Efficacy of Chloramine-T for Control of Mortality Associated with Bacterial Gill Disease and Flexibacteriosis in a variety of Fish Species.

Protocol #4000 - 1

Sponsor:
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Pivotal Clinical Field Efficacy Protocol

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1. INTRODUCTION:

1.1 Objective:

1. To evaluate the efficacy of chloramine-T administered as a bath solution every other day for 60 minutes for a total of three treatments (in a flow-through system) to control mortality caused with bacterial gill disease (BGD) and flexibacteriosis associated with flavobacters in a variety of fish species (species of fish will be dependant upon study site - see Table 5.1.2).

2. H_o : Mortality caused by BGD or flexibacteriosis caused by external infections of flavobacters is equal between fish treated with 10 - 20 mg/L chloramine-T on alternate days for 60 minutes for a total of 3 treatments using a standing bath or flow-through method, and fish that receive no chloramine-T treatment.

3. H_a : Mortality associated with BGD or flexibacteriosis caused by external infections of flavobacters will be lower among fish treated with 10 - 20 mg/L chloramine-T on alternate days for 60 minutes for a total of 3 treatments using a standing bath or flow-through method than of mortality of untreated fish.

4. Investigator, study monitor and hatchery personnel involved in the conduct of the study will be thoroughly familiar with both the Guide for Implementing the Chloramine-T INAD by the USFWS and Study Protocol for a Compassionate Aquaculture Investigational New Animal Drug Exemption - Chloramine-T INAD #4000 (see Attachment I); and the Study Protocol for Efficacy of Chloramine-T To Control Mortality Associated with Bacterial Gill Disease and Flexibacteriosis in a variety of Fish Species - Protocol #4000 - 1; and with Conduct of Clinical Investigations: Responsibilities of Clinical Investigators and Monitors for Investigational New Animal Drug Studies (FDA document dated Oct. 1992 or later - see Attachment II).

1.2 Background and Justification:

Chloramine-T has historically been the drug of choice when diagnostic evidence shows salmonids to have either bacterial gill disease (BGD) or flexibacteriosis. These diseases can pose a serious threat to fish survival in intensive culture programs. Although death is usually a result of damage caused by massive infection of the gills, stressors associated with intense fish culture often predispose fish to infection. No single pathogen appears to be responsible for BGD or flexibacteriosis, but all

known agents are gram-negative bacteria, including flexibacteria and flavobacteria. Integrated fish health management practices usually prevent the occurrence of these diseases. However, numerous factors can lead to severe disease outbreaks requiring prompt treatment to prevent losses of fish valuable to natural resource stewardship. Results of ancillary chloramine-T studies conducted by the U.S. Fish and Wildlife Service (Service) under INAD exemption during the 1996 reporting year have shown that therapeutic treatment at 10 or 15 mg/L chloramine-T administered three times on alternate days for 60 minutes appeared efficacious in approximately 87% of treatment trials. Treatment at lower concentrations, or when chloramine-T was administered fewer than three times also appeared efficacious, although not to the degree of the above treatment regime.

The objective of these field based clinical efficacy trials is to evaluate the efficacy of a single therapeutic chloramine-T treatment regime to control mortality in a variety of fish species caused by BGD and flexibacteriosis associated with flavobacters at a number of study sites. Fish species that may be tested include, but are not limited to, *Oncorhynchus mykiss* (rainbow trout), *Salvelinus fontinalis* (brook trout), *Oncorhynchus apache* (Apache trout), *Oncorhynchus keta* (chum salmon), *Oncorhynchus nerka* (kokanee salmon), *Salmo salar* (Atlantic salmon), *Acipenser oxyrhynchus* (Atlantic sturgeon), *Acipenser brevirostrum* (shortnose sturgeon), *Acipenser fluvescens* (lake sturgeon), *Polyodon spathula* (paddlefish), *Morone saxatilis* (striped bass) and *Lepomis macrochirus* (bluegill). Fish size in most studies will ranged from 1 - 6 inches. Fish in this size range are typically reared during a time of year in which the prevalence of pathogens associated with BGD and flexibacteriosis (e.g. flavobacters) is highest, and disease outbreak is most likely to occur. Studies will be conducted at the following U.S. Fish and Wildlife Service facilities: the Bozeman Fish Technology Center - Bozeman, MT; Neosho National Fish Hatchery (NFH) - Neosho, MO; Quilcene NFH - Quilcene, WA; Alchesay-Williams Creek NFH Complex - Whiteriver, AZ; Jones Hole NFH - Jones Hole, UT; Hotchkiss NFH - Hotchkiss, CO; Jordan River NFH - Elmira, MI, and Creston NFH - Creston, MT. These sites were selected as potential pivotal study sites for the following reasons: 1) each has historical, predictable, recurring outbreaks of the disease or are able to induce the disease; 2) each has space in their hatchery buildings to dedicate to conducting studies; 3) each has additional resources available to conduct studies (i.e. - test units, test animals, staff to monitor study; 4) and each has demonstrated commitment to adherence to protocols and guidelines. Studies will be conducted under compassionate Investigational New Animal Drug exemption (#4000) and are intended to

conducted under compassionate Investigational New Animal Drug exemption (#4000) and are intended to provide the U.S. Food and Drug Administration/Center for Veterinary Medicine with field based clinical efficacy data.

2. INVESTIGATIONAL DRUG AND CONTROL:

2.1 Test Substances:

2.1.1 Trade name:
Chloramine-T (halamide)

2.1.2 Chemical name (active component(s):
a) Sodium p-toluenesulfonchloramide
b) N-chloro-4-methylbenzenesulfonamide sodium salt

2.1.3 Molecular formula

$C_7H_7ClNNaO_2S$

2.1.4 Appearance and odor:
White crystalline powder with weak chlorine odor

2.1.5 Active/inactive ingredients
Benzenesulfonamide, N-chlorami-4-methyl-, sodium salt. This is the final formula.

2.1.6 Dosage Form
Chloramine-T for use in treating BGD and flexibacteriosis in fish is a pure, water soluble compound and is not formulated in any way. During use, Chloramine-T is dissolved in water and applied as a bath solution at a specific concentration for 1 hour and then flushed from the fish-holding container, or metered for 1 hour at a flow adequate to achieve the desired treatment concentration in a flowing system.

2.1.7 Dose(s) to be tested
10 mg/L chloramine-T

2.1.8 Manufacturing site
Akzo Chemical, Inc.
300 South Riverside Plaza

Chicago, IL 60606

2.1.9 Lot Number

Studies will be conducted at numerous sites that have already purchased their own supply of chloramine-T. The table below lists participating facilities and corresponding chloramine-T lot number. Chloramine-T lot numbers will also be listed in the methods section of the final report submitted to FDA/CVM following each field based clinical study.

Table 2.1.9 Participating facilities and corresponding chloramine-T lot numbers

Facility	Chloramine-T Lot Number
Bozeman Fish Technology Center	0299303520272
Quilcene National Fish Hatchery (NFH)	0299302530118
Alchesay-Williams Creek NFH Complex	0299302520118
Neosho NFH	0299302520199 358151 (1994)
Northeast Fisheries Center	To Be Determined (TBD)
Creston NFH	0299303520272
Hotchkiss NFH	0299408520652 or 0299302520101
Jones Hole NFH	0299302520118
Jordan River NFH	**0299302520118**

2.1.10 Packaging

Chloramine-T will be stored in its original packaging, which consists of a durable paper sack placed in a heavy cardboard barrel. Barrels are equipped with metal locking rings and lids for security.

2.1.11 Drug storage during study.

Chloramine-T will be stored in the original container supplied by the Manufacturer with the appropriate investigational label attached. The container will be stored in a cool, dry location away from direct sunlight. The container will NOT be stored in a refrigerator since opening a cold container can cause

condensation of moisture on Chloramine-T. Chloramine-T should be stored in a secure location such as in a locked cabinet.

2.1.12 Drug handling procedures:

Each Study Monitor and Investigator will be provided with a current copy of the Material Safety Data Sheet (MSDS) for Chloramine-T (Appendix I). Each person involved with the study and each person who may be present during the use of Chloramine-T shall be required to read the MSDS. Safety precautions as outlined in the MSDS will be followed at all times when working with Chloramine-T. **As a special precautionary note, Chloramine-T may explode violently when heated above 130° C.**

2.1.13 Verification of drug integrity/strength:

The Manufacturer (Akzo Chemicals, Inc.) will provide the analytical data necessary to establish purity of each lot of Chloramine-T supplied. The lot number and date of manufacture for each batch of Chloramine-T will be placed on the label of each container. The form "Guide for Reporting Investigational New Animal Drug Shipments for Poikilothermic Food Animals" (Form 1) will clearly identify the lot number and date of manufacture of Chloramine-T shipments. If the integrity of the Chloramine-T is compromised (i.e., by spilling or contamination of the stock container) the event will be carefully recorded, dated, and signed in the Chemical Use Log (Form 2). The Study Monitor assigned to the Investigator involved will be immediately notified and the remaining material will be returned to the Study Monitor along with the properly recorded Form 1.

2.1.14 Investigational labeling:

Copies of the labels to be attached to each container of Chloramine-T will be provided to all facilities participating in pivotal field based clinical efficacy trials. It will be the responsibility of the Investigator to ensure proper labeling of all containers of Chloramine-T. Copies of investigational labels (See Appendix II) will be included in the final report.

2.1.15 Accountability:

Each Investigator will notify FDA prior to any shipment of Chloramine-T for use under this INAD. Immediately upon placing an order with the approved supplier, the Investigator will complete Form 1, "Guide for Reporting Investigational New Animal Drug Shipments for Poikilothermic Food Animals" and send it to the

Study Monitor. The Study Monitor will then send the original plus two copies to the FDA. Both the Investigator and the Study Monitor are required to sign Form 1. The Study Monitor will send a single copy of Form 1 to the Study Director at the Bozeman National INAD Office. The Investigator will keep one copy of the completed Form 1 for the facility's INAD file. Arrangements should be made between Investigators and Study Monitors to insure completed Form 1's are received by the FDA within 7 days of the date an order was placed. If chloramine-T is presently on-station, a copy of the Form 1 on file at the National INAD Office (NIO) will be attached to the final report.

Investigators are also responsible for maintaining an accurate inventory of Chloramine-T on-hand. A Chemical Use Log (Form 2) will be supplied to each Investigator. Each time Chloramine-T is used, it must be reported by the Investigator on Form 2.

At the conclusion of the study, all remaining Chloramine-T will remain on site for further use. The properly recorded Chemical Use Log (Form 2) will be sent to the Study Monitor.

2.1.16 Material Safety Data Sheet (MSDS)

A complete MSDS is included in Appendix I.

2.2 Controls:

Controls will be fish that receive no chloramine-T treatments. Water will be metered into non-treated test units, in a manner identical to the treatment method.

3. STUDY SCHEDULES:

3.1 Proposed date(s) of initiation:

Proposed dates of initiation will be study site specific. Studies will be conducted at several U. S. Fish and Wildlife Service National Fish Hatcheries and Technology Centers. These locations have a history of predictable, recurring outbreaks of bacterial gill disease (BGD) and/or flexibacteriosis. Studies will be initiated when there is a disease outbreak and the Investigator is available to initiate chloramine-T treatment. There is a very good probability that more than one site will experience a disease outbreak at approximately the same time, and both of the Investigator will be unable to be on-site to conduct the study. In the event that one of the above, or a similar scenario surfaces, initiation of studies will be addressed on a case-by-case basis. Table 3.1 lists facility name

and the time of year in which an outbreak is expected to occur. This table does not commit the listed facilities to conduct a field based clinical efficacy trial during the proposed dates of initiation in both 1997 & 1998. It is merely a guideline as to potential proposed dates of initiation.

Table 3.1 Proposed date(s) of initiation at the pivotal study sites.

Facility	Proposed Date(s) of Initiation
Bozeman Fish Technology Center	January - December, 1997 & 1998
Quilcene National Fish Hatchery (NFH)	March - April, 1997 & 1998
Alchesay-Williams Creek NFH Complex	June - July, 1997 & 1998
Neosho NFH	February - June, 1997 & 1998
Northeast Fisheries Center	June - July, 1998
Creston NFH	March - May, 1997 & 1998
Hotchkiss NFH	December - May, 1997 & 1998
Jones Hole NFH	February - May, 1997 & 1998
Jordan River NFH	**May - June, 1997 & 1998**

3.2 Schedule of events:

3.2.1 Transfer of test animals:

When a raceway or tank of fish held at a participating facility begin to show disease symptoms (described in Section 9.2 Inclusion criteria) indicating a disease outbreak, fish will be transferred to test units. Fish will be transferred into test units approximately 24 hours prior to initiation of treatment. Because fish will be transferred only when they become diseased, there is no way to accurately predict the date when fish will be transferred. The disease episode will be site specific. This information will be described in detail in the methods section of the final report.

3.2.2 Initiation of treatment:

Treatment will begin one day following transfer of all test fish into test units. As stated in Section 3.2.1, there is no way to predict the date when treatment will be initiated. This information will be described in the methods section of the final report

3.2.3 Treatment period duration:

The treatment period will begin the day treatment is initiated (day 1) and will last 5 days (day 5).

3.2.4 Post-treatment period duration:

Duration of the post-treatment period will be 14 days. The post-treatment period will begin on day 6 of the study and will end on day 19.

3.2.5 Data analysis and report writing:

Data analysis and preparation of final report will require a maximum of 6 months to complete. Within 6 months of completion of post-treatment period, a final report will be submitted to the Food and Drug Administration/Center for Veterinary Medicine.

3.3 Proposed date(s) of completion:

The data collection portion of individual studies will be completed within 20 days of the initiation of treatment. Final date of completion of individual studies will be approximately 9 months after initiation of treatment. Because potential pivotal study sites will initiate studies based on when there is a disease outbreak at their facility, it is not feasible at this time to propose individual dates of completion. The proposed date for completion of all studies is December, 1998.

4. STUDY DESIGN:

4.1 Treatment groups:

Each study will consist of a single treatment group and a single non-treated (control) group. All fish for a particular study will consist of diseased fish from the same fish lot. Study fish will be reared in either a single common rearing unit or in multiple experimental test units. Treated groups will be treated at 10 -20 mg/L chloramine-T. Control groups will receive no chemical treatment, but will receive a pure water "sham" treatment. There will be three replicates per treatment group.

4.2 Experimental design:

The experimental design used will be completely randomized.

4.3 Blocking factor(s):

No blocking factor(s) will be used in the study design. All rearing units will be plumbed with the same water supply in a confined area in a hatchery building. All units will be exposed to the same ambient temperature and photoperiod, and fed the same diet. Water chemistry conditions, such as temperature, dissolved oxygen, pH, and hardness should be consistent among all tanks.

4.4 Randomization procedures:

4.4.1 Allocation of animals to test units:

Animals will be randomly placed in one of six test units in such a manner as to minimize bias. The six test units will be numbered 1 through 6. Fish will be distributed into individual test units in stages to minimize bias. At each stage, a given test unit will receive approximately 25% of the total number of fish to be ultimately transferred to the test unit. A random number table will be used to determine which test unit will receive the first group of fish, which will receive the second, and so on until fish have been transferred into the sixth test unit. Following this procedure, each test unit will receive a second group of fish following the same order as described above, again constituting approximately 25% of the total number of fish to be ultimately transferred. Following the transfer of the second portion of fish, each test unit will hold approximately 50% of the total number of fish to be used in the study. This procedure will be repeated two more times, for a total of four times. At this point, 100% of the fish to be used in the study will have been transferred to their respective test units. This procedure will minimize potential bias with regards to health of fish in that not all healthy or sick fish (fish taken from the head or near the bottom of the tank/raceway as opposed to near the surface or tail-end of the tank/raceway) will be placed in one tank and not another.

4.4.2 Allocation of treatment groups to experimental units:

After all fish have been transferred to test units, units will be randomly assigned as either Treatment or Control. A random number table will be used to assign treatment condition to each test unit.

4.5 Configuration of experimental units:

Configuration may be different for each participating facility. Most likely, the configuration will be a single row of 6 test units, two rows of 3 test units, or three rows of 2 test units. Each test unit will be configured and plumbed in the same manner, each will receive water from the same source and hold equal numbers of test fish. However, because none of the participating hatcheries are set-up to run small-scale controlled, replicated clinical field trials, test units will have to be plumbed into in-flowing water lines and effluent waste-water lines to conduct these studies As a result, each facility will have a different configuration. The configuration of experimental test units will be described in detail in the methods section of the final report.

5. STUDY PROCEDURES:

5.1 Test Animals:

5.1.1 Description:

5.1.1.1 Age/Size:

Fish used in the studies will be of varied ages, depending on which fish become diseased at a particular facility. Although at this time the age of the test fish to be used is not known, in most cases fish will be less than one year old and between 1 - 6 inches in length (see Table 5.1.2). Fish in this size range are characterized as fingerlings, and have not yet reached a harvestable size. Because of the uncertainty of the age and size of fish at this time, fish age and size, and whether fish are in the category of fry, fingerling or adult, will be described in detail in the final report.

5.1.1.2 Sex:

Sex of fish used during the study will not be determined. It is assumed that approximately 50% of the test fish will be males and 50% of the test fish will be females. It is also assumed that sex will not be a factor in treatment efficacy.

5.1.1.3 Breed/Class:

Table 5.1.2 lists the potential fish species that may be used at a particular facility. Fish species will be confirmed by the Hatchery Manager.

5.1.1.4 Initial body weight:

As explained in Section 5.1.1.1, the age and size of fish is not precisely known at this time. Initial body weight will be dependant upon when fish become diseased, and at which facility they are reared. As a result, initial body weight will be described in the final report.

5.1.1.5 Physiological state:

Test fish will be in no particular physiological state. In most cases, test fish will not be sexually mature (hatchery reared fish less than 6 inches in length).

5.1.1.6 History of test animals:

Test fish will most likely be from eggs incubated at the study site. Test fish will be reared on site under standard hatchery conditions as described by Piper et al. (1981). A brief description will be included in the final report detailing egg source, egg incubation procedures, management practices and environmental conditions under which test fish were reared prior to study (e.g., type and size

of fish, flow and density index values, water turnover rates, rearing temperature, pH, hardness, dissolved oxygen), and any therapeutic chemical treatment required prior to the study. If such treatment has been deemed necessary, a justification statement for the treatment will be included in the final report.

5.1.2 Number of test animals:

The number of test animals used in each study will be predicated on achieving a flow index and density index that approximates normal production conditions. Use of these indices, which are described below (see Tables 5.1.2.1 & 5.1.2.2), will be used instead of absolute fish numbers. This will be done to achieve a degree of uniformity among all study sites. It is likely that the various study sites will use various sizes and shapes of rearing units (e.g. 2' circular tanks or 4' rectangular troughs) and this will ensure that all studies will be conducted under approximately the same conditions. Table 5.1.2 lists participating facilities, test species and the approximate flow and density index ranges under which clinical trials will be run. If any other species are used as the test animal, the flow index and density index values used and justification for the selected levels will be described in the final report. In the case of sturgeon, numbers of test fish per square foot of bottom surface will be used instead of flow index and density index. Since sturgeon are generally bottom oriented fish, flow index and density index values are not indices typically associated with rearing these fish. The description and application of flow and density indices below are for all test fish species except for sturgeon.

5.1.2.1 Flow index:

A flow index (FI) range, in combination with a density index range, will be used to describe the number of test animals used. Note that ranges will differ for different fish species at a given facility. Flow index is the relationship of fish size/weight to the water flow (flow rate) to a rearing unit, calculated by the formula:

$$FI = \frac{(\text{total number of fish})*(\text{mean weight of fish (lbs)})}{(\text{mean length of fish (in.)})*(\text{water flow rate (gal./minute)})}$$

As the number of fish decreases in a particular test unit as a result of mortality, the flow index will also decrease. No measures will be taken to maintain flow index as the study progresses to the level set at the start of the study. Although fish will be fed during the course of study, no appreciable growth or weight gain is expected. Water flows will be readjusted periodically throughout the study to maintain constant water flow.

5.1.2.2 Density index:

A density index range, in combination with a flow index range, will be used to describe the number of test animals used. Note that ranges will differ for different fish species at a given facility. Density index is the relationship of fish size/weight to water volume of a rearing unit, calculated by the formula

$$\frac{\text{(total number of fish)*(mean weight of fish (lbs))}}{\text{(mean length of fish (inches))*(size of test unit (in cubic feet))}}$$

As the number of fish decreases in a particular test unit as a result of mortality, the density index will also decrease. No steps will be taken to lower the volume of water in the test unit to maintain the density index as the study progresses at the level set at the start of the study. Although fish will be fed during the course of study, no appreciable growth or weight gain is expected.

5.1.3 Source of animals:

Source of test animals will be recorded in Form 3. At this time the source of test animals is not known and may be different for each participating facility.

5.1.4 Identification method if not client-owned companion animals:

Test animals will not possess any artificial or man-made identification. Measures will be taken to ensure test animals from one test unit do not mix with test animals from another test unit. This will be accomplished by methods such as covering test units with screens or ensuring the distance from one test unit to another is sufficient enough so that test fish cannot jump from one to another. Because methods may differ among facilities, the method used to prevent fish from moving from one test unit to another will be described in the final report, .

Table 5.1.2 Fish species,size range, and target flow index and density index range

Facility	Fish Species	Size Range (inches)	Flow Index Range	Density Index Range
Hotchkiss NFH	Rainbow Trout	1 - 6	0.8 - 1.4	0.1 - 0.75
Neosho NFH	Rainbow Trout	2 - 4	1.5 - 3.0	0.3 - 0.7
	Brook Trout	2 - 4	1.5 - 3.0	0.3 - 0.7
	Paddlefish	2 - 4	1.5 - 3.0	< 0.1
	Lake Sturgeon	2 - 4	20 - 50/square ft. bottom area	

Table 5.1.2 (continued) Fish species, size range, and target flow index and density index range

Facility	Fish Species	Size Range (inches)	Flow Index Range	Density Index Range
Northeast Fisheries Center	Rainbow Trout	4 - 6	0.5 - 2.0	0.1 - 1.0
	Atl. Sturgeon	4 - 6	1.0 - 2.5	0.1 - 1.0
	Shortnose Sturgeon	3 - 5	1.0 - 2.5	0.1 - 1.0
	Atl. Salmon	4 - 6	0.5 - 2.0	0.1 - 1.0
	Bluegill	1 - 3	1.0 - 2.5	0.1 - 1.0
	Striped Bass	2 - 4	1.0 - 2.5	0.1 - 1.0
Jones Hole NFH	Rainbow Trout	1 - 3	1.0 - 1.2	0.3 - 0.4
	Rainbow Trout	6 - 8	1.2 - 1.4	0.4 - 0.5
Quilcene NFH	Fall Chum Salmon	1 - 2	1.5 - 2.2	0.5 - 1.0
Alchesay-Williams Creek NFH Complex	Apache Trout	1 - 3	1.0 - 2.5	0.2 - 0.5
Jordan River NFH	Lake Trout	1.5 - 3	0.75 - 2.0	0.2 - .06
Bozeman FTC	Rainbow Trout	1 - 6	0.75 - 1.5	0.4 - 0.8
Creston NFH	Kokanee Salmon	1 - 2	1.0 - 2.0	0.4 - 0.6

5.2 Inclusion criteria:

The entrance criteria for inclusion in the study will include the following: 1) a holding tank of fish are diagnosed with BGD and/or external flexibacteriosis, and the diagnosis is confirmed by the presence of external infections of flexibacteria/flavobacteria; 2) the study investigator is on site to initiate transfer of fish and initiate start of treatment; and 3)

enough test units and test animals are available to conduct the study in triplicate under production-like conditions. It will be assumed that the study monitor, investigator and other personnel involved in the study are thoroughly familiar with protocols and guidelines listed in Section 1.1 paragraph 4, and are committed to following through with study until completion.

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

Investigator will be fully capable of ensuring all requirements of the protocol are fulfilled. Study sites were selected, in part, because of historical recurring outbreaks of BGD/flexibacteriosis, and also because Investigator (s) has experience using chloramine-T in accordance with the compassionate INAD protocol to treat for the disease. Not only will the investigator be thoroughly familiar with the Study Protocol for a Compassionate Aquaculture Investigational New Animal Drug Exemption - Chloramine-T INAD #4000 (see Attachment I), but also the Study Protocol for Efficacy of Chloramine-T Treatment for the Control of Mortality Associated with Bacterial Gill Disease and Flexibacteriosis in a variety of Fish Species - Protocol #4000 - 1; and with Conduct of Clinical Investigations: Responsibilities of Clinical Investigators and Monitors for Investigational New Animal Drug Studies (dated Oct. 1992 or later - see Attachment II). Although the investigator will be capable of determining whether fish are infected with flavobacteria/flexibacteria from past experience, a Fish Health Biologist will evaluate prepared slides and confirm findings of investigator by examining wet mount or prepared gill squash slides.

5.2.2 Diagnosis of BGD:

Diagnosis of BGD will consist of observation of bacterial masses (filamentous bacteria) in a direct gill squash. A description of how to prepare a gill squash is detailed in Appendix III. Preparation of a gill squash involves removing several gill filaments with scissors and placing them on a clean microscope slide. Another clean slide is used to "squash" and macerate the gill tissue; larger material is scraped off until a thin preparation has been made. The preparation is allowed to dry completely. One of two methods can be used to fix the slide: 1) slide is passed through a flame a few times to heat fix; or 2) 3-6 drops of 90% methyl alcohol are added to the slide, a match is used to ignite it and it is allowed to cool. After fixing, the slide is then stained with methylene blue (60 seconds), rinsed thoroughly with water, blotted gently, air dried, and examined under oil immersion. Standard procedures will be followed in preparing and storing methylene blue, as described in Appendix IV.

Observation of long bacterial filaments in gill squashes are adequate for routine diagnosis of BGD.

5.2.3 Diagnosis of Flexibacteriosis:

5.2.3.1 Early signs of flexibacteriosis:

An early sign of flexibacteriosis is a thickening of mucous at various spots on the head, opercula, fins or around skin injuries. Fish go "off feed" and fins may develop necrotic lesions on the outer edges.

5.2.3.2 Diagnosis of flexibacteriosis:

The presumptive diagnosis of flexibacteriosis is based on the presence of long, thin, gram negative rods taken from necrotic lesions on the surface of the skin, or by squash preparations made from the affected areas on the gills (see Appendix III). Examination of gill arches will reveal high numbers of bacterial cells massed on the tissue surface as detected in a wet mount preparation under a microscope. Further diagnosis of flexibacteriosis requires isolation of the bacterium on Cytophaga, TYES, Brain-Heart Infusion Agar, Shiehs, or a similar medium. Identification of the colonies isolated on the media is accomplished by standard biochemical characteristics for each species, although this motile gram-negative bacteria is typically difficult to culture on routine media (FWS 1995), and definitive identification may not be possible (Spear 1995).

5.2.4 Level of disease:

Ideally, there should be increased morbidity or mortality rates among fish with disease signs typical of bacterial infections that cannot be treated by other means. Typical disease signs should be detectable in at least a few fish and the causative bacterial agent identified. However, the level of BGD or flexibacteriosis must be relatively low or in the early stages of development to obtain control. If the level of the disease is far advanced, complete mortality may result. Therefore, prompt diagnosis and treatment is imperative. In general, the investigator will respond with diagnosis and initiation of fish transfer into test units when daily mortality rates exceed approximately 0.1% of the total fish in a holding unit. Unlike BGD, which is an external infection, flexibacteriosis starts out externally and, will become systemic and cause blood septicemia if no treated promptly.

5.2.5 Uniformity of disease - BGD only:

Gill squashes will be prepared and evaluated from 5 fish from each test unit immediately prior to initiation of treatment. Comparison of disease level may only be used to support the assumption that because all test fish were originally from the same rearing unit and/or fish lot, that the level of infection will be the same. Evaluation of gill squashes will confirm level of disease uniformity. However, data may also be used as a covariate in data analyses where the mortality data may be pooled across locations and time. The data collected will be of useful quality. The procedure to be used to select and remove fish from each tank to evaluate level of bacterial presence/absence from gills is as follows: Fish will be crowded into the tail-end of the rearing unit as tightly as possible. Fish will be scooped up into a small net and the first 5 that voluntarily fall from the net as it is "tipped" will constitute a sample. Each fish will receive a fish health evaluation and results will be recorded on Form 11 to document overall fish health. A wet mount gill slide will be prepared using the first gill from each fish. Wet mounts will be examined at 400x magnification. Ten to fifteen fields will be evaluated for presence/absence of bacteria and results from the evaluation will be recorded on Form 12. Presence will be noted if a single filamentous bacteria is present. At least one wet mount sample from each test unit will be stained with methylene blue or crystal violet for confirmation. Bacterial evaluations will be conducted by either a fish health biologist or the investigator. The number of fish sampled from each test unit and the number of fields of view evaluated for each fish will be consistent among all test units for a particular study.

5.3 Exclusion criteria:

A test unit will be excluded from the study if conditions such as multiple disease outbreaks occur, water flow is interrupted unintentionally for more than a few minutes, a standpipe is left out, or some other similar incident occurs. 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 pathogens are present (see Appendix V). If it is discovered that mortality among diseased fish may not be attributable to external infections of flavobacteria/flexibacteria, then steps for exclusion will be taken. If pre-treatment mortality in any unit is too high, which may possibly jeopardize the integrity of the experimental units with respect to extrapolation to the fish populations sampled, may be excluded from statistical analysis. If exclusion of any test unit is deemed necessary, a full explanation will be described in the final report, as will modifications in the data analysis. If an incident occurs during the course of the study which may introduce variability but does not result in exclusion, it will also be described in the final report.

5.4 Acclimation of test animals:

5.4.1 Duration:

Test conditions will be nearly identical to conditions under which fish became diseased. The 24-hour period between transfer of fish to test units and initiation of treatment will be termed the acclimation period. If a longer acclimation period is used, the duration and reasons for instituting the lengthened acclimation period will be described in the final report.

5.4.2 Medication and/or vaccination during acclimation period:

During the acclimation period, no medication and/or vaccinations will be administered to the test animals. In the event medication and/or vaccinations are deemed necessary, the test unit will be excluded from the study or the study will be terminated.

5.4.3 Baseline data collected prior to initiating study:

Disease level and uniformity, described in Section 5.2.5, will be evaluated prior to initiating study, as will fish length and weight. Mortality data will also be collected from all rearing units prior to initiation of study.

5.5 Blinding of study:

5.5.1 Extent of blinding:

Either the Hatchery Manager or Asst. Hatchery Manager will be the only study participant who has prior knowledge regarding experimental unit treatment condition. This same person will also be the only person on-site with access to the codes indicating which test units are treated and which are controls. A person other than the Hatchery Manager/Asst. Hatchery Manager (re:Investigator - Jim Bowker) will prepare three containers (e.g. erlenmeyer flask) with the appropriate concentration of chloramine-T to deliver 10 mg/L of drug to the test unit (for description of 'appropriate' concentration of chloramine-T see Section 5.6.1 Dosing regime), and three containers with water only. All containers will be identified and will contain the same solution volume. At this time, the person with access to treatment codes will remove the label describing the containers contents and replace it with the tank identification number which corresponds to whether it is a treated or control test unit. The container will then be placed on the test unit with the matching identification number/letter, and treatment initiated. For example, if test units #2, #3 and #5 are to receive chloramine-T treatments and test units #1, #2 and #6 are controls, then the Hatchery Manager/Asst. Manager will remove the chloramine-T treatment tags from the containers containing chloramine-T and replaced with tags marked either tank #2, #3 or #5.

The control label will be removed from the containers holding only water and replace them with tags marked either tank #1, #4 or #6. This way, only the Hatchery Manager/Asst. Manager knows which test unit is receiving which treatment. An inherent blinding problem encountered when using chloramine-T dissolved in water is the discernable chlorine smell. Efforts will be made to minimize the opportunities that those other than the Hatchery Manager/Asst. Manager discover which treatment condition is being administered to which test unit based on residual chlorine smell. The disclosure of who will know treatment codes and who actually administers the chloramine-T treatment will be described in the final report.

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 rationale:
Table 10.2 lists the co-investigators and study monitor for each participating facility. The co-investigator other than Jim Bowker and the study monitor will have access to the treatment codes. The co-investigator other than Jim Bowker will be either the Hatchery Manager or Asst. Hatchery Manager. This person will be in a position of authority and with sufficient scientific background to assist in the study, but will not play a role in the day-to-day activities of the study. The other person with access to treatment codes will be Dave Erdahl, who will act as co-Study Monitor on all Service studies, and also will have access to the codes. Dave Erdahl is the Services National INAD Coordinator, and as one of the two study monitors will have knowledge of each study conducted by the Service, but will not play a role in conducting the study. Treatment condition codes will be recorded on Form 8 by the co-investigator other than Jim Bowker. When this form has been completed, it will be stored in a secure location not accessible to anyone except the listed person. At study termination, this form will be included with other data/information forms.

5.6 Drug Administration:

5.6.1 Dosing regime:
The dosing regime for all studies will be 10 - 20 mg/L chloramine-T every other day for 60 minutes for a total of three treatments using a standing bath or flow-through treatment method. Initiation of treatment will begin on day 1. Fish will be treated again on days 3 and 5. Treatment period is over following the treatment on day 5.

5.6.1.1 Procedures for determining the amount of chloramine-T to be administered:

As with most water-soluble crystals, chloramine-T will be dissolved in an aliquot of water prior to bringing solution up to final volume. The amount of chloramine-T to be dissolved and administered to each treated test unit will be determined using the following formula (Piper et al. 1982):

<u>For a flow through treatment method</u>
[water flow (gpm) x treatment duration (min) x final concentration desired (ppm) x correction factor] ÷ *purity* of chemical

For example, under test conditions in which 10 ppm chloramine-T is to be administered using a flow through method for 60 minutes at a water flow of 1 gallon per minute:

(1 gpm x 60 minutes x 10 ppm x 0.0038) ÷ 1 = 2.3 g chloramine-T

<u>For a standing bath treatment method</u>
[Water volume (gallons) x target treatment concentration (ppm) x correction factor] ÷ purity of chemical

For example, under test conditions in which 10 ppm chloramine-T is to be administered using a standing bath method for 60 minutes in a tank with 2 cubic feet of rearing capacity:

[(2 cubic feet x 7.58 gallons/cubic foot) x 10 ppm x 0.0038] ÷ 1 = 0.6 g chloramine-T.

5.6.1.2 Procedure for administering chloramine-T solution to test units:

5.6.1.2.1 Flow Through Treatment Method

Flow through treatments will actually be charged flow-through treatments. Water will be shut off to each test unit and a measured amount of chloramine-T solution added to achieve the desired target concentration. Water will then be turned back on to the approximate flow rate and chloramine-T administered to test units using a multichannel peristaltic pump (Bran & Lubbe Proportioning Pump, Model II). Water flows will then be readjusted to exactly the predetermined rate. With regard to the flow-through portion of the treatment, the volume of water flowing into each test unit, as well

as the stock solution of chloramine-T metered into the in-flowing water, must be determined accurately in order to maintain the desired treatment concentration. Chloramine-T solutions (see example in Section 5.6.1.1) will have been prepared with precautions to assure the amount of chloramine-T used does not exceed the solubility limits of chloramine-T in water. Chloramine-T solutions will be pumped directly into the area where source water enters the test unit to ensure maximum mixing. Pump delivery volume will be set initially prior to the study. The calibration method used will be to pump pure water from a flask into a graduated cylinder for 15 minutes. The volume delivered in 15 minutes will be multiplied by 4 to get volume delivered in 60 minutes. The pump will be calibrated prior to each treatment to ensure that the pre-determined volume of solution is delivered in 60 minutes.

5.6.1.2.2 Standing Bath Treatment Method

Chloramine-T will be administered to test units using a commercially available plastic watering can. An appropriate amount of chloramine-T (see example in Section 5.6.1.1) will have be added to a 1+ gallon bucket of water. The chloramine-T solution will then be transferred to a plastic watering can. After the water flow to the test unit has been stopped, and with standpipe in place, the contents of the watering can will be poured into the test unit and stirred to ensure thorough mixing. Sham (water) controls will be administered in the sam way.

5.6.1.3 Method for preparing chloramine-T solution:

The pre-determined amount of chloramine-T will be weighed out on an analytical balance capable of weighing material to 0.01 g. Balance manufacture, model number and calibration service will be described in detail in the methods section of the final report. Chloramine-T will then be transferred into 200 - 500 mL capacity flasks containing 100 mL deionized water. Thorough mixing is ensured when a crystalline powder is dissolved in a small amount of water prior to dilution to final volume. Chloramine-T powder will be mixed thoroughly into solution, and deionized water will be added to bring treatment solution up to final volume. Final volume for flow-through treatments will be dictated by pump delivery rate, which will be described in the final report (delivery rate for the Bran & Lubbe Multistaltic pump is 117.5 mLs per hour). For static bath treatment, final volume will exceed the minimum volume of water

required to dissolve the chloramine-T. Solubility of chloramine-T is 150 g per liter at 25° C.

5.6.2 Route of administration:
Water bath/standing bath or flow through treatment

5.6.3 Investigational withdrawal period:
45 days (see Appendix VI)

5.6.4 Proposed withdrawal period:
45 days

5.7 Removal of subjects from study:

5.7.1 Criteria for removal of subjects from the study:
Only dead fish will be removed from the test units.

5.7.2 Procedures for removal of subjects from the study:
Dip nets will be used to remove dead animals from test units. Net(s) will be sanitized after each use.

5.7.3 Fate of removed study animals:
Animals removed from the study will be disposed of in a local landfill or incinerated (Form 13).

5.8 Concurrent/concomitant medications/therapies:

There will be no concurrent/concomitant medication/therapy administered during the course of the study. If concurrent/concomitant medication/therapy is deemed necessary, then the test unit will be removed from the study as described in section 5.3.

5.9 General management practices:

5.9.1 Site visits:
Hatchery personnel (listed in Section 10.2) involved in the study will be on station daily during the course of the study. Jim Bowker, the co-Investigator from the Service's National INAD Office (NIO), will be on station one day prior to the beginning of treatment, and will remain on site during the treatment period of the study. Following treatment, Mr. Bowker will no longer remain on site. The other co-Investigator, listed in Table 10.1, being the Hatchery Manager/Asst. Hatchery Manager, will be on site on weekdays during the course of the study. The co-study monitors will

be Dr. Dave Erdahl, the Services National INAD Coordinator, and the regional Fish Health Biologist (see Table 10.1 Investigators, study monitor and fish health biologists involved in proposed field based clinical efficacy trials). The regional Fish Health Biologist and/or Dr. Erdahl will conduct the site visit according to Conduct of Clinical Investigations: Responsibilities of Clinical Investigators and Monitors for Investigational New Animal Drug Studies (dated Oct. 1992 or later).

5.9.2 Data Collection:
Data will be collected by only the study investigator (other than the investigator with access to treatment codes) and hatchery staff listed in Table 10.2 as other study participants, according to the schedule outline in the protocol.

5.9.3 Frequency of monitoring water chemistry parameters, such as water temperature and dissolved oxygen:
Tables 5.9.3A and 5.9.3B list when and where water chemistry parameters will be measured during the course of the study. To summarize, water temperature and dissolved oxygen will be measured twice daily, at the beginning and end of each workday (e.g.~ 8 a.m. and 3 p.m.). Water hardness and pH will be measured twice during the study, at the beginning and end of the study. Chloramine-T levels in the test units will be measured three times during the study, on each day of treatment.

5.9.3.1 Parameters to be measured prior to initiation of study:
Prior to initiation of treatment, the following will be measured and recorded in Form 3: (1) rearing unit #; (2) treatment condition code; (3) mean fish weight (grams); (4) mean fish length (inches); (5) rearing unit configuration (i.e. - circular, rectangular); (6) rearing unit dimensions; (7) rearing unit size (ft^3); (8) number of fish; and (9) water flow (gallons/minute). Flow index and density index values will be calculated based on the above measured parameters.

5.9.3.2 What, when and where parameters will be measured during study:
Tables 5.9.3A and 5.9.3B describe when and where treatment parameters will be measured during the course of the study. Twice daily indicates at the beginning and end of the work day; daily indicates at the beginning of the work day; twice during study indicates at the beginning and end of the study; during treatment only indicates a single time, 45 minutes into the 60 minute

treatment period. Dissolved oxygen will be recorded on Form 5, water temperature will be recorded on Form 6. Water pH and hardness will be recorded on Form 7. Space will be provided on Form 7 to record actual mLs of titrant used to change sample color and show calculations to determine mg/L $CaCO_s$.

Table 5.9.3A. Parameter/schedule/location for circular test units.

Parameter	Schedule	Location
Dissolved Oxygen	Twice Daily[1]	Mid-Depth - any location
Water Temperature	Twice Daily	Mid-Depth - any location
Water Flow	Daily[2]	at test unit source
pH	Twice during Study[3]	Source water
Hardness (as $CaCO_3$)	Twice during Study	Source water
Chloramine-T	During treatment only[4]	Surface - any location

1. at ~ 8 am and 3 pm
2. at ~ 8 am
3. at the beginning and end of the study
4. three times during the study, on each day of treatment

Table 5.9.3B. Parameters/schedule/location for rectangular test units.

Parameter	Schedule	Location
Dissolved Oxygen	Twice Daily[1]	Mid-Depth - tail-end
Water Temperature	Twice Daily	Mid-Depth - any location
Water Flow	Daily[2]	at test unit source
pH	Twice during Study[3]	Surface - any location
Hardness (as $CaCO_3$)	Twice during Study	Surface - any location
Chloramine-T	During Treatment Only[4]	Surface - any location

1. at ~ 8 am and 3 pm
2. at ~ 8 am
3. at the beginning and end of the study
4. three times during the study, on each day of treatment

5.9.3.3 Procedures and equipment for assessing treatment parameters:

Tables 5.9.3.3 lists the treatment parameters to be measured, the equipment that will be used to measure these parameters, and the cited reference material used to conduct the measurements. All cited reference material for measuring treatment parameters are included in Appendix VII. Water flows will be set at a predetermined rate by calibrating flow from water-pipes plumbed to each tank. Flow will be measured by collecting water in a 1,000 mL graduated cylinders for 15 seconds and extrapolating total volume delivered over 60 seconds.

Table 5.9.3.3. Equipment and Reference Material for Measurement of Treatment Parameters.

Parameter	Equipment	Reference
Dissolved Oxygen	D.O. Meter (YSI model 55)	Owners manual
Water Temperature	Thermometer (Mercury thermometer or Barnstead Thermolyne Instruments)	Owners manual
Water Flow	Stop watch and container marked every 1/2 gallon	n/a
pH	pH Meter Accumet Portable AP5	Owners Manual
Total Hardness (as $CaCO_3$)	Hach kit reagents	See Appendix VII
Chloramine-T	Hach Test Kit for Chlorine, Pocket Colorimeter (Cat. #46700-00)	See Appendix IX - XI

5.9.3.4 Calculations for derived data:

Calculations are described in the reference material (see Appendix VII for total hardness & Appendix IX, X and XI for chloramine-T).

5.9.4 Frequency of feeding:

Test animals will not on treatment days. They will, however, be fed on non-treatment days during the treatment period. They will be fed daily during the 14-day post-treatment period. Feed brands and feed size, amounts of feed and feeding frequency will be specific to the participating facility and fish species used as the test animal. At this time, these

parameters are not known. Feeding frequency will be the same as that received by other production fish reared on site. Trout and salmon will be fed by 2 - 5 times daily by hand or by belt feeders over a 12 hour period, lake sturgeon will be fed 7 times daily by hand, and paddlefish will be fed every 15 minutes by autofeeder. Because it is difficult to firmly establish feed brand and size, amount of feed and daily feeding frequency at this time, they will be described in detail in the methods section of the final report. Whatever the feed brand, size, amount and frequency used at a particular facility, fish in all test units will by fed the same feed in the same amounts in the same manner.

5.9.5 Frequency of monitoring and adjusting water flow:

Water flow will be checked daily and adjusted to pre-study flow rates (see Table 5.9.3A and 5.9.3B).

5.10 Environmental conditions:

Trials will be conducted under environmental conditions which will be unique for each participating facility. Table 5.10 below describes the environmental conditions under which fish will be are reared for each participating facility. Fish species at each facility are also listed.

Table 5.10 Environmental conditions at study sites.

Facility	Fish Species	Dissolved Oxygen (mg/L)	Water Temp. (C)	pH	Hard-ness (ppm CaCO3)
Hotchkiss NFH	Rainbow Trout	6 - 8	13.1 - 13.4	7.7- 7.8	321
Bozeman FTC	Rainbow Trout	5 - 8	8.8 - 12.8	7.6	230
Neosho NFH	Rainbow & Brook Trout, Paddlefish, Lake Sturgeon	5 - 7.5	11.5 - 15.5	6.6 - 7.2	150
Jones Hole	Rainbow Trout	7 - 9	12.0 - 12.4	7.5 - 8.2	unknown
Creston NFH	Kokanee Salmon	9 - 11	8 - 9	7.4 - 7.8	unknown
Northeast FC	Rainbow Trout, Atlantic Salmon	4 - 9	12 - 14	6.9 - 7.4	75
	Atlantic & Shortnose Sturgeon, Striped Bass, Bluegill	3 - 10	16 - 24	6.9 - 9.5	115
Alchesay-Williams Creek NFH Complex	Apache Trout	5 - 9	12 - 14	7.2	42

Facility	Fish Species	Dissolved Oxygen (mg/L)	Water Temp. (C)	pH	Hard-ness (ppm CaCO3)
Quilcene NFH	Fall Chum Salmon	9.0 - 11.5	2.6 - 9.0	7.8	35
Jordan River NFH	Lake Trout	8.0 - 11.5	4.4 - 10.0	7.7 - 8.4	190

5.11 Tank Cleaning:

Tanks will be cleaned at the beginning of each day. Cleaning at the beginning of the day will ensure that when test units are treated with chloramine-T there will be minimum amount of accumulated organic material present, such as uneaten food and test animal waste. Tank cleaning will entail brushing the bottom of test units. Standpipes will be loosened to draw re-suspended material down the drain. When standpipes are loosened during cleaning, no more than 1/2 the total water volume will be allowed to drain from the test unit before standpipes are refitted into the bottoms of tanks. Tank cleaning will be complete when standpipes are refitted into tank drains.

5.12 Provisions for necropsy and disposal of expired test subjects:

Test animals will not be necropsied. For disposal of expired test subjects see Section 5.7.3.

5.13 Fate of living test animals after study completion:

The fate of all remaining test animals from studies conducted at Service facilities will be left to the discretion of the Hatchery Manager. However, if post-study fish are to be used as part of the facilities production program, they will be subjected to a 45-day withdrawal period prior to release. Control fish which still may be diseased may be treated according to the Study Protocol for a Compassionate Aquaculture Investigational New Animal Drug (INAD) Exemption, Chloramine-T INAD #4000. In most cases, fish will likely be transferred back to the original production lot from which they came. If post-study fish are not to be part of a facilities production program, they will be disposed of in a local landfill or incinerated, their fate will be denoted in Form 13. At this time, it is not possible to forecast the exact fate of living test animals after study completion at a particular facility. The fate of living test animals after study completion at a particular facility will be described in the final report.

5.14 Test unit configuration:

Test units may be different for each participating study site, depending upon a number of factors including species, fish size and tank availability. Test unit dimensions, as well as standpipe height, test unit volume in cubic feet and in gallons will be recorded on Form 3. Prior to transfer of fish into test units, standpipes will be put into place, water turned on and water flow rate adjusted to achieve desired flow index. Once a test unit is full, depth measurements will be taken at several locations to get an average depth. In circular test units, depth measurements will be taken at the outside edge of the tank, next to the standpipe, and midway between the outside edge and standpipe. In rectangular test units, depth measurements will be taken at the head, tail and midpoint of the tank. All measurements will be made near the mid-line of the test unit.

5.15 Owner consent:

Not applicable.

6 SPECIFICATIONS OF VARIABLES:

6.1 Primary variable to be measured for evaluating labeled claim:

Mortality will be the primary response variable. This is consistent with addressing the "proposed" label claim, which will read "to control or prevent mortality associated with Bacterial Gill Disease and Flexibacteriosis in a variety of Fish Species.

6.1.1 When primary variables will be assessed:

Mortalities will consist of dead fish. Mortalities will be removed, counted and recorded daily, beginning the first day of the treatment period and ending at 14-days post treatment. This covers the entire 19-day study period. Mortalities will be removed and counted from each test unit at the beginning and end of each day.

6.1.2 Procedures for assessing primary variable:

Fish will be evaluated as either 'dead' or 'alive' by the investigator and/or hatchery biologist by observation. The study participant responsible for assessing which fish are dead fish will have had extensive experience in evaluating whether fish are dead or not based on experience working at a fish hatchery (see Section 10 for list of Study Personnel and Appendix XIV for CV's).

6.1.3 Equipment used for assess primary variable:

No specialized equipment will be required to evaluate or remove dead test animals.

6.1.4 Calculation of derived data:

Data used for statistical analysis will be total mortality during the 19-day study period. Total mortality will be the sum of the daily mortality during the 19-day study period.

6.1.5 Forms for retention of source data:

Form 4 will be used to record mortality 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 response variable (mortality).

6.2 Other variables to be recorded during the study:

Presence or absence of external bacteria on the gills will be evaluated to address uniformity of disease infection prior to study and after treatment. In addition, kidney/spleen squeezes/blots or smears will be made to ensure that no other confounding pathogen may be contributing to mortality. This data may not be used to support treatment efficacy. See Section 6.2.2 for a description of procedures to be used to prepare gill slides, kidney/spleen squeezes/blots or smears and methods for evaluation.

6.2.1 When other variables will be assessed:

Gills will be evaluated for presence/absence of external bacteria no more than 24 hours prior to the initiation of treatment, and again within three days post-treatment.

6.2.2 Procedures for assessing other variables:

Three to five fish will be randomly selected and removed from each tank to evaluate level of bacterial presence/absence on gills. Fish will be crowded as tightly as possible in the rearing unit, and a small net used to scoop a subsample of fish. The first three to five fish that voluntarily fall/flop from the net as it is "tipped" will constitute a sample. Each fish will receive a fish health evaluation and results will be recorded on Form 11 to document overall fish health. A wet mount gill slide will be prepared using the first gill arch from each fish. Wet mounts will be examined at 400x magnification. Ten fields of view will be evaluated for presence/absence of bacteria. Presence will be noted if a single rod shaped/filamentous motile/non-motile bacteria is present. At least one wet mount sample from each tank will be stained with methylene blue or crystal violet (see appendix III) for confirmation by a fish health biologist. In addition, a standard fish health examination will be conducted to

ensure no other infectious pathogen is present that may be responsible for mortality (see appendix V). The fish health biologist who will evaluate prepared slides for confirmation/diagnosis and conduct the fish health evaluation is listed in Table 10.1.

6.2.3 Equipment used to assess other variables:

Equipment and supplies necessary to evaluate secondary variables will include:

1. Microscope with 400X magnification
2. microscope slides and cover slips
3. dissecting equipment including scalpel, scissors, forceps and probes
4. 95% ethyl alcohol, distilled water, methylene blue or crystal violet (see appendix VII for stain preparation directions and storage instructions).
5. Net, bucket or small container, 5% MS222
6. Sterile loops and culture media (e.g. Shiehs, TYES, TSA (Tryptic Soy Agar) or Cytophaga Agar).

6.2.4 Calculation of derived data:

The number of fields viewed in which bacteria believed to be the causative agent (flavobacteria) are present will be summed for each fish evaluated. Ten fields of view will be examined per fish gill. For example, if flavobacters were present in 7 fields of view and absent from 3 fields of view, the total for that fish would be 7. This value will be summed for each fish evaluated from a particular test unit.

6.3 Adverse reactions:

Adverse reactions will be recorded and reported immediately to study monitor. Adverse reactions will also be recorded on Form 9.

6.4 Study facilities:

Study facilities refer to the placement and location of test units at a particular study site. All test units will be within a covered hatchery building, regardless of study site. At a particular study site, all test units will be under the same conditions within the hatchery building.

6.4.1 Containment equipment:

Test fish will be contained in either fiberglass, aluminum or cement tanks or troughs, but most probably in **aluminum** test units. Each facility may use a different test unit, depending upon species requirements and tank availability. However, all test units used for a study will be the same.

Type of test unit, dimensions and test unit volume will be recorded on Form 3

6.4.2 Lighting equipment:
No specialized lighting equipment will be used. Photoperiod may be different for each participating facility, and will be described in the final report.

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:
In some cases, automatic belt feeders may be used.

6.4.6 Watering equipment:
No specialized watering equipment will be used.

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

6.4.8 Space allocation of test units:
All test units will be in the same immediate area, probably along one wall or in a corner area of the hatchery building. Test units will be placed within a small area to ensure minimizing variability in conditions, which may affect the test unit or test animals within.

6.4.9 Pasture allocation:
Not applicable

6.4.10 Facility diagram:
Not applicable

6.5 Experimental diets:
All diets used will be commercial diets normally used at the site where the study is to be conducted.

7 DATA ANALYSIS:

7.1 Define the experimental unit:
The experimental unit will be the test unit, not the individual fish.

7.2 Define the number of replicates per treatment:

There will be three replicates per treatment, and there will be two treatment conditions (treated and control). There will be a total of six test units used per study.

7.3 Define statistical methodology:

7.3.1 Null hypothesis:

H_o : $u_1 = u_2$; Mortality caused by BGD or flexibacteriosis associated with external infections of flavobacters is equal between fish treated with 10 mg/L chloramine-T for 60 minutes on alternate days for a total of 3 treatments using a flow-through method, and fish which receive no chloramine-T treatment.

7.3.2 Alternate (research) hypothesis:

H_a : $u_1 \leq u_2$; Mortality caused by BGD or flexibacteriosis associated with external infections of flavobacters will be lower among fish treated with 10 mg/L chloramine-T for 60 minutes on alternate days for a total of 3 treatments using a flow-through method than of mortality of untreated fish.

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:

An independent t-test will be used to detect differences between treated and untreated fish with regards to total fish mortality/test unit . Where differences are stated to be significant, a level of $p \leq 0.05$ is implied. Bartletts test for homogeneity of variance will be used to test for equality of variance. Where variances are equal, results from the pooled variances t-test will be used. Where variances are unequal, results from the separate variances t-test will be used. The separate variances t-test adjusts the degrees of freedom to account for unequal variances (see appendix VIII, page 601). If there is a potential for pooling data from different studies involving different fish species, discussions with CVM's Biometrics Division will be initiated to explore other statistical analyses.

7.3.5 Statistical data software to be used:

The statistical software package to be used will be SYSTAT (Wilkinson 1990).

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

Differences in mortality will be detected using an independent t-test. Where differences are stated to be significant, a level of $p \leq 0.05$ is implied. If total mortality among non-treated control groups is higher than total mortality among the treated groups, and the calculated p value is less than 0.05, then the conclusions drawn will state that the chloramine-T treatment regime used was effective in controlling mortality caused by BGD or flexibacteriosis associated with external infections of flavobacters in test fish species used. If total mortality among non-treated groups is higher than the total mortality among treated groups, but the calculated p value is greater or equal to 0.05, or if the total mortality among non-treated groups is less than or equal to the total mortality among treated groups, then the conclusions drawn will state that the chloramine-T treatment regime used was not effective in controlling mortality caused by BGD or flexibacteriosis associated with external infections of flavobacters in the test fish.

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 chloramine-T dose verification 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. Measurements will be made using a Pocket Colorimeter (Cat. # 46700-00). 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 IX for method description.

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

Water samples will be collected from each treated test unit. Samples will be collected 45 minutes into the 60 minute treatment period. A single sample from a treated unit will be split and analyzed in duplicate. Sample size will be approximately 100 mL and will be collected from near the middle of the test unit and taken from the surface. Samples will be analyzed within 30 minutes of collection

8.2.3 Evidence of methods validation:

See appendix X for 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:

See appendix XI.

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

The pocket colorimeter will be zeroed using a blank solution immediately prior to analyzing test unit samples. A duplicate will be run with every sample batch. A sample batch will consist of all water samples taken during a single treatment period for dose verification. Raw data for both free and total chlorine and calculations to determine concentration of chloramine-T will be recorded on Form 9a. Dose verification results and target concentrations will be recorded on Form 9b.

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

See appendix XII for reference to study titled A Simple Analytical Procedure to Replace HPLC for Monitoring Treatment Concentrations 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 XIII.

9 STUDY LOCATIONS:

Table 9. List of study location, address, phone number, and contact person (Hatchery Manager/Asst. Manager).

Facility	Address	Phone Number	Contact
Bozeman Fish Technology Center	4050 Bridger Canyon Rd Bozeman, MT 59715	(406) 587-9265	Ron Zitzow
Quilcene National Fish Hatchery (NFH)	281 Fish Hatchery Rd. Quilcene, WA 98376	(360) 765-3334	Larry Telles
Alchesay-Williams Creek NFH Complex	P.O. Box 398 Whiteriver, AZ 85941	(520) 338-4901	Bob David
Neosho NFH	520 E. Park St. Neosho, MO 64850	(417) 451-0554	Doug Aloisi

Creston NFH	780 Creston Hatchery Rd. Creston, MT 59901	(406) 755-7870	Don Edsall
Hotchkiss NFH	807 - 3150 Lane Hotchkiss, CO 81419	(970) 872-3170	David Oviedo
Jones Hole NFH	266 W. 100 North #2 Vernal, UT 84078	(801) 789-4481	Lloyd Strobeck
Northeast Fisheries Center	P.O. Box 75 Lamar, PA 16848	(717) 726-4247 (717) 726-6611	Bill Fletcher John Coll
Jordan River NFH	6623 Turner Road Elmira, MI 49730	(616) 584-2461	David Huntley

10 PERSONNEL:

Personnel will be debarred from participating in the proposed field based clinical efficacy trials if they take a new position within the Service, or take a new position outside the Service. It is not anticipated that justification for debarment will occur in the near future.

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

See table 10.1 below. See appendix XIV for Curriculum Vitaes for Investigators, Study Monitor and Fish Health Biologists involved in the proposed field based clinical efficacy trials.

Table 10.1 Investigators, study monitor and fish health biologists involved in proposed field based clinical efficacy trials

Facility	Investigators and Phone Number	Study Monitor and Phone Number	Fish Health Biologist and Phone Number
Bozeman Fish Technology Center	Jim Bowker - (406) 587-9265	Dave Erdahl (406) 587-9265	Beth MacConnell (406) 587-9265 or Andy Anderson (970) 867-3773
Quilcene NFH	Jim Bowker - (406) 587-9265 Larry Telles - (360) 765-3334	Dave Erdahl (406) 587-9265	Joy Evered and/or Ray Brunson (360) 753-9046
Alchesay-Williams Creek NFH Complex	Jim Bowker - (406) 587-9265 Bob David - (520) 338-4901	Dave Erdahl (406) 587-9265	Jerry Landye (520) 367-1953
Neosho NFH	Jim Bowker - (406) 587-9265 Doug Aloisi - (417)451-0554	Dave Erdahl (406) 587-9265	Terry Ott (608) 783-8444
Creston NFH	Jim Bowker - (406) 587-9265 Don Edsall - (406) 755-7870	Dave Erdahl (406) 587-9265	Andy Anderson (970) 867-3773
Hotchkiss NFH	Jim Bowker - (406) 587-9265 David Oviedo - (970) 872-3170	Dave Erdahl (406) 587-9265	Andy Anderson (970) 867-3773

Jones Hole NFH	Jim Bowker - (406) 587-9265 Lloyd Strobeck - (801) 789-4481	Dave Erdahl (406) 587-9265	Andy Anderson (970) 867-3773
Northeast Fisheries Center	Jim Bowker - (406) 587-9265 Bill Fletcher - (717) 726-4247	Dave Erdahl (406) 587-9265	John Coll (717) 726-6611
Jordan River NFH	Jim Bowker - (406) 587-9265	Dave Erdahl (406) 587-9265	Terry Ott (608) 783-8444

10.2 Other personnel involved in study:

See Table 10.2 below. See appendix XIV for Curriculum Vitaes for other personnel involved in the proposed field based clinical efficacy trials.

Table 10.2. List of other study participants

Facility	Other Study Participants
Bozeman Fish Technology Center	Steve Murray
Quilcene NFH	Paul Keiser, Ron Wong, Bud Young
Alchesay-Williams Creek NFH Complex	Michael A. Ternes, Sherry White, Bradley Clarkson
Neosho NFH	Peter J. Walters, Jeffery L Massens, Ralph W. Simmons
Creston NFH	Mark Maskill, Garr Holmes, Dave Bermel, Jim Till, John Scott
Hotchkiss NFH	Adam Mendoza, Durand Dickman
Jones Hole NFH	Ken Blick, Lee Bender
Northeast Fisheries Center	Jerre W. Mohler, Kim King
Jordan River NFH	TBD

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 National INAD Office (NIO) in Bozeman, MT in a secure area which protects the source data and records from deterioration, destruction, tampering and vandalism in accordance to Section D-4 paragraphs (a) and (b) of the Conduct of Clinical Investigations: Responsibilities of Clinical Investigators and Monitors for Investigational New Animal Drug Studies (dated Oct. 1992 or later - see Attachment II). Also,copies of all source data will be stored in a similar manner at the original study site.

12 ADDENDUM/DEVIATIONS TO THE PROTOCOL:

12.1 Protocol addendums:

Protocol addendums will be forwarded to FDA/CVM and referenced to the Efficacy of Chloramine-T for Control of Mortality Associated with Bacterial Gill Disease and Flexibacteriosis a variety of Fish Species, Protocol #4000 - 1. Cover letters accompanying submitted addendums will reference the submittal date of the above protocol. Addendums will also be attached to this protocol (see Appendix XV).

12.2 Protocol amendments:

A signed copy of the Study Protocol must be retained by each Investigator. At any time before a study begins, desired changes in the Study Protocol should be brought to the attention of the Study Monitor (Dave Erdahl). The desired changes will be fully described in the form of an amendment along with the reason for the change. The amendment will be signed by Dave Erdahl. Copies of the signed amendment will be attached to each copy of the Study Protocol. Amendments will also be attached to this protocol (see Appendix XV).

12.3 Protocol deviations:

Deviations from the established Study Protocol occasionally cannot be avoided. If deviations occur, the Study Monitor will be contacted immediately for advice. Protocol deviations will be documented fully and accompanied by a written explanation of what happened, why, and what steps were taken to mitigate the deviation. Deviation statements will be signed and dated. These statements will be forwarded to the Study Monitor and then submitted with the protocol as part of the final report. Deviations will also be attached to this protocol (see Appendix XV).

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

Unused drug will be kept on site for future use according to the Service's Study Protocol for a Compassionate Aquaculture Investigational New Animal Drug Exemption - Chloramine-T INAD #4000 (see Attachment I). Fate of unused living test animals will be handled in a manner identical to the fate of living post-study test animals (see Section 5.12 Fate of living test animals after study completion). In most cases, unused living fish will be returned to the general hatchery population.

REFERENCES

Amos, Kevin H. , editor. 1985. Procedures for the Detection and Identification of Certain Fish Pathogens. Third Edition. Fish Health Section, American Fisheries Society. Corvallis, Oregon.

Piper, R.G., I.B. McElwain, L.E. Orme, J.P. McCraren, L.G. Fowler and J.R. Leonard. 1982. Fish Hatchery Management. United States Department of the Interior, U.S. Fish and Wildlife Service, Washington, DC., 517 pp.

Spear, D.J., R.J.F. Markham, B. Despres, K. Whitman and N. MacNair. 1995. Examination of Gills from Salmonids with Bacterial Gill Disease using Monoclonal Antibody probes for *Flavobacterium branchiophilum* and *Cytophaga columnaris.* Journal of Veterinary Diagnostic Investigations. 7:500-505

United States Fish and Wildlife Service (USFWS). 1995. Introduction to Fish Health Management. United States Department of the Interior, Washington, DC 139 pp.

Wilkinson, L. 1990. SYSTAT: The System for Statistics. SYSTAT, Inc. Evanston, IL 677 pp.

FORMS

1. Shipment of drug - Section 2.1.11
2. Drug log - Section 2.1.11
3. Environmental and culture conditions prior to treatment initiation - Section 6.3.1
4. Daily mortality - space for recording mortality twice daily and daily total - Section 6.1.5
5. Daily dissolved oxygen - Section 5.9.3.2
6. Daily water temperature - Section 5.9.3.2
7. pH and hardness - Section 5.9.3.2
8. Treatment Condition Code

9a. Raw data record for concentrations of free and total chlorine for samples and duplicates for each of the treatment periods.

9b. Chloramine-T concentration record for samples and duplicates, and target doses

10. Adverse reactions Section 6.4
11. Fish Health Evaluation
12. Gill Evaluation for Presence/Absence of Bacteria
13. Disposal Record for Animals from Clinical Field Trials

APPENDICES:

I. Chloramine-T MSDS

II. Chloramine-T Investigational Label

III. Procedure for fixing and staining gills with methylene blue

IV. Procedure for methylene blue stain preparation and storage instructions

V. Standard fish health diagnostic scheme to determine if other infectious pathogens are present.

VI. Investigational Withdrawal Period

VII. Reference material for the following: 1) YSI - 55 DO meter, 2) procedure for determining pH, and 3) procedure for determining hardness (as $CaCO_3$).

VIII. Reference for appropriateness of using pooled and separate variances when using an independent t-test to detect differences between treated and untreated fish with regards to total fish mortality/test unit.

IX. Abstract of the DPD colorimetric method to determine concentration of chloramine-T for dose verification.

X. Evidence of DPD colorimetric method validation.

XI. Description of validation DPD colorimetric method plan when method is being developed for the study.

XII. Relevant scientific literature supporting the use of the DPD Colorimetric method for the intended measurements.

XIII. Certification that all needed validations for the DPD Colorimetric method will be done before the initiation of the study.

XIV. Curriculum vitaes for Study Monitors, Investigators and other Personnel involved in study.

XV. Addendums/deviations to protocol.

FORM 1. GUIDE FOR REPORTING INVESTIGATIONAL NEW ANIMAL DRUG SHIPMENTS FOR POIKILOTHERMIC FOOD ANIMALS

Department of Health and Human Services
Center for Veterinary Medicine, HFV-199
Food and Drug Administration
7500 Standish Place
Rockville, Maryland 20855

Date:
INAD No: 4000
Name of Drug: Chloramine-T
Trial Number:
Lot Number:

The sponsor, U.S. Fish and Wildlife Service, submits a notice of claimed investigational exemption for the shipment or delivery of a new animal drug under the provisions of Section 512 of the Federal Food, Drug, and Cosmetic Act. The following information is submitted in triplicate (original and two copies):

Name of Drug: Chloramine-T

Proposed Use of Drug: Treatment of bacterial gill disease and flexibacteriosis in a variety of cultured fish species

Date of CVM Authorization Letter: April 18, 1994

Date of Drug Order: _____

Amount of Drug Ordered: _____

Name of Investigator: _____
(typed or printed)

Location of Trial: _____

Pivotal (intended for support of NADA) X or nonpivotal _____ study

Approximate Number of Animals: Treated _____ Controls _____

Protocol (*pivotal studies only*): Date submitted to CVM and/or number: January 4, 1994

Approximate Date of Trial: Start: _____ End: _____

Species, Size, and Type of Animals: _____

Maximum dose and duration: 20 mg/L for 1 hour - up to 3 times per week

Method(s) of Administration: Immersion bath or constant flow

Withdrawl Period: 45 days

If the investigation is discontinued, the Food and Drug Administration will be notified, giving the reason and disposition of the drug.

Investigator: _____
Signature and Date

Study Monitor: _____
Signature and Date

Form 2. Chemical Use Log for Clinical Field Trials Using Chloramine-T Under INAD #4000

Instructions:

1. Initiate Form 2 immediately upon receipt of Chloramine-T.
2. Only a single shipment/lot number of Chloramine-T should be used per each copy of Form 2.
3. A signed copy of Form 2 should be sent to the Study Monitor at the end of the Study Year.
4. Original Form 2 should be archived at the investigating facility.

Facility: _____ **Trial Number:** _____

Lot Number of Chloramine-T: _____ **Date Received:** _____

Amount Chloramine-T Received (g)	Container and Chloramine-T Weight Before Use (g)	Chloramine-T Container Weight (g)

Date Chl-T Used	Amount Chl-T Used (g)	Chl-T On-hand (g)	Dosage and # of treatments (mg/L : reps)	Species Treated	Inventoried by (initials)

Investigator: _____
Signature and Date

Study Monitor: _____
Signature and Date

Form 3. Test Site, Species Tested, Environmental and Culture Conditions, and Water Quality Parameters

Test Site: _____

_____

_____

Chloramine-T Lot Number _____ Test Site Elevation: _____

Fish Species (inculding scientific name): _____

Fish Source (originating facility/body of water): _____

Fish Lot Number: _____ Fish Length (in): _____ Fish Weight (g): _____

Test Unit Type (e.g. fiberglass/circular, aluminum/trough, concrete/raceway): _____

_____

Test Unit Dimensions (ft) : _____ Standpipe Heigth[1] (in): _____

Test Unit Volume[2] (cu. ft): _____ Test Unit Volume[2] (gal): _____

Number of Test Units:

Number of Control Test Units: _____ Number of Treated Test Units: _____

Number of Fish/Test Unit: _____ Total Number of Fish in Study: _____

Flow Index[3]: _____ Density Index[3]: _____

Water Temperature (°C): _____ Water Flow per Test Unit (gpm): _____

Dissolved Oxygen (mg/L): _____ Dissolved Oxygen (% saturation): _____

pH: _____ Water Hardness (mg/L $CaCO_3$): _____

Water Source (e.g. well, spring, creek, resorvoir, etc): _____

NOTE: It is assumed that test conditions are identical for each test unit in the study. If not, please specify any irregularities on a separate sheet and attach to this form.

[1] Standpipe height refers to distance standpipe extends above bottom of test unit.
[2] See Section 5.14 for description of measuring test unit.
[3] See Section 5.1.2.1 and 5.1.2.2 for description of flow index and density index calculations. Assume first pass water.

Investigator: _____ **Study Monitor:** _____
Signature and Date **Signature and Date**

Form 4. **Daily Mortality Record for Pivotal Studies Evaluating the Efficacy of Chloramine-T to Control Mortality Associated with Bacterial Gill Disease and Flexibacteriosis**

Daily Mortality

Date	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Initials
	AM / PM / Total	AM / PM / Total	AM / PM /Total	AM / PM /Total	AM / PM /Ttotal	AM / PM / Total	
	/ /	/ /	/ /	/ /	/ /	/ /	
	/ /	/ /	/ /	/ /	/ /	/ /	
	/ /	/ /	/ /	/ /	/ /	/ /	
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Total Mortality							

NOTE: Mortality should be recorded twice daily, once early in the morning and again late in the afternoon (see Section 6.1). The sum of the two values should then be recorded as total daily mortality. Total Mortality for the entire study period should include the sum of all values for the 5-day treatment period and the 14-day post-treatment period

Study Monitor ______________________________ Date __________
Signature

Investigator ______________________________ Date __________
Signature

Investigator ______________________________ Date __________
Signature

Form 5. **Daily Record for Dissolved Oxygen Levels in Pivotal Studies Evaluating the Efficacy of Chloramine-T to Control Mortality Associated with Bacterial Gill Disease and Flexibacteriosis**

Dissolved Oxygen (mg/L)

Date	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Initials
	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM	
	/	/	/	/	/	/	
	/	/	/	/	/	/	
	/	/	/	/	/	/	
	/	/	/	/	/	/	
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NOTE: Dissolved oxygen levels should be recorded twice daily, once early in the morning and again late in the afternoon (see Tables 5.9.3A,5.9.3B, and 5.9.4).

Study Monitor ______________________ Date ____________
Signature

Investigator ______________________ Date ____________
Signature

Investigator ______________________ Date ____________
Signature

Form 6. **Daily Record for Water Temperature in Pivotal Studies Evaluating the Efficacy of Chloramine-T to Control Mortality Associated with Bacterial Gill Disease and Flexibacteriosis**

Water Temperature (°C)

Date	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Rearing Unit #	Initials
	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM	AM / PM	
	/	/	/	/	/	/	
	/	/	/	/	/	/	
	/	/	/	/	/	/	
	/	/	/	/	/	/	
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NOTE: Water temperature should be recorded twice daily, once early in the morning and again late in the afternoon (see Tables 5.9.3A, 5.9.3B, and 5.9.4).

Study Monitor ______________________ Date ____________
Signature

Investigator ______________________ Date ____________
Signature

Investigator ______________________ Date ____________
Signature

Form 7. **Record of pH and Water Hardness in Pivotal Studies Evaluating the Efficacy of Chloramine-T to Control Mortality Associated with Bacterial Gill Disease and Flexibacteriosis**

pH and Water Hardness

Date	pH	Water Hardness (mg/L $CaCO_3$)	Initials

NOTE: pH and water hardness should be recorded once at the beginning of the study, and again at study termination (see Tables 5.9.3A, 5.9.3B, and 5.9.4).

Study Monitor _____ Date _____
Signature

Investigator _____ Date _____
Signature

Investigator _____ Date _____
Signature

Raw Data: Water Hardness - Record number mLs titrant used to change sample color, and show calculations used to derive mg/L hardness (as CaCO3).

Hardness #1

Hardness #2

Form 8. Treatment Condition Code for Pivotal Studies Evaluating the Efficacy of Chloramine-T to Control Mortality Associated with Bacterial Gill Disease and Flexibacteriosis

Treatment Condition Code

Treated Tank #1: _____	Control Tank #1: _____
Treated Tank #2: _____	Control Tank #2: _____
Treated Tank #3: _____	Control Tank #3: _____

NOTE: Once this form has been completed, it must be stored in a secure location not accessible to anyone except the listed hatchery manager/assistant manager. At study termination, this form is to be included with other data/information forms.

Study Monitor _____ Date _____
Signature

Investigator _____ Date _____
Signature

Investigator _____ Date _____
Signature

Form 9a. Chloramine-T Analysis Raw Data Record for Pivotal Studies Evaluating the Efficacy of Chloramine-T to Control Mortality Associated with Bacterial Gill Disease and Flexibacteriosis.

Chloramine-T Analysis Record - 1st Chloramine-T Treatment

Treatment Date ____________________

Sample Identification	A	B	C	D	E	F	G
Free Chlorine							
Total Chlorine							
Total - Free Chlorine							
Multiplication Factor	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97
Chloramine-T (mg/L)							

Chloramine-T Analysis Record - 1st Chloramine-T Treatment

Treatment Date ____________________

Sample Identification	A	B	C	D	E	F	G
Free Chlorine							
Total Chlorine							
Total - Free Chlorine							
Multiplication Factor	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97
Chloramine-T (mg/L)							

Chloramine-T Analysis Record - 1st Chloramine-T Treatment

Treatment Date ____________________

Sample Identification	A	B	C	D	E	F	G
Free Chlorine							
Total Chlorine							
Total - Free Chlorine							
Multiplication Factor	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97
Chloramine-T (mg/L)							

Form 9b. Chloramine-T Analysis Summary Record for Pivotal Studies Evaluating the Efficacy of Chloramine-T to Control Mortality Associated with Bacterial Gill Disease and Flexibacteriosis.

Chloramine-T Analysis Record - 1st Chloramine-T Treatment

Treatment Date _____

Sample Identification	Tank #1	Tank #2	Tank #3	Tank #4	Tank #5	Tank $6	Duplicate
Concentration (Target)							
Concentration (Observed)							

Chloramine-T Analysis Record - 2nd Chloramine-T Treatment

Treatment Date _____

Sample Identification	Tank #1	Tank #2	Tank #3	Tank #4	Tank #5	Tank #6	Duplicate
Concentration (Target)							
Concentration (Observed)							

Chloramine-T Analysis Record - 3rd Chloramine-T Treatment

Treatment Date _____

Sample Identification	Tank #1	Tank #2	Tank #3	Tank #4	Tank #5	Tank $6	Duplicate
Concentration (Target)							
Concentration (Observed)							

Study Monitor _____ Date _____
Signature

Investigator _____ Date _____
Signature

Analyst _____ Date _____
Signature

Form 10. Record of Adverse Reactions and/or Chloramine-T Toxicity

It is imperative that a complete record of possible adverse reactions and/or drug toxicity is established with respect to chloramine-T treatment. This should include a description of all pertinent events before, during, and after treatment. The following space is provided for such information. If more space is required, please attach supplemental sheets to this form.

Date	Tank ID	Adverse Reactions / Cloramine-T Toxicity

Date	Tank ID	Adverse Reactions / Cloramine-T Toxicity

Date	Tank ID	Adverse Reactions / Cloramine-T Toxicity

Investigator: _____ _____
Signature Date

Study Monitor: _____ _____
Signature Date

Form 11. Fish Health Evaluation

Tank No. _____
Fish No. _____
Date _____

Fish species _____ Wt. (g) _____ Length (inches) _____

Body Surface: ( ) Normal ( ) Excess Mucus ( ) Irregular color
( ) Gross Pathology _____
( ) Micro Wet _____

Fins: ( ) Normal ( ) Gross Pathology: Frayed P1 P2 Ad C D An
Hemorrhaged P1 P2 Ad C D An
Eroded P1 P2 Ad C D An
( ) Micro Wet _____

Gills: ( ) Normal ( ) Pale ( ) Hemorrhagic ( ) Necrotic
( ) Micro Wet: ( ) Mucus _____ ( ) Hypertrophy F L _____
( ) Edema _____ ( ) Hyperplasia F L _____
( ) Embolism _____ ( ) Telangiectasis _____
( ) Bacteria _____
( ) Parasites _____

Liver: ( ) Normal ( ) Pale ( ) Edematous ( ) Mottled ( ) Fatty
( ) Hemorrhagic ( ) Necrotic
( ) Parasites _____

Spleen: ( ) Normal ( ) Pale ( ) Enlarged ( ) Edematous ( ) Granulated
( ) Parasites _____

Posterior Kidney: ( ) Normal ( ) Pale ( ) Edematous ( ) Necrotic
( ) Parasites _____

Dermal Lesion: ( ) None ( ) Ulceration ( ) Hemorrhagic ( ) Necrotic
( ) Furuncle ( ) Marginal ( ) Marginal
( ) Central ( ) Central
( ) Closed ( ) Open
( ) Location: ( ) Dorsal ( ) Caudal ( ) Lateral
( ) Ventral ( ) Cranial ( ) Isthmus
( ) Base of fins P1 P2 Ad C D An
( ) Description: _____
( ) Micro Wet _____

Notes and Comments on other organs and tissues

Eyes _____ Stomach _____
Body Cavity _____ Gastrointestinal Tract _____
Gall Bladder _____ Gas Bladder _____
Adipose Tissue _____ Musculature _____
Gonads _____ Brain _____
Other _____

Study Monitor _____ Date _____

Investigator _____ Date _____

Form 12. Presence/Absence of External Bacteria on Gills

Tank No. _____

Date _____

	Fish #1	Fish #2	Fish #3	Fish #4	Fish #5
Length (")					
Wt. (g)					
Field #1					
Field #2					
Field #3					
Field #4					
Field #5					
Field #6					
Field #7					
Field #8					
Field #9					
Field #10					
Field #11					
Field #12					
Field #13					
Field #14					
Field #15					
Sum					

Investigator: _____ _____
Signature Date

Study Monitor: _____ _____
Signature Date

Form 13. Disposal Record for Animals from Clinical Field Trials Using Chloramine-T Under INAD 4000-1

Facility name and trial number: _____

Chloramine-T Lot No.: _____ Date Received: _____

Fish Lot No.: _____ Date of Disposal: _____

Number of Fish: _____ Size of Fish: _____

Date of Last Treatment: _____

Disposal Method: ☐ Stocking ☐ Burial ☐ Incinerator

_____

_____

Disposal Site:

Body of Water: _____

Location of Burial Site: _____

Location of Incinerator: _____

Investigator: _____ _____
Signature Date

Study Monitor: _____ _____
Signature Date