ORIGINAL

A RESEARCH PROTOCOL TO DETERMINE:

THE SAFETY OF CHLORAMINE-T (Trihydrate) TO VARIOUS RAINBOW TROUT LIFE STAGES

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Proposed Starting date:	April 1999
Proposed Ending date:	December 2000
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1. INTRODUCTION:

1.1 Objective:

1. To evaluate the toxicity of 0, 20, 60, and 100 mg chloramine-T per L of water administered as a bath solution daily on three alternate days for 3 hours using a static bath treatment method on four life stages (fry, fingerling, juvenile, and adult) of rainbow trout (*Oncorhynchus mykiss*).

2. H_o : $u_1 = u_2 = u_3 = u_4$; Mortality within specific life stages of rainbow trout is equal when exposed to 0, 20, 60, or 100 mg/L chloramine-T administered as a bath solution daily on three alternate days for 3 hours using a static bath treatment method.

3. H_a : $u_1 = u_2 \neq u_3 \neq u_4$; Mortality within specific life stages of rainbow trout will be different when exposed to 0, 20, 60, or 100 mg/L chloramine-T administered as a bath solution daily on three alternate days for 3 hours using a static bath treatment method.

4. The Study Director, Investigator and other personnel involved in the conduct of the study will be thoroughly familiar with this protocol and with the document titled "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:

Bacterial gill disease (BGD) is one of the most common diseases of hatchery reared salmonids (Bullock 1991) and causes more fish losses than any other bacterial disease (Bills et al. 1988). In Ontario, Canada, BGD accounts for about 21% of all diagnostic submissions from fish farms to the Fish Pathology Laboratory of the Ontario Veterinary College (Ferguson et al. 1991). Death is generally not a direct result of the infection, but is a consequence of the infection. In the case of BGD, death is most likely the result of asphyxiation from lack of adequate oxygen exchange at severely congested gills. Stressors associated with intense fish culture may predispose fish to infection. Proliferation of gill epithelial tissue and subsequent loss of gill surface by clubbing and fusing of lamellae are often associated with BGD infections (Bullock 1990). The disease is characterized by an acute onset, flared opercula, increased branchial rate and decreased fright response. In addition, fish spaced equidistantly from each another, reject feed, and high mortality is observed (Lumsden et al. 1994; Lasee 1995). If BGD, which is

horizontally transmitted, is not diagnosed and treated during the early stages of infection, thousands of fish may die within a 24-h period (Bullock et al. 1990).

Historically, several chemicals, including benzalkonium chloride (available as Hyamine 1622 and 3500), diquat, and chloramine-T (Bullock et al. 1990) have been used to control mortality caused by BGD. However, none of these available chemicals are approved by the U.S. Food and Drug Administration (FDA) to control mortality in freshwater fish caused by BGD or any other infectious pathogen. Because chloramine-T seems to be the most effective therapeutant when salmonids have BGD (From 1980; Bullock et al. 1990), it has become one of the prime candidates for FDA approval as a bath treatment. Chloramine-T has been characterized as a non-selective sanitizing agent and has been shown to "clean up" gills infested with bacteria and coated with excess mucus. In order for a chemical to be approved under an Investigational New Animal Drug (INAD) exemption, it must be demonstrated to be efficacious in field trials, and pose no toxicological effects to exposed fish at the proposed therapeutic levels. Both efficacy and target animal safety studies, conducted to meet the FDA Center for Veterinary Medicine (CVM) minimum requirement for clinical and non-clinical field trials, are necessary components of a New Animal Drug Application submission. Efforts have been underway since 1997 to generate clinical field efficacy trials, however, no data exists with respect to toxicity of chloramine-T based on target animal safety studies. Efficacy data generated to date, including data from both pivotal and non-pivotal trials, indicate chloramine-T treatment concentrations up to 20 mg/L to be efficacious while showing no indication of toxicological effects. Additionally, a target animal safety pilot study conducted at the Bozeman Fish Technology Center (BFTC), which evaluated mortality among rainbow trout fingerlings exposed to 0, 20, 60, and 100 mg/L chloramine-T for 3 hours at both 9.8 and 15.9° C, also demonstrated lack of toxicity at 20 mg/L chloramine-T.

As a result of needed non-clinical chloramine-T target animal safety data, a study protocol was developed to determine toxicity of this chemical in various fish species. Chloramine-T will be tested at 1, 3 and 5 times the upper efficacious treatment level for three times the standard treatment duration on various life stages of rainbow trout. Rainbow trout were chosen for this study as a representative salmonid species because they are a frequently cultured species in the United States and relatively easy to obtain, culture, and hold. Rainbow trout are also widely recognized in aquatic toxicology as a species that is representative of all salmonids.

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. Chloramine-T is a pure compound with no inactive ingredients.

2.1.6 Dosage Form:
Chloramine-T is a pure, water soluble compound and is not formulated in any way. During therapeutic or prophylactic 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:
0, 20, 60 and 100 mg/L chloramine-T dissolved in water.

2.1.8 Manufacturer:
Akzo Chemical, Inc.
300 South Riverside Plaza
Chicago, IL 60606
1-800-662-8170

2.1.9 Lot Number:
0299303520272

2.1.10 Packaging:

Test article will either be stored in its original packaging, which consists of a durable paper sack placed in a heavy cardboard barrel, or in comparable packaging, such as stiff plastic bags in large plastic containers. Containers will be labeled in accordance with GLP requirements.

2.1.11 Drug storage during study:

Test article will either be stored in the original container supplied by the manufacturer or in a different, similar container, with the appropriate investigational label attached. The container will either be stored in the BFTC HazMat building (a secured building), or in a locked cabinet/refrigerator in the bioassay. Both are cool, dry locations away from direct sunlight. If a portion of the bulk test article is stored in the Drug Registratin Lab (DRL), it will be properly labeled and will include an INAD label.

2.1.12 Drug handling procedures:

The Study Director, Investigator and all other personnel involved in the study will be provided with (and shall be required to read), a current copy of the Material Safety Data Sheet (MSDS) for Chloramine-T (Appendix I).

2.1.13 Verification of drug integrity/strength:

The Manufacturer (Akzo Chemicals, Inc.) will provide a certificate of analysis documenting the authenticity and purity of each lot of the test chemical. The lot number and date of manufacture for each batch of Chloramine-T will be placed on the label of each container. A sample of the test article will be archived according to SOP No. GEN 011.0.

2.1.14 Investigational labeling:

The Study Director will be responsible to ensure proper labeling of the container of Chloramine-T to be used.

2.1.15 Accountability:

All chemical used for studies will be from chloramine-T currently stored at the BFTC that is used under a compassionate INAD exemption to treat fish for BGD and external. The Study Director will be responsible for coordinating chloramine-T use with the BFTC chloramine-T INAD Investigator to accurately maintain the

chemical inventory of chloramine-T on-hand. The Chemical Use Log provided to the Investigator will be used to log each use of chloramine-T. Each time Chloramine-T is used, it will be reported by the Study Director.

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:
A series of up to eight studies will be conducted under this protocol. Each rainbow trout life stage (fry, fingerling, juvenile and adult) will be test at two water temperatures. Studies will begin no sooner than April 1, 1999, and be conducted over a period extending until no later than December 2000.

3.2 Schedule of events:

Listed in Table 3.2 is a schedule of significant events for BFTC-99-CHLT-TAS, including transfer of fish to test units, initiation of study, chloramine-T exposure period duration, and post-exposure observation period duration.

Time (Day)	Event
7 days before start	Randomly transfer test fish to test tanks
1 day before start	Randomly assign treatment condition to each test tank; pre-study fish health evaluation of 3 - 5 fish per tank; histological samples collected from fish evaluated above for pre-exposure condition.
Day 1	Expose fish to chloramine-T for the first time.
Day 3	Expose fish to chloramine-T for the second time.
Day 5	Expose fish to chloramine-T for the third and final time
Day 6	Beginning of post-exposure daily observation for effects of chemical exposure.

Time (Day)	Event
Day 19	Final daily observations of chemical exposures cease; final sample collection of 2 fish per tank for histological evaluation. Fewer fish will be sampled if ≤ 1 fish remain per tank.

3.2.1 Post-treatment period duration:

Duration of the post-exposure period will begin on day 6 of the study and will end no sooner than on day 19 (total of 14 days). Results from three pilot chloramine-T target animal safety studies conducted at the BFTC using fingerling and juvenile rainbow trout showed that nearly all mortality that occurred during the study, regardless of temperature, occurred during or immediately following the first exposure of chloramine-T. Although some mortality occurred on days 2 through 4, virtually no mortality occurred after day 4. Based on these data, the post-exposure observation period was set at 14 days, the same duration as used in chloramine-T pivotal efficacy trials.

3.2.5 Data analysis and report writing:

Data analysis and preparation of final report will require a maximum of 12 months to complete. Within 12 months of completion of the post-exposure period, a final report will be submitted to the Food and Drug Administration/Center for Veterinary Medicine, Public Master File number PMF 005-637, or submitted to other Agencies involved in consolidating chloramine-T target animal safety data packets (e.g., Upper Midwest Environamental Science Center, USGS, BRD).

3.3 Proposed date(s) of completion:

The data collection portion of individual studies will be completed within 20 days of the initiation of chemical exposure. Final date of completion of individual studies will be approximately 12 months after this date. The proposed date for completion of all studies and study termination is December 2000.

4. STUDY DESIGN:

4.1 Exposure groups:

Each study will consist of four exposure groups — three groups to be exposed to different concentrations of chloramine-T and a single group to receive no chloramine-T (control). Exposed groups will be exposed to either 20, 60 or 100 mg/L chloramine-T. Control groups will receive no chemical treatment, but will receive a pure water "sham" treatment. Three

test units will be set up for each exposure group for a total of 12 test units. Individual test units will be considered the experimental unit for each study.

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. No factors have been identified that would require blocking. All rearing units will be plumbed with the same water supply in a confined area in the BFTC DRL. 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, hardness, and alkalinity will 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 12 test units according to SOP No. MISC 205.0. The 12 test units will be numbered 1 through 12, and fish will be distributed into individual units in stages. 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. Although all fish to be used in the studies will be healthy, with no known pathogens, this randomization procedure will ensure that allocation of fish to test units does not become a variable.

4.4.2 Allocation of treatments to experimental units:

After all fish have been transferred to test units, units will be randomly assigned to an exposure condition according to SOP No. MISC 206.0. Exposure concentrations (w/v) will be:

Control	0 mg/L chloramine-T	(0x)
Exposure #1	20 mg/L chloramine-T	(1x)
Exposure #2	60 mg/L chloramine-T	(3x)
Exposure #3	100 mg/L chloramine-T	(5x)

4.5 Configuration of experimental units:

Configuration of experimental units is described in Figure 1. Studies will be conducted in one of three banks of available test units, identified by color coding with either red, black, or green.

5. STUDY PROCEDURES:

5.1 Test Animals:

All test fish in a given study will be from the same fish lot. Rainbow trout fry, fingerling, and juvenile fish used in all studies will be from eyed eggs obtained from the Ennis National Fish Hatchery, Ennis, MT and hatched and reared at the BFTC under conditions identified in SOP No. GEN 030.0. Studies involving adult rainbow trout may not be done at the BFTC because policies involving importation/transfer of live fish and space constraints. Therefore, rearing conditions for these studies will be consistent with the hatchery/lab identified as the study site and will be described in the final report. All test fish will be acclimated in spring water (or source water if study site is other than the BFTC) at the study temperature ± 2 ^{0}C for 1 week prior to initiation of chemical exposure.

5.1.1 Description:

5.1.1.1 Age/Size:

Fish used in studies will be identified according to size class as fry, fingerling, juveniles, or adults. Test animal size used in the studies will correspond to the ranges listed below:

Fry	0.75 - 1.5 inches
Fingerling	2.0 - 4.0 inches
Juvenile	5.0 - 7.0 inches
Adult	> 12 inches

5.1.1.2 Sex:

Sex of the test fish will not be determined or considered.

5.1.1.3 Breed/Class:

The species of test fish will be determined by the hatchery manager according to Eddy and Underhill (1986) or a comparable reference. In addition, egg delivery documentation that includes information pertaining to species, strain and age of broodstock, egg lot number, and fish health reports for the broodstock will be used as a test fish species identification tool. Receipt and distribution of eggs at the BFTC for TAS studies will follow procedures practiced by fish culturists at the BFTC .

5.1.1.4 Initial body weight:

Body weight of individual fish may not be determined prior to transfer of fish to test units. Fish will be sample counted to

determine an estimated fish weight according to Piper et al. (1982). Weights will also be estimated from length/weight tables (Piper et al. 1982) based on mean lengths measured during the pre-study fish health evaluation.

5.1.1.5 Physiological state:
Physiological state of test fish will not be determined nor evaluated.

5.1.1.6 History of test animals:
All life stages of fish with the exception of adults will be held/reared at the BFTC according to SOP No. GEN 030.0. Mortality and maintenance/care records will be kept for at least 1 month prior to fish transfer for fingerling and juvenile studies. Mortality and maintenance/care records will be kept from the date fry were transfered from egg incubation units to rearing tanks for the studies. If any fish receive therapeutic treatment prior to the start of the study, such treatment will be described in the final report.

5.1.2 Number of test animals:
Numbers of test animals per test unit will be dependant on size classification of fish and are listed below. Studies involving adults will not likely be conducted at the BFTC, so number of fish/tank will likely be dependant on test unit size and available test animals. However, justification for the number of adults per test unit will be fully described in the final report. Based on results from pilot chloramine-T target animal safety studies conducted during the past 2 years indicate the numbers of test fish listed below are adequate.

Size classification	Numbers of fish/test unit
Fry	75 - 150
Fingerling	50 - 100
Juveniles	30 - 75
Adult	10 - 50

5.1.3 Source of animals:
Source of fry, fingerling, and juvenile test animals will be from the Ennis NFH. Adults may not be from the Ennis NFH, but the source will be described in the final report.

5.1.4 Identification method if not client-owned companion animals:
Not applicable

5.2 Inclusion criteria:

The entrance criteria for inclusion in the study will depend upon whether healthy fish of the desired size classification are on station and have been acclimated for 1 week at the study temperature.

5.2.1 Ability of Study Director and Investigator to fulfill all the requirements of the protocol:

The Study Director and Investigator will be fully capable of ensuring that all requirements of the protocol are fulfilled. Both have extensive research backgrounds, experience using chloramine-T in accordance with the compassionate INAD protocol (#4000) to treat fish for BGD or other external flavobacteria, and have been involved in conducting/coordinating chloramine-T pivotal field efficacy trials. Both Study Director and Investigator will also have reviewed and understood concepts presented in the document titled "Conduct of Clinical Investigations: Responsibilities of Clinical Investigators and Monitors for Investigational New Animal Drug Studies" (dated October 1992 or later - see Attachment II).

5.2.2 Evaluation to determine health of fish prior to study and samples to be collected at the end of the study:

Methods to evaluate pre-study fish health may include microscopic examination of gill wet mounts and skin scrapes, observation of external and internal tissue characteristics, culture of kidney or spleen innocula on recommended culture media, and/or standard virology testing. Results from the fish health evaluation will be documented on the appropriate forms. All procedures and media used will be used in accordance to FWS/AFS Blue Book Procedures (Amos 1985; see Appendix III for pertinent pages). Pre and post-study fish will be sampled for histological evaluation. Gill (and possibly kidney) tissue from both pre- and post-study fish will be collected, preserved, and processed according to standard procedures for histological examination (Sheehan and Hrapchak 1980; also see SOPs No. MISC 210, INST 115.0, INST 113 and INST 114) .

5.3 Exclusion criteria:

A test unit will be excluded from the study if a disease outbreak occurs, water flow is interrupted unintentionally for more than 1 hour causing DO levels to drop below 5 - 6 mg/L (Davis 1975; Westers and Pratt 1977; Klontz et al. 1983), a standpipe is not refitted after tank cleaning, water flow is not shut off during the standing bath chemical exposure period, or some other similar incident(s) occur. Any incident that may warrent exclusion from the study will be described in the final report.

5.4 Acclimation of test animals:

5.4.1 Duration:
Fish will be acclimated to test water conditions, including temperature, for 1 week to the start of the study.

5.4.2 Medication and/or vaccination during acclimation period:
Fish will receive no medication/vaccination or other type of drug/therapeutant during the acclimation period.

5.4.3 Baseline data collected prior to initiating study:
Daily mortality records for test populations will be maintained prior to transfer of fish, as will weekly water temperature and dissolved oxygen (DO) concentrations measured from head boxes supplying water to rearing/holding tanks. Following transfer of fish to test units, daily mortality, water temperature, and DO concentrations will be collected from each test unit prior to starting the study.

5.5 Blinding of study:

5.5.1 Extent of blinding:
Both the Study Director and Investigator, as well as personnel involved in daily data collection duties, will be blinded with respect to test unit exposure condition. Either the Asst. Center Director (Mr. Greg Kindschi, MS) or Hatchery Manager (Mr. Ron Zitzow, MS) at the BFTC, or both, will serve as non-blinded study participants. They will be responsible for assigning exposure conditions to each test unit, coordinating dosage of test units with chemical solution, collecting water samples during the exposure period, and diluting samples prior to analysis for dose verification. Exposure conditions assigned to test units will be recorded by non-blinded study participants, and this information will be stored in a secure place until completion of the study.

5.5.2 Blinding method:
A single blinding method will be used.

5.5.3 List of personnel with access to treatment codes and rationale:
Only either Mr. Kindschi or Mr. Zitzow will have access to treatment codes. Both will serve as non-blinded study participants who will play key roles in ensuring test units are properly dosed with chloramine-T, but who will not be involved in collection of any raw data.

5.6 Drug Administration:

5.6.1 Dosing regime:

The dosing regime for all studies will be 0, 20, 60, and 100 mg/L (w/v) chloramine-T daily on three alternate days for 3 hours using a standing bath treatment method. Initiation of exposure will begin on day 1.

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):

> [Water volume (gallons) x target treatment concentration (mg/L) x correction factor] ÷ purity of chemical (purity assumed to be 100%).

Studies conducted in 48 x 14 x 7.4" aluminum rectangular tanks dedicated to target animal safety studies will use the following amounts of chloramine-T to achieve 20, 60 or 100 mg/L chloramine-T:

> 20 mg/L = [(2.9 cubic feet x 7.48 gallons/cubic foot) x 20 mg/L x 0.00378] ÷ 1 = 1.64 g chloramine-T.
>
> 60 mg/L = [(2.9 cubic feet x 7.48 gallons/cubic foot) x 60 mg/L x 0.00378] ÷ 1 = 4.92 g chloramine-T.
>
> 100 mg/L = [(2.9 cubic feet x 7.48 gallons/cubic foot) x 100 mg/L x 0.00378] ÷ 1 = 8.20 g chloramine-T.

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

Chloramine-T will be prepared and administered to test units according to SOP No. MISC 202.0. Note that the solubility of chloramine-T in water is 150 g per liter at 25° C.

5.6.2 Route of administration:

Water bath/standing bath treatment.

5.6.3 Proposed withdrawal period:

Fish used in the studies will not be part of any fish production program, and will either remain on station or be disposed of in the local landfill. Therefore, withdrawal periods will not be considered.

5.7 Removal of subjects from study:

5.7.1 Criteria for removal of subjects from the study:

Fish that are either sampled for post exposure fish health evaluations, moribund and sampled for histological evaluation, or that die during acclimation or after initiation of the study will be physically removed from test units.

5.7.2 Procedures for removal of subjects from the study:

Dip nets will be used to collect samples and remove dead fish from test units. Net(s) will be sanitized before and after each use in disinfectant prepared according to SOP No. MISC 203.0.

5.7.3 Fate of removed study animals:

Fish removed for pre-study fish health evaluation, for evaluation of damage to gills after exposure and all mortalities will be disposed of in a local landfill.

5.8 Concurrent/concomitant medications/therapies:

There will be no medication/therapy administered during the course of the study. If fish become diseased, either individual test units will be excluded from the study, or if more than three test units become diseased, the study will be terminated.

5.9 General management practices:

5.9.1 Site visits:

The Study Director and Investigator are stationed at the study site, thus rendering site visits unnecessary.

5.9.2 Data Collection:

Data will be collected by the Study Director, Investigator and/or study participants listed below in section 10.

5.9.3 Frequency of monitoring water chemistry parameters, such as water temperature, dissolved oxygen, hardness, pH, and alkalinity:

Water temperature and dissolved oxygen will be measured twice daily, at the beginning and end of each workday (i.e.,~ 7 a.m. and 3 p.m.), in each

test unit at mid-depth behind the tail screen. On weekends and holidays such data may be collected earlier or later in the day.

Water hardness, pH, and alkalinity will be measured twice during the study. Parameters will be measured once during the first 5 days of the study and again on the last day of the study.

Chloramine-T levels in each test unit will be measured 2 hours into each exposure period during the study.

All measurements will be recorded as raw data on appropriate data collection forms listed below:

Dissolved Oxygen
Water Temperature
Hardness, pH and Alkalinity
Chloramine-T

Where calculations are required to convert digital readings to concentration of desired parameter, space will be provided on data collection forms to show calculations.

Water flows will be adjusted prior to the initiation of the study, after each standing bath exposure, and at least once weekly during the post-exposure period.

5.9.3.1 Parameters to be measured prior to initiation of study:
Before treatment, the following will be measured or calculated and recorded: (1) average fish length (inches); (2) approximate fish weight (grams); (3) test unit volume (gallons); (4) test unit size (ft^3); (5) number of fish/tank; and (6) water flow (gallons/minute); (7) dissolved oxygen (mg/L); (8) water temperature (° C); (9) hardness, pH, and alkalinity. Parameters 6 - 9 will be measured from samples taken from the head box.

5.9.3.3 Procedures and equipment for assessing treatment parameters:
Water temperature will be measured with a YSI Model 55 DO/Temperature meter calibrated against a certified liquid filled thermometer according to SOP No. INST 106.0, and DO concentrations will be measured with a YSI Model 55 DO/Temperature meter according to SOP No. INST 103.0.

Hardness and alkalinity will be measured using Hach Kit Reagents and a Digital Titrator according to SOP Nos. INST 105.0 and INST 104.0, and pH will be measured using a HACH Co. EC10 pH Meter according to SOP No. INST 102.0.

Chloramine-T will be measured using a HACH Test Kit Pocket Colorimeter according to SOP No. INST 101.0. See Appendix IV and V for Draft of General Instructions and FDA Letter of Acceptance of the Method.

Water flows will be set at a predetermined rate (1 - 2 gpm ± 10%) by calibrating flow from gravity fed head boxes plumbed to each test unit according to SOP No. MISC 207.0.

5.9.3.4 Calculations for derived data:
Calculations to determine hardness, alkalinity, and chloramine-T are described in their respective SOP.

5.9.4 Feed type and frequency of feeding:

Test fish will be fed a standard commercial trout grower diet (either from Rangen Inc., P.O. Box 706 (115 13th Avenue S.), Buhl ID, or Nelson and Sons, Inc., 118 West 4800 South, Murray, UT). Fish will be fed 2 - 4 times daily by hand throughout the acclimation and study period. Feed used contains no known contaminants. Dry feed has a shelf life of 3 months if stored unrefrigerated and 6 months if refrigerated. Test fish will not be fed on exposure days. Feed size will be dependant upon life stages and adjusted according to the following schedule:

Fry	Starter or #1 granules
Fingerling	#2 or #3 granules
Juvenile	#4 granules
Adult	1/8" or 3/16" pellets

5.10 Environmental conditions:
Environmental conditions will be consistent for all studies. Listed below are the anticipated conditions (i.e water temperature, DO, pH, hardness and alkalinity) under which studies will be conducted:

	Cold water studies	Warm water studies
Temperature	8° C ± 2° C	14° C ± 2.5° C
DO (mg/L)	9.5 ± 2 mg/L	8.5 ± 2 mg/L
pH	7.8 ± 0.5	8.0 ± 0.5
Hardness (mg/L CaCO3)	206 ± 20	208 ± 20

Alkalinity (mg/L CaCO3)	170 ± 20	160 ± 20

Environmental conditions will be measured prior to the start and at specific times during each study. Water temperature during the three hour static bath chemical exposure may be somewhat higher than the levels described above, but this will be considered acceptable. Water temperature will be measured and recorded throughout the chemical exposure period.

5.11 Tank Cleaning:

Tanks will be cleaned at the beginning of each day according to SOP No. GEN 030.0 and SOP No. INST 112.0.

5.12 Provisions for necropsy and disposal of dead test subjects:
Test animals will not be necropsied, and all mortalities will be disposed of in the local landfill.

5.13 Fate of living test animals after study completion:
All fry, fingerling and juvenile rainbow trout used in studies will be disposed of after the study in the local landfill. Adult fish may or may not be disposed of in a similar manner. If adult fish are not disposed of immediately following study completion, they will remain at the study site. Fate of living test animals after the study will be recorded.

5.14 Test unit configuration:
Test units are configured as described in Figure 1. Each bank of 12 test tanks are color coded, and test tanks used for each study will be denoted in the final report.

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.

6.1.1 When primary variables will be assessed:
Mortalities will be removed, counted, and recorded daily, beginning one the first day of the exposure period and ending at 14 days post exposure.

6.1.2 Procedures for assessing primary variable:

Fish will be classified as either "'dead'" or "alive'" by the Study Director, Investigator, or other personnel who are involved in the study and trained to identify dead fish.

6.1.3 Equipment used for assessing 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 this period.

6.1.5 Forms for retention of source data:
Appropriate forms will be used to adequately record mortality data.

6.1.6 Name(s) and address(es) of outside labs used for analysis of the primary response variable:
No outside labs will be used for analysis of the primary response variable (mortality).

6.2 Other variables to be recorded during the study:
Gill, kidney, and possibly other tissue will be collected for histological evaluation before the first exposure, possibly during the study and at the completion of the study.

6.2.1 When other variables will be assessed:
Samples will be collected from the study population prior to moving fish to test units and on the final day of the study. In addition, moribund fish may be sampled during the course of the study.

6.2.2 Procedures for assessing other variables:
A total of 10 to 20 fish from the reference study population will be sampled before the study starts. Post-study samples will consist of $\leq$ 5 fish per tank. Samples will be processed, embedded, and sectioned according to SOP No. MISC 210, No. INST 113, No. INST 114 and No. INST 115. Gill condition will be described in the final report by the histopathologist assessing damage to gill tissue or lack thereof, and whether damage was reversible. The certified histopathologist will prepare and submit a report describing the assessment, as well as a description of techniques and criteria for assessment used during the evaluation. All preserved tissue, blocks, and slides will be considered raw data and will be archived along with study log books.

6.2.3 Equipment used to assess other variables:
Equipment used to process and stain gill tissue is listed in SOP No. MISC 210, No. INST 113, No. INST 114 and No. INST 115.

6.2.4 Calculation of derived data:
Assessment of gill tissue will be descriptive and require no calculations of derived data.

6.3 Adverse reactions:
Adverse reactions will be noted and recorded by the Study Director, Investigator, or other personnel involved in the study who are trained to detect adverse reactions to chemical exposure. Any adverse reactions not noted by the Study Director will be reported immediately to the Study Director and documented.

6.4 Study facilities:
The BFTC will be the study site when chemical exposure is conducted on fry, fingerling, and juvenile rainbow trout. Exposure studies carried out on adult fish may or may not be conducted at the BFTC. In the event studies using adult fish are carried out at a different facility, study site location and justification for its selection will be described in the final report.

6.4.1 Containment equipment:
Test fish will be contained in the DRL by using covers on test tanks to prevent escapement.

6.4.2 Lighting equipment:
No specialized lighting equipment will be used. Photoperiod will be natural, enhanced by overhead lights whenever they are turned on.

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:
No specialized feeding equipment will 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:
No consideration will be given to space allocation because test tanks will be the experimental unit.

6.4.9 Pasture allocation:
Not applicable

6.4.10 Facility diagram:
Not applicable

6.5 Experimental diets:
No experimental diets will be used during these studies.

7. DATA ANALYSIS:

7.1 Define the experimental unit:
Individual test units will be the experimental unit.

7.2 Define the number of replicates per exposure:
There will be three replicates per exposure and four exposure conditions for each study, for a total of 12 test tanks per study.

7.3 Define statistical methodology:

7.3.1 Null hypothesis:
H_o : $u_1 = u_2 = u_3 = u_4$; Mortality is equal among rainbow trout exposed to 0, 20, 60 or 100 mg/L chloramine-T daily on 3 alternate days for 3 h using a standing bath exposure method.

7.3.2 Alternate (research) hypothesis:
H_a : $u_1 = u_2 \neq u_3 \neq u_4$; Mortality within specific life stages of rainbow trout will be different when exposed to 0, 20, 60, or 100 mg/L chloramine-T administered as a bath solution daily on three alternate days for 3 hours using a static bath treatment method.

7.3.3 Assumptions:
1) Four normally distributed populations.
2) Equality of variances is known.
3) Independent random samples of size n_1, n_2, n_3, and n_4.

7.3.4 Biostatistical procedures used:

A logistic regression analysis will be used to detect effects of chloramine-T exposure concentrations and total mortality. Where differences are stated to be significant, a level of $p \leq 0.05$ is implied.

7.3.5 Statistical data software to be used:
Two statistical software packages will be used: SYSTAT Ver. 8.0 (Wilkinson 1990) and SIGMASTAT Ver. 2.0.

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 logistical regression. Where differences are stated to be significant, a level of $p \leq 0.05$ is implied. If total mortality among any group exposed to chloramine-T is higher than total mortality among the control group, and the calculated *p*-value is less than 0.05, then the conclusions drawn will state that exposure levels that resulted in significantly higher mortality than controls were unsafe (toxic) levels of chloramine-T. If total mortality among chloramine-T exposure groups is higher than the total mortality among the control group, but the calculated *p* value is greater or equal to 0.05, or if the total mortality among chloramine-T exposure groups is less than or equal to the total mortality in the control group, then the conclusions drawn will state that the chloramine-T concentrations test fish were exposed to were not unsafe (non toxic).

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. The method to be used is a DPD colorimetric method using a HACH Co. Test Kit for Chlorine using reagents for both free and total chlorine. A multiplying factor is applied to the measured chlorine level to calculate the concentration of chloramine-T in mg/L. Measurements will be made using a Pocket Colorimeter (Cat. # 46700-00) according to procedures described in SOP No. INST 101.0.

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

8.2.1 An abstract of the method:
See appendix IV 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 in bottles rinsed sufficiently with spring/deionized water using standard analytical chemistry techniques to ensure all chlorine residuals have been flushed from the sample bottles. Samples will be collected at time = 2 h into the 3 h exposure period. One sample will be collected from each test unit. However, a single test unit that received chloramine-T at a target concentration of ≥ 20 mg/L will be sampled in triplicate. Sample volume will be approximately 200 mL and will be collected from near the surface at the middle of the test unit. Because of quantification limitations of the colorimeter, samples will be diluted according to the following scheme:

20 mg/L samples will be diluted 1:1 with distilled water (v:v)
60 mg/L samples will be diluted 4:1 with distilled water (v:v)
100 mg/L samples will be diluted 9:1 with distilled water (v:v)

After sample and distilled water have been combined, the sample must be shaken to ensure thorough mixing. Calculations to determine chloramine-T concentrations must include dilution factor. Concentration of chloramine-T in samples diluted 1:1 will be multiplied by 2; those diluted 4:1 will be multiplied by 5; and those diluted 9:1 will be multiplied by 10. Following analysis all samples will be discarded. Storage of samples for re-analysis is not recommended because chloramine-T breaks down readily under certain circumstances, such as excess organic material in the water. Samples will be analyzed within 3 h of collection.

8.2.3 Evidence of methods validation:
Methods have been developed and accepted for use by the FDA as described in a letter to Dr. Meg Oeller, D.V.M., FDA Liason to NRSP-7 from Dr. Nicholas E. Webber, Leader, Residue Chemistry Team, HFV-151, Office of New Animal Drug Evaluation, Center for Veterinary Medicine (see Appendix V).

8.2.4 Description of validation method plan when method is being developed for the study:
Method had been developed for chloramine-T efficacy trials that were started in 1997.

8.2.5 Quality control procedures for the method and criteria used to assess analytical results:
The pocket colorimeter is factory calibrated and will be zeroed prior to analysis of each sample according to SOP No. INST 101.0. A single test unit that has been dosed with chloramine-T at a concentration of ≥ 20

mg/L will be collected and analyzed in triplicate with each set of samples. A set of samples will consist of all water samples taken during a single exposure period for dose verification. Raw data for both free and total chlorine and calculations to determine concentration of chloramine-T will be recorded. Dose verification results and target concentrations will also be documented.

8.3 Relevant scientific literature supporting the use of the analytical method for the intended measurements:
See **appendix VI** 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 letter of method acceptance in Appendix V.

9. STUDY LOCATIONS:

Studies using fry, fingerling, and juvenile fish will be conducted at the Bozeman Fish Technology Center, U.S. Fish and Wildlife Service, Department of the Interior, 4050 Bridger Canyon Road, Bozeman, Montana 59715.

10. PERSONNEL:

Curriculum Vitae's of all study participants are in Appendix VII. Personnel will be debarred from participation in the proposed studies if they leave the BFTC because of retirement or another job opportunity. It is not anticipated that justification for debarment will occur during the testing period.

10.1 Study Director, Investigator, and Histopathologist involved in studies:

Study Director	James D. Bowker, MS
Investigator	Daniel Carty, MS
Histopathologist	Charlie E. Smith

10.2 Other personnel involved in study:

Bonnie Elliott
Ron Zitzow
Greg Kindschi
Joe Townsend*

Note - Joe Townsend currently serves as the Quality Assurance Officer. In the event of his departure, a substitute QAO will be assigned and denoted in a study protocol amendment.

11. COLLECTION AND RETENTION OF SOURCE DATA:

All data generated in the studies will be recorded in bound laboratory data books or kept in file folders. All data sheets and file folders, preserved tissue, sample blocks and prepared slides, and laboratory data books will be encoded with the study number when the data are generated and stored in secure files. Because of limited space, slides and tissue blocks may have another unique number that can be traced to the study number. Raw data, laboratory data books, and the final report will be archived in the archives of the USFWS National INAD Office (NIO) in Bozeman, MT, before the final report is signed by the Study Director.

12. GOOD LABORATORY PRACTICES:

Data collection, storage, and retrieval procedures for the study will be conducted in compliance with FDA regulations for Good Laboratory Practices (21 CFR 58). The study protocol and progress of the study will be reviewed at the start of the study and periodically throughout the study by the Quality Assurance Officer. The study director has the responsibility of ensuring that all procedures used in conjunction with the study conform with Good Laboratory Practices.

13. ADDENDUM/DEVIATIONS TO THE PROTOCOL:

13.1 Protocol addendums:

Protocol addendums will be forwarded to FDA/CVM and referenced to The Safety of Chloramine-T (Trihydrate) to Various Rainbow Trout Life Stages, Study Protocol Number BFTC-99-CHLT-TAS. Cover letters accompanying submitted addendums will reference the submittal date of the above protocol. Addendums will also be attached to this protocol (see Appendix VIII).

13.2 Protocol amendments:

A signed copy of the study protocol will be retained by the Study Director at the BFTC and at other testing sites that may be used to test adult fish. At any time before a study begins, desired changes in the study protocol will be discussed among the Study Director, Investigator, and Quality Assurance Officer. 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 the Study Director. 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 VIII**).

13.3 Protocol deviations:

Deviations from the established study protocol occasionally cannot be avoided. If deviations occur, the Study Director will make a judgement on the impact of the deviation(s). 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. These statements will be submitted with the protocol as part of the final report.

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

Unused drug will be kept on site for future use according to The USFWS Study Protocol for a Compassionate Aquaculture Investigational New Animal Drug Exemption - Chloramine-T INAD #4000. 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 reference population for use in other research.

REFERENCES

Amos, K. H. , editor. 1985. Procedures for the detection and identification of certain fish pathogens. Third Edition. Fish Health Section, American Fisheries Society. Corvallis, Oregon.

Bills, T. D., L. L. Marking, V. K. Dawson, and J. J. Rach. 1988. Effects of environmental factors on the toxicity of chloramine-T to fish. U.S. Fish and Wildlife Service, Investigations in Fish Control 96, Upper Mississippi Science Center, LaCrosse, Wisconsin.

Bullock, G. L., R. L. Herman, and C. Waggy. 1991. Hatchery efficacy trials with chloramine-T for control of bacterial gill disease. Journal of Aquatic Animal Health 3:48-50.

Davis, J. C. 1975. Minimal dissolved oxygen requirements of aquatic life with emphasis on Canadian species: a review. Journal of Fisheries Research Board of Canada 32:2295 - 2332.

Ferguson, H. W., V. E. Ostland, P. Byrne, and J. S. Lumsden. 1991. Experimental production of bacterial gill disease in trout by horizontal transmission and by bath challenge. Journal of Aquatic Animal Health 3:188:123.

From, J. 1980. Chloramine-T for control of bacterial gill disease. Progressive Fish-Culturist 42:85-86.

Klontz, G. W., P. C. Downey and R. L. Focht. 1983. A manual for trout and salmon production. Sterling H. Nelson, Murray, Utah.

Lasee, B. A., Editor. 1995. Introduction to fish health management, 2nd edition. U.S. Department of the Interior, Fish and Wildlife Service. LaCrosse Fish Health Center, Onalaska, Wisconsin.

Lumsden, J. S., V. E. Ostland, D. D. MacPhee, J. Derksen, and H. W. Ferguson. 1994. Protection of rainbow trout from experimentally induced bacterial gill disease caused by *Flavobacterium branchiophilum*. Journal of Aquatic Animal Health 6:292-302.

Piper, R. G., I. B. McElwain, L. E. Orme, J. P. McCraren, L. G. Fowler and J. R. Leonard. 1982. Fish hatchery management. U.S. Department of the Interior, Fish and Wildlife Service, Washington, DC.

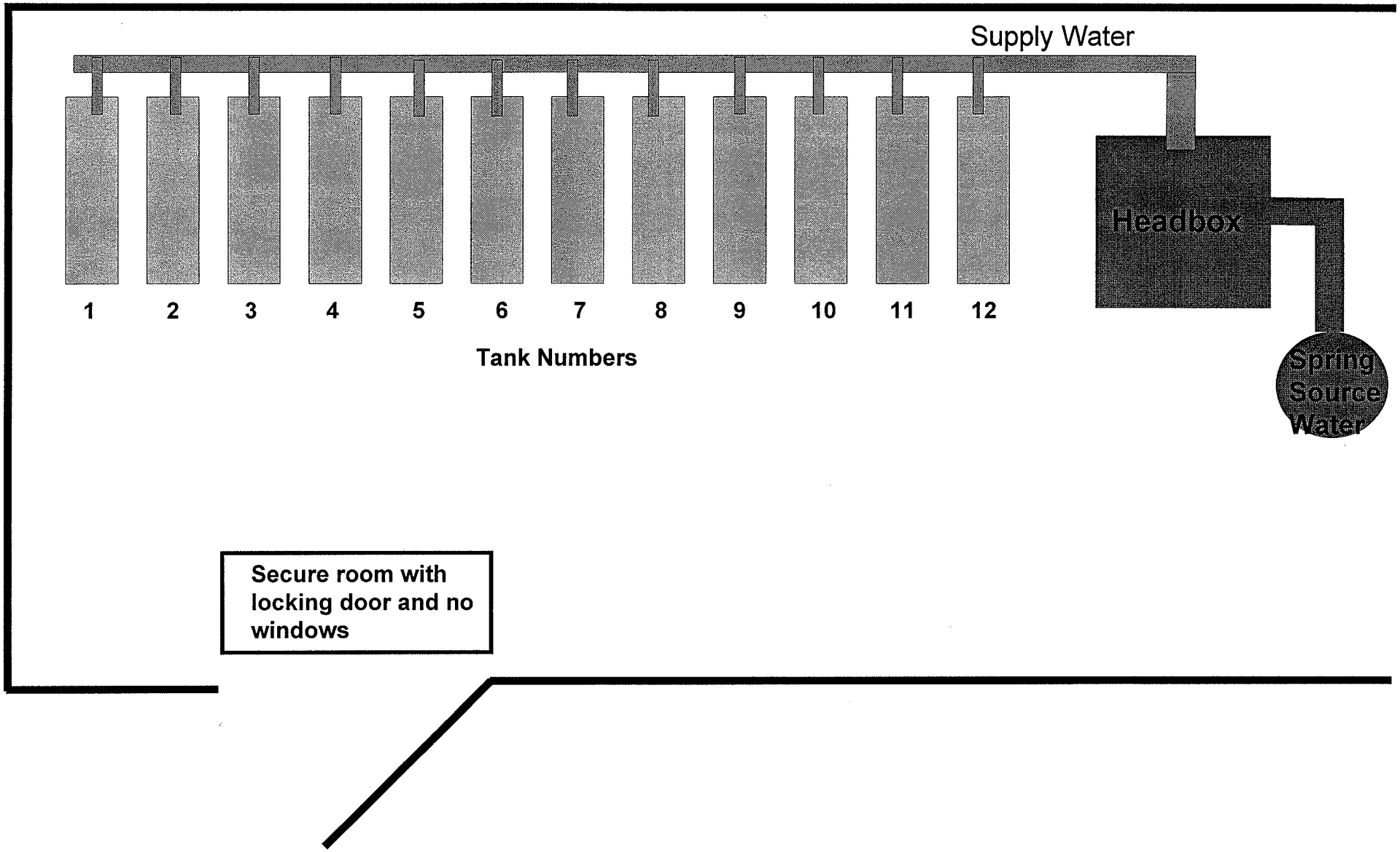
Sheehan, D. C. and B. B. Hrapchak. 1980. Theory and practice of histotechnology, 2nd edition. The C.V. Mosby Co., St. Louis, Missouri.

Westers, H and K. M. Pratt. 1977. Rational design of hatcheries for intensive salmonid culture, based on metabolic characteristics. Progressive Fish-Culturist 39:157-165.

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

Figures

Figure 1. Schematic of Drug Registration Labs Target Animal Safety tank room



Standard Operating Procedures

1.	SOP No. GEN 011.0	Archive of Test Substance
2.	SOP No. MISC 205.0	Randomization Procedures for Distributing Fish to Test Tanks and Transferring Fish from Holding Tanks to Test Tanks
3.	SOP No. MISC 206.0	Randomization Procedure for Assigning Treatments and Controls to Test Tanks
4.	SOP No. GEN 030.0	Test System Care, Maintenance, Disposal
5.	SOP No. MISC 202.0	Handling, preparation and application of Chloramine-T and controls
6.	SOP No. MISC 203.0	Disinfectant Solution
7.	SOP No. INST 106.0	Liquid-Filled Thermometer
8.	SOP No. INST 103.0	Dissolved Oxygen and Water Temperature Meter
9.	SOP No. INST104.0	Hach Digital Titrator to Measure Alkalinity
10.	SOP No. INST105.0	Hach Digital Titrator to Measure Water Hardness
11.	SOP No. INST 102.0	Portable pH/mV/Temperature Meter (pH mode only)
12.	SOP No. MISC 207.0	Method to Measure and Adjust Water Flow
13.	SOP No. INST 101.0	Chlorine Pocket Colorimeter
14.	SOP No. MISC 210.0	Method for Collecting, Processing, Embedding, Cutting, and Staining Histological Samples
15.	SOP No. INST 112.0	Test Tanks
16.	SOP No. INST 113.0	Tissue-Tek III Tissue Embedding Console System
17.	SOP No. INST 114.0	Rotary Microtome
18.	SOP No. INST 115.0	Hypercenter XP tissue Processing System

APPENDICES:

I. Chloramine-T MSDS
II. Chloramine-T investigational label
III. Standard fish health diagnostic scheme to determine if other infectious pathogens are present
IV. Abstract of the DPD colorimetric method to determine concentration of chloramine-T for dose verification
V. Evidence of DPD colorimetric method validation
VI. Relevant scientific literature supporting the use of the DPD Colorimetric method for the intended measurements
VI. Certification that all needed validations for the DPD Colorimetric method will be done before the initiation of the study
VII. Curriculum vitaes for Study Director, Investigator and other personnel involved in study
VIII. Addendums/deviations to protocol

Form 2. Chemical Use Log for Clinical Field Trials Using Chloramine-T Under INAD #4000 for Target Animal Safety Studies following Protocol BFTC-99-CHLT-TAS

Instructions: 1. A copy of this form is to be forwarded to the INAD Investigator at the study site to update the facilities inventory

Facility: _____ **Study Number:** _____

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

Date Chl-T Used	Amount Chl-T Used (g)	Chl-T On-hand (g)	Target Dosage	Species Treated	Used by (initials)

Study Director: _____

Signature and Date

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

Test Site: _____

Address _____

_____

Chloramine-T Lot Number _____ Concentration Tested _____ Test Site Elevation: _____

Fish Species (including 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): _____

Total Number of Test Units: _____

Number of Replicates per Exposure Condition: _____

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 $CaCO_3$): _____

Alkalinity: _____

Water Source (e.g. well, spring, creek, reservoir, 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.

Study Director: _____
Signature and Date

Form 4. **Daily Mortality Record for Target Animal Safety (BFTC-99-CHLT-TAS)**

Daily Mortality

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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	/	/	/	/	/	/	
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NOTE: Mortality should be recorded twice daily, once early in the morning and again late in the afternoon

Study Director: ______________________________ Date ____________

Form 4. **Daily Mortality Record for Target Animal Safety (BFTC-99-CHLT-TAS)**

Daily Mortality

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: Mortality should be recorded twice daily, once early in the morning and again late in the afternoon

Study Director: ______________________________ Date ____________

Form 5. **Daily Record for Dissolved Oxygen Levels for Target Animal Safety (BFTC-99-CHLT-TAS)**

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.

Study Director ______________________ Date ____________

Signature

Form 5. **Daily Record for Dissolved Oxygen Levels for Target Animal Safety (BFTC-99-CHLT-TAS)**

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.

Study Director ______________________ Date ____________
Signature

Form 6. **Daily Record for Water Temperature for Target Animal Safety (BFTC-99-CHLT-TAS)**

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.

Study Director ____________________ Date ____________

Signature

Form 6. Daily Record for Water Temperature for Target Animal Safety (BFTC-99-CHLT-TAS)

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.

Study Director ______________________ Date ____________

Signature

Form 7. **Record of pH, Alkalinity, and Water Hardness for Target Animal Safety (BFTC-98-CHLT-TAS)**

pH and Water Hardness

Date	pH	Alkalinity (mg/L $CaCO_3$)	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.

Study Director _____ Date _____
Signature

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

Hardness #1

Alkalinity #1

Hardness #2

Alkalinity #2

Form 8. Exposure Condition Code for Target Animal Safety (BFTC-98-CHLT-TAS)

Exposure Condition Code

Enter tank number selected into blank space

1x Replicate #1: _____	3x Replicate #1: _____
1x Replicate #2: _____	3x Replicate #2: _____
1x Replicate #3: _____	3x Replicate #3: _____
5x Replicate #1: _____	0x Replicate #1: _____
5x Replicate #2: _____	0x Replicate #2: _____
5x Replicate #3: _____	0x Replicate #3: _____

NOTE: Fill this table out after the end of the study using information from the Random Assignment of Treatments to Test Tanks form filled out by non-blinded study participants.

Study Director _____ Date _____
Signature

Form 8a. Chloramine-T identification code (BFTC-98-CHLT-TAS)

Weighing out chloramine-T
Exposure #1; Date _____

Chloramine-T Concentration	Tank Number	Number Designation	Chloramine-T Concentration	Tank Number	Number Designation
0 mg/L			60 mg/L		
0 mg/L			60 mg/L		
0 mg/L			60 mg/L		
20 mg/L			100 mg/L		
20 mg/L			100 mg/L		
20 mg/L			100 mg/L		

Sample designation code - Chloramine-T samples collected at t = 2 h

Target Chloramine-T Concentration	Tank Number	Letter Designation	Chloramine-T Concentration	Tank Number	Letter Designation
0 mg/L			60 mg/L		
0 mg/L			60 mg/L		
0 mg/L			100 mg/L		
20 mg/L			100 mg/L		
20 mg/L			100 mg/L		
20 mg/L			Triplicate #1		
60 mg/L			Triplicate #2		

Note - Non-blinded study participant, using Form 8, will label bottles with one of the following letters: A through L for weighing out chloramine-T; and A through N for collecting water samples for dose verification. Letter designation will be unique for each tank and will be recorded in the above tables.

Study Director _____ Date _____
Signature

Form 8b. Chloramine-T identification code (BFTC-98-CHLT-TAS)

Weighing out chloramine-T
Exposure #2; Date _____

Chloramine-T Concentration	Tank Number	Number Designation	Chloramine-T Concentration	Tank Number	Number Designation
0 mg/L			60 mg/L		
0 mg/L			60 mg/L		
0 mg/L			60 mg/L		
20 mg/L			100 mg/L		
20 mg/L			100 mg/L		
20 mg/L			100 mg/L		

Sample designation code - Chloramine-T samples collected at t = 2 h

Target Chloramine-T Concentration	Tank Number	Letter Designation	Chloramine-T Concentration	Tank Number	Letter Designation
0 mg/L			60 mg/L		
0 mg/L			60 mg/L		
0 mg/L			100 mg/L		
20 mg/L			100 mg/L		
20 mg/L			100 mg/L		
20 mg/L			Triplicate #1		
60 mg/L			Triplicate #2		

Note - Non-blinded study participant, using Form 8, will label bottles with one of the following letters: A through L for weighing out chloramine-T; and A through N for collecting water samples for dose verification. Letter designation will be unique for each tank and will be recorded in the above tables.

Study Director _____ Date _____
Signature

Form 8c. Chloramine-T identification code (BFTC-99-CHLT-TAS)

Weighing out chloramine-T
Exposure #3; Date _____

Chloramine-T Concentration	Tank Number	Number Designation	Chloramine-T Concentration	Tank Number	Number Designation
0 mg/L			60 mg/L		
0 mg/L			60 mg/L		
0 mg/L			60 mg/L		
20 mg/L			100 mg/L		
20 mg/L			100 mg/L		
20 mg/L			100 mg/L		

Sample designation code - Chloramine-T samples collected at t = 2 h

Target Chloramine-T Concentration	Tank Number	Letter Designation	Chloramine-T Concentration	Tank Number	Letter Designation
0 mg/L			60 mg/L		
0 mg/L			60 mg/L		
0 mg/L			100 mg/L		
20 mg/L			100 mg/L		
20 mg/L			100 mg/L		
20 mg/L			Triplicate #1		
60 mg/L			Triplicate #2		

Note - Non-blinded study participant, using Form 8, will label bottles with one of the following letters: A through L for weighing out chloramine-T; and A through N for collecting water samples for dose verification. Letter designation will be unique for each tank and will be recorded in the above tables.

Study Director _____ Date _____
Signature

Form 9a. Chloramine-T Analysis Raw Data Record for Target Animal Safety (BFTC-99-CHLT-TAS).

Chloramine-T Analysis Record - 1st Chloramine-T Exposure

Exposure Date _____

Sample Identification	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Free Chlorine														
Total Chlorine														
Dilution Factor														
Multiplication Factor	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97	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 - 2nd Chloramine-T Exposure

Exposure Date _____

Sample Identification	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Free Chlorine														
Total Chlorine														
Dilution Factor														
Multiplication Factor	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97	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 - 3rd Chloramine-T Exposure

Exposure Date _____

Sample Identification	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Free Chlorine														
Total Chlorine														
Dilution Factor														
Multiplication Factor	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97	x 3.97	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 Target Animal Safety (BFTC-99-CHLT-TAS).

Chloramine-T Analysis Record - 1st Chloramine-T Exposure

Exposure Date _____

Sample Identification	Tank #1	Tank #2	Tank #3	Tank #4	Tank #5	Tank #6	Tank #7	Tank #8	Tank #9	Tank #10	Tank #11	Tank #12	Dup. #1	Dup. #2
Concentration (Target)														
Concentration (Observed)														

Chloramine-T Analysis Record - 2nd Chloramine-T Exposure

Exposure Date _____

Sample Identification	Tank #1	Tank #2	Tank #3	Tank #4	Tank #5	Tank #6	Tank #7	Tank #8	Tank #9	Tank #10	Tank #11	Tank #12	Dup. #1	Dup. #2
Concentration (Target)														
Concentration (Observed)														

Chloramine-T Analysis Record - 3rd Chloramine-T Exposure

Exposure Date _____

Sample Identification	Tank #1	Tank #2	Tank #3	Tank #4	Tank #5	Tank #6	Tank #7	Tank #8	Tank #9	Tank #10	Tank #11	Tank #12	Dup. #1	Dup. #2
Concentration (Target)														
Concentration (Observed)														

Study Director _____ 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 / Chloramine-T Toxicity

Date	Tank ID	Adverse Reactions / Cloramine-T Toxicity

Date	Tank ID	Adverse Reactions / Cloramine-T Toxicity

Study Director: _____ _____
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 _____
( ) Cultured on _____

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

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 Director _____ Date _____

Form 13. Disposal Record for Animals for Target Animal Safety (BFTC-99-CHLT-TAS).

Study No: _____ Date of Disposal: _____

Number of Fish: _____ Size of Fish: _____

Date of Last Treatment: _____

Disposal Method: ☐ Burial ☐ Incinerator

_____

_____

Disposal Site:

Location of Burial Site:_____

Location of Incinerator:_____

Study Director: _____ _____
Signature Date