entire in-life phase of the study. The SM (Dr. Tom Bell or a qualified fish health professional – are required to be familiar with this protocol and Guidance for Industry Document #85 Good Clinical Practice) will also conduct a site visit before the study starts. If the SM can not make a physical site visit, a “phone site-visit” will be convened to ensure that the SM has an opportunity to provide input into the planned study.

5.9.2 Data collection:

The SD and OSI will assign study personnel to collect data. Study personnel responsible for collecting mortality/behavior data, confirming Chl-T doses, or measuring water quality parameters will be adequately trained by the SD or designee.

5.9.3 Frequency of monitoring water chemistry parameters:

Water temperature and dissolved oxygen (DO) concentration will be measured once daily at the tail end of rectangular experimental units or raceways or at any location in circular experimental units. Water hardness, alkalinity, and pH will be measured at least twice over the course of the study.

If measuring hardness, alkalinity, or pH is not possible, it will be acceptable to report historical data.

5.9.3.2 Parameters to be measured prior to initiating study:

Prior to initiating the study, the following will be counted/measured and documented: 1) number of experimental units (i.e., test tanks) used in the study; 2) number of treated and untreated replicate experimental units; 3) mean fish weight (based on sample counts); 4) mean fish length (based on actual measurements); 5) experimental unit shape and dimensions; 6) mean test fish loading density in experimental units; 7) number of test fish per experimental unit; 8) water inflow to experimental units (gpm or Lpm).
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5.9.3.3 What, when, and where parameters will be measured during the study:

Water temperature and DO concentration will be measured once daily in the morning (Form 2). Water hardness, alkalinity, and pH will be measured at least twice during the study (Form 3).

5.9.3.4 Procedures and equipment for assessing treatment parameters:

Water temperature and DO concentration will be measured with a YSI Model 550 DO Meter (or equivalent) according to SOP No. INST 131. Water hardness and alkalinity will be measured using HACH Co. (Loveland, CO) reagents and titrators according to procedures described in SOP No. INST 104 and 105. Water pH will be measured with a YSI Model 60 pH Meter (or equivalent) according to procedures described in SOP No. INST 125. Use of equivalent indicates that study personnel at the study site used their own equipment to measure the variables. Use of instruments other that those described in this section of the protocol will be documented and described in the FSR. Please see Appendix J for suggested water quality parameters.

5.10 Environmental conditions:

Field efficacy studies conducted under this research protocol will be done under environmental conditions similar to those in which fish from the reference population were reared. In addition, such conditions will be nearly identical among all experimental units. Environmental conditions will be different at each study location. Therefore, environmental conditions and physical experimental unit layout will be documented and described in the FSR.

5.11 Tank cleaning:

Experimental units will be cleaned according to routine test site procedures, preferably once daily (in the morning). Tank cleaning is typically done by loosening/removing standpipe and using a brush to re-suspend uneaten feed/fecal material. Re-suspended material will be drawn down the standpipe drain in the tank. Water should be drawn down no further than to a level that will

not unduly stress test fish. Standpipes will then be refitted into experimental units and water level will return to full depth.

5.12 Provisions for necropsy and disposal of expired test fish:

Moribund fish (which should include fish showing clinical signs of BGD or EC; Appendix B) from the reference population will be sampled before the start of the study to presumptively confirm presence of the pathogen of concern (Note – live, healthy-appearing fish will not be sampled during the course of the study). Moribund fish will be those showing clinical signs of disease (Appendix B), not considered "healthy-appearing," and death is imminent. A representative number of fish from the reference population (10 fish) will be sampled and necropsied before the start of the study (see Section 5.2 Inclusion Criteria). During the treatment and post-treatment periods, no fish will be sampled for fish health to re-affirm the diagnosis. If an unexpected rise in mortality occurs (indicating a possible re-infection or another etiological agent), several moribund fish (≥5 fish) should be sampled and necropsied to confirm cause of mortality/morbidity (these moribund fish will be recorded as mortalities for that experimental unit). Necropsies will include using appropriate diagnostic techniques to (1) presumptively confirm presence of external bacterial infections and (2) assess whether there is a systemic bacterial infection that could confound test results (such as microscopic examination of kidney imprints or culturing kidney streaks on appropriate media). Necropsies will be performed by trained study personnel (training documentation to be included in the FSR) following guidelines outlined in references such as Fish Disease, Diagnosis and Treatment (Noga 2000; Appendix B). All fish health results will be documented on Form 4 (or equivalent) and will be included in the FSR.

5.13 Fate of living animals after study completion:

Fate of living test fish after study completion will be at the discretion of the hatchery owner or manager. In most cases, it is presumed fish will be euthanized and discarded according to routine hatchery procedures.

5.14 Test unit configuration:

Configuration of experimental units could be different at each study site. The SD (or designee) and the OSI will discuss potential experimental unit configuration design. An experimental unit configuration, as well as dimensions, standpipe height, water depth, and rearing volume will be documented and described in the FSR.

5.15 Owner consent:

If privately owned, a written letter of consent will be provided to the SD and submitted in the FSR. No such letter will be required at studies conducted by federal or state natural resource agencies.

6. SPECIFICATION OF VARIABLES:

6.1 Primary variable to be measured for evaluating labeled claim:

Mortality will be the primary response variable to be measured for evaluating the label claim. The proposed label claim will read, in part "… to control mortality caused by BGD or EC in a variety of freshwater-reared finfish…"

6.1.1 When primary variables will be assessed:

Mortalities (dead or moribund fish) will be collected, counted, and recorded twice daily.

6.1.2 Procedures for assessing primary variable:

No special procedures are necessary for assessing the primary variable. Dead fish will include moribund fish. Moribund fish will be defined as, in the opinion of study personnel, those approaching death. All study personnel involved in collecting mortality data will have had previous experience.

6.1.3 Equipment used for assessing primary variable:

No specialized equipment will be required to assess the primary variable.

6.1.4 Calculation of derived data:

First, for each experimental unit, daily mortality during the treatment and post-treatment periods will be summed to determine total mortality. Second, total mortality will (typically) be re-expressed as percent total mortality, which will be calculated by dividing total mortality by the number of fish per experimental unit at the start of the study. Third, percent total mortality will be transformed to degrees or radians via an arcsine square root transformation (Zar 1984). Fourth, means (treated vs. control) of the transformed data will (typically) be statistically compared ($\alpha = 0.05$) via a t-test or ANOVA .

Number of fish at the start of the study will be determined using one of the following procedures:

6.1.4.1 Sample counts

A routine hatchery procedure called "sample counting" will be used to determine fish size (Appendix I; Form 11). A subsample of fish from the reference population will be weighed and counted, and then an estimated individual fish weight will be calculated. Based on calculated individual fish weight, a pre-determined weight of fish will be collected, weighed, and transferred to experimental units to achieve target fish numbers. On rare occasions, it may be necessary to separately weigh out all fish in experimental units at

the end of the study. In the event that this procedure is done, a sample count will be performed to estimate mean fish weight at the end of the study, then all fish from each experimental unit will be weighed, and the number of fish remaining in each experimental unit will be estimated by dividing the total fish weight/unit by the mean individual fish weight.

6.1.4.2 Actual counts

One of the following two procedures will be used to determine the number (i.e., actual counts) of fish stocked in each experimental unit.

Actual counts during allocation of fish to tanks: In this procedure, fish will be hand-counted from the reference population into a bucket partially filled with water. The contents of the bucket will then be transferred into an experimental unit. This is an accurate procedure to use when the number of test fish does not exceed 400 fish/experimental unit.

Actual counts at the end of the study: This procedure is bettered suited when transferring large numbers of fish to experimental units, an estimated number of fish will be transferred into an experimental unit using the sample count procedure described in Section 6.1.4.1. At the end of the study, fish remaining alive in each experimental unit will be hand-counted. Results from hand-counting fish from experimental units will be added to the total mortality in each of the experimental units, resulting in an accurate number of fish/experimental unit at the start of the study.

Missing fish: In the event that a discrepancy (i.e., missing fish) in the number of fish/unit is discovered at the end of the study (i.e., when fish are hand-counted into experimental units at the beginning of the study and then hand-counted at the end of the study), all missing fish will be considered mortalities. As such, missing fish will be included when cumulative mortality is calculated in each experimental unit. For ease of reporting (and to minimize confusion when graphing daily mortality), missing fish will be added to cumulative mortality one day after the end of the post-treatment period.

6.1.5 Forms for retention of source data:

Forms used for recording source data will provide space to record data for each experimental unit for each day of the study (Appendix C). There will

be sufficient space on the form so that each entry may be printed clearly and legibly. Space will be provided for the initials of the individual recording data.

6.1.6 Name(s) and address(es) of outside labs used for analysis:

No outside labs will be used for analysis of the primary variable.

6.2 Other variables to be recorded during the study:

No other variables critical to addressing the study objective will be recorded. Other variables to be recorded during the study, such as water quality parameters, have been described elsewhere in the protocol (Appendix J; Section 5.9.3: Frequency of monitoring water chemistry parameters).

6.3 Adverse reactions:

General fish behavior data, feeding behavior/appetite data, and any potential adverse reactions will be documented on appropriate data collection forms (Forms 5 and 6). General fish behavior data will be documented as either normal or abnormal. If abnormal behavior is noted, then a description of that behavior (i.e., hyperactive, lethargic, position in rearing unit, flashing, etc.) will be documented. An ordinal scale with 3 defined categories will be used to document feeding behavior/appetite. Data collection forms will include a definition of ordinal scales used to characterize feeding behavior/appetite, and will be designed to make the collection of data as objective as possible. Adverse reactions will include any observation in test fish that is unfavorable and unintended and occurs after exposure to Chl-T, whether or not the observation is considered to be product related. Other adverse reactions could include excessive mortality, power outages, pump failures, water heater failure, and loss of water to describe a few. Other adverse reactions should be documented in detail on Form 10. General fish behavior, feeding behavior/appetite data, and other adverse reactions will be summarized and discussed in the FSR.

6.4 Study facilities:

The name and address of each study facility (i.e., the hatchery or research center where a clinical field efficacy study takes place) will be documented and described in the FSR. A number of locations, including federal, state, private, and tribal hatcheries, are potential study facilities. All study facilities will be suitable for conducting adequate and well-controlled pivotal field efficacy studies.

6.4.1 Containment equipment:

Test fish will be contained in suitable fish tanks or raceways. Fish tanks will be constructed of fiberglass, plastic, aluminum, or cement. Raceways will typically be constructed of cement. Type and/or size of containment equipment should not be of critical importance to the successful completion of a pivotal field efficacy study. The containment equipment (i.e., the experimental unit or test tank) used in each study will be documented and described in the FSR.

6.4.2 Lighting equipment:
No specialized lighting equipment will be used. Photoperiod will be similar among all test units at a particular facility, but may differ from facility to facility. When studies are conducted outdoor, it is unlikely that artificial light will be used (security lights may be on). When studies are conducted indoor, artificial lighting will supplement natural light.

6.4.3 Heating equipment:
No specialized heating equipment will be used.

6.4.4 Cooling equipment:
No specialized cooling equipment will be used.

6.4.5 Feeding equipment:
Typically, fish will be fed by hand. However, automatic feeders, such as Ziegler belt feeders, may be used. Feeding methods and equipment used will be described in the FSR.

6.4.6 Watering equipment:
Water inflow to experimental units will be gravity-fed or pumped. No other specialized water equipment will be used.

6.4.7 Ventilation equipment:
No specialized ventilation equipment will be used.

6.4.8 Space allocation of test units:
Space allocation of test units will be sufficient so that study personnel can adequately feed fish and collect data. To ensure minimizing variability between test units, all test units will typically be positioned side-by-side in the same physical location.

6.4.9 Pasture allocation:
N/A

6.4.10 Facility diagram:
The layout of the complete study facility will not have an impact on study results. Consequently, a facility diagram for each study site will not be documented or included in the FSR. However, a diagram of the specific study layout will be included in the FSR.

6.5 Experimental diets:
N/A

7. DATA ANALYSIS:

7.1 Define the experimental unit:

The experimental unit will be the test tank.

7.2. Define the number of replicates per treatment:

There will be a minimum of three replicates per treatment group.

7.3 Define statistical methodology:

7.3.1 Null hypothesis:

H_o: $\mu_{\text{treated}} \geq \mu_{\text{non-treated (control)}}$

Mean percent total mortality in experimental units of test fish treated with Chl-T at a dosage of 20 mg/L for 60 min three times on alternate or consecutive days *is equal to or greater than that* in experimental units of test fish not treated with Chl-T.

7.3.2 Alternative (research) hypothesis:

H_a: $\mu_{\text{treated}} < \mu_{\text{non-treated (control)}}$

Mean percent total in experimental units of test fish treated with Chl-T at a dosage of 20 mg/L for 60 min three times on alternate or consecutive days *is less than that* in experimental units of test fish not treated with Chl-T.

7.3.3 Assumptions:

1) Two normally distributed populations.
2) Equality of variances is known.
3) Independent random samples of size n_1 and n_2.

7.3.4 Biostatistical procedures used:

A generalized linear model will be used to analyze mortality. The analysis will be done using Proc GLIMMIX or equivalent (SYSTAT or SIGMASTAT equivalent to SAS Proc GLIMMIX). The model will include treatment as a fixed effect and other effects related to the design (e.g., random block effects) will be included when appropriate. Where differences are stated to be significant, an alpha level of 0.05 is implied.

7.3.5 Statistical data software to be used:

The statistical software packages to be used will be SYSTAT (SYSTAT 2006a), SigmaPlot (SYSTAT 2006b), SigmaStat (SYSTAT 2006c), or equivalent.

7.4 Define how the statistical results will be used to draw conclusions about the study's objective:

If mean percent total mortality in the treated tanks is significantly less than that in the non-treated (control) tanks, then we will conclude that the Chl-T treatment regimen administered to the treated tanks was effective in controlling mortality caused by BGD or EC. However, if mean percent total mortality in the treated tanks is not significantly less than that in the non-treated (control) tanks, then we will conclude that the Chl-T treatment regimen administered to the treated tanks was not effective in controlling mortality caused by BGD or EC.

8. ANALYTICAL METHODS:

8.1 Describe the analytical measurement to be made and the relevance to the protocol objective:

The only analytical measurement to be made is to confirm Chl-T dose during the treatment period. This method is a DPD colorimetric method using a Hach Test Kit for Chlorine and reagents (powder pillows) for both free and total chlorine (SOP No. INST 101 and MISC 202). Measurements will be made using a Chlorine Pocket Colorimeter (HACH Co, Loveland, CO). Analytical confirmation of treatment dose is important to verify the working treatment concentration.

8.2 Specify the analytical plan to be used for the protocol measurements:

8.2.1 An abstract of the method:

See Appendix F for a description of the method.

8.2.2 Description of procedures for sample selection, preparation, and storage:

Approximately 30 - 45 min into the 60-min treatment period, water samples will be collected for Chl-T dose-verification. One water sample will be collected from each experimental unit. One randomly selected sample collected from treated tanks and one randomly selected sampled collected from control tanks will be measured on each treatment day. In the event that results from the dose verification of the one treated sample is greater than ± 25% of the target dose, then all samples collected on that day will be analyzed. In the event that results from the dose verification of the one control sample is greater than 1 ppm, then all samples collected on that day will be analyzed. Before the study starts, a non-blinded study participant will determine which samples to analyze using a completely randomized procedure following SOP MISC 237 (samples to analyze on the three different treatment days will be selected using a "new" randomization procedure). Water samples will be held until analysis is completed (within 2 h of collection; Appendix F, Dawson et al 2003) and

then will be discarded. Storing samples longer is not recommended as Chl-T breaks down relatively quickly under certain circumstances (SOP MISC 202).

8.2.3 Evidence of methods validation:

See Appendix F for the FDA Letter of Approval of Simple Colorimetric Analysis of Chloramine-T.

8.2.4 Description of validation method plan when method is being developed for the study:

The analytical method used to verify Chl-T concentrations was developed by researchers at the USGS Upper Midwest Environmental Sciences Center (LaCrosse, WI) prior to the beginning of Chl-T field efficacy trials. See Appendix F.

8.2.5 Quality control procedures for the method and criteria used to assess analytical results:

The Chlorine Pocket Colorimeter (SOP No. INST 101) will be zeroed using a blank solution immediately before analyzing samples. A sample batch will consist of all water samples taken during a single treatment event for dose-verification. Raw data for both free and total chlorine and calculations to determine concentration of Chl-T will be recorded on Form 8. Dose verification results and target concentrations will be recorded on Form 9.

8.3 Relevant scientific literature supporting the use of the analytical method for the intended measurements:

See Appendix F for reference titled, “A simple analytical procedure to replace HPLC for monitoring treatment concentration of chloramine-T on fish culture facilities.”

8.4 Certification that all needed validations will be done before the initiation of the study:

See Appendix F.

9. STUDY LOCATIONS:

At this time, study sites have not been definitively identified. Due to the nature of BGD and EC, the fish species that need to be tested, and the willingness of hatchery owners or managers to participate in field effectiveness trials, any list of potential study locations would be inaccurate. As such, study site location will be fully described in the FSR.

10. PERSONNEL:

The SD (or designee) will have had past experience conducting pivotal field efficacy trials using Chl-T (or other chemotherapeutant). The SD and OSI will have had, in many cases, past experience conducting pivotal field efficacy trials. Other study personnel will typically be hatchery biologist or technicians fully capable of collecting and recording mortality data, measuring water temperature and DO concentration, cleaning tanks, and feeding fish. It is not anticipated that study participants will be debarred from participating in a clinical field efficacy trial. However, if this is the case, information related to their debarment will be documented and described in the FSR. A copy of each study participant's curriculum vitae will be included in the FSR.

10.1 Investigators, study monitor, and fish health biologist involved in proposed clinical field efficacy trials:

The names of the OSI and SM (i.e., fish health biologist) will depend on the location of the study site. Potential study sites are in different regions of the USFWS, in different states, or are private or tribal hatcheries or research facilities. The OSI and SM will be fully capable of fulfilling their study responsibilities. The names of the OSI and SM will be included in the FSR.

10.2 Other personnel involved in study:

Other personnel involved in studies will be documented and their names and study involvement will be included in the FSR.

11. COLLECTION AND RETENTION OF SOURCE DATA:

All source data, including those produced electronically, and a copy of all applicable reports will be retained at the Service's AADAP Program in Bozeman, MT in a secure area which protects the source data and records from deterioration, destruction, tampering, and vandalism in accordance to Section 8.4 of the Guidance for Industry #85, Good Clinical Practice (Appendix D).

12. ADDENDUM/DEVIATIONS TO THE PROTOCOL:

12.1 Protocol addendums:

Protocol addendums will be attached to the original version of study protocol CHLT-07-EFF.1, "The efficacy of chloramine-T to control mortality due to bacterial gill disease or external columnaris in cool and warmwater finfish." If a protocol addendum is sent to FDA's Center for Veterinary Medicine, a cover letter will accompany the addendum, referencing the submittal date of the above protocol, describing the purpose of the addendums. Addendums and copies of the cover letter sent to FDA will be kept with the original study protocol in Appendix H.

12.2 Protocol amendments:

A signed copy of the Study Protocol will be retained by the OSI during the entire study period. At any time before a study begins, desired changes to the Study Protocol should be brought to the attention of the SD, OSI, and SM. The desired changes will be documented. Documentation will include date of amendment, reason for change, and the amended text, and kept with the original study protocol in Appendix H. The amendment will be signed by the SD. Copies of the signed amendment will be attached to the original Study Protocol. All study personnel will be made aware or protocol amendments prior to starting a study.

12.3. Protocol deviations:

Deviations from the established Study Protocol will be documented in the FSR. If deviations occur, the SD and OSI should be contacted immediately for advice. The SD will let the SM know of the deviation and course of action. Protocol deviations will be documented fully when they occur, accompanied by a written explanation of what happened, why, and what steps were taken to mitigate the deviation (Form 10). If deviation statements are entered in log books as raw data, they will be signed and dated.

13. DRUG DISPOSITION/ANIMAL ACCOUNTABILITY/FEED DISPOSITION/FEED ACCOUNTABILITY:

Unused drug remaining at the end of a study can be kept on-site for future use according to the Service's Study Protocol for a Compassionate Aquaculture INAD Exemption – Chloramine-T INAD #9321.

14. REFERENCES:

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Zar, J.H. 1984. Biostatistical analysis, 2nd Edition. Prentice-Hall, Inc., Englewood Cliffs, NJ.

15. FORMS

Form 1. Test site information, species tested, and culture conditions.
Form 2. Daily mortality, temperature, and dissolved oxygen concentration record for pivotal studies evaluating the efficacy of chloramine-T
Form 3. Record of water hardness, alkalinity, and pH in pivotal studies evaluating the efficacy of chloramine-T to control mortality

Form 4. Fish health evaluation sheet
Form 5. General fish behavior observation record during chloramine-T pivotal efficacy studies
Form 6. Feeding observation record during chloramine-T pivotal efficacy studies
Form 7. Chloramine-T identification codes.
Form 8. Chloramine-T analysis raw data record.
Form 9. Chloramine-T analysis summary record for pivotal efficacy studies.
Form 10. Deviation and adverse event documentation.
Form 11. Sample counting information and fish lengths for Chloramine-T pivotal efficacy studies.