

ORIGINAL

THE SAFETY OF CHLORAMINE-T TO VARIOUS LIFE STAGES OF RAINBOW TROUT (*Oncorhynchus mykiss*)

Study Protocol Number: BFTC-99-CHLT-TAS

Summary Report

Experiments 01 - 08 and 10

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Abstract

The United States Fish and Wildlife Service's National Investigational New Animal Drug Office conducted a target animal safety study (Study Protocol Number BFTC-99-CHLT-TAS) designed to generate data needed to obtain U.S. Food and Drug Administration approval for the use of chloramine-T to control mortality in hatchery-reared fishes diagnosed with bacterial gill disease or other Flavobacterial infections of the gills. The study consisted of 10 biologically and statistically independent experiments (Experiments 01 - 10) in which groups of healthy rainbow trout *Oncorhynchus mykiss* fry, fingerlings, or juveniles were acclimated to a water temperature of either approximately 8 or 14°C and then exposed three times to chloramine-T at concentrations ranging from 0 to 100 mg/L (i.e., at concentrations up to five times greater than the proposed maximum therapeutic concentration of 20 mg/L). Nine of the experiments (Experiments 01 - 08 and 10) were conducted to evaluate mortality of test fish resulting from exposure to chloramine-T, and one of the experiments (Experiment 09) was conducted to evaluate histological effects on test fish resulting from exposure to chloramine-T. The objective of this report is to summarize data generated during the nine "mortality" experiments (Experiments 01 - 08 and 10). In these experiments, blinding techniques were employed to ensure that study participants involved in day-to-day data collection did not know which exposure concentrations of chloramine-T were administered to which test tanks. In all nine experiments, completely randomized design procedures were used

to allocate test fish to test tanks. Either completely randomized design procedures or randomized block design procedures were used to assign chloramine-T exposure concentrations to test tanks. Each experiment consisted of a pre-exposure phase, an exposure phase, and a post-exposure phase. During the pre-exposure phase, test fish were acclimated to environmental conditions in the test tanks. Also, during the pre-exposure phase, test fish found dead in test tanks or confirmed missing from test tanks were replaced with live fish from a reference population so that the appropriate number of test fish was present in each test tank at the start of the exposure phase. During the exposure phase, chloramine-T exposures were administered three times on either alternate days (Experiments 01 - 08) or consecutive days (Experiment 10). Exposures were administered as static-bath treatments that lasted 3 h each (i.e., three times longer than the proposed standard therapeutic treatment duration of 1 h), and each chloramine-T concentration tested was administered in triplicate. During the exposure and post-exposure phases, mortality of test fish was the primary response variable. For each test tank, "total mortality" was calculated by adding the number of dead fish removed from the tank during the exposure and post-exposure phases to the number of fish missing and unaccounted for when test fish were counted out of the tank on the last day of the experiment. In each experiment, mean total mortality was (when possible) compared statistically among exposure groups; in addition, for each exposure group, mean total mortality was subjectively ranked as being "low," "moderate,"

"moderately high," or "high." Mortality data generated during Experiments 01 - 08 and 10 indicated that:

1. The proposed maximum therapeutic treatment concentration of 20 mg/L chloramine-T, when administered as a static-bath treatment three times on alternate or consecutive days, is safe for use on rainbow trout fry, fingerlings, and juveniles being reared at water temperatures ranging from ≈ 8 to $\approx 14^{\circ}\text{C}$;
2. For rainbow trout fry being reared at water temperatures ranging from ≈ 8 to $\approx 14^{\circ}\text{C}$, the margin of safety extends to nearly 100 mg/L chloramine-T when the drug is administered three times as a static-bath treatment;
3. For rainbow trout fingerlings being reared at water temperatures ranging from ≈ 8 to $\approx 14^{\circ}\text{C}$, the margin of safety extends to at least 60 mg/L chloramine-T when the drug is administered three times as a static-bath treatment;

4. For rainbow trout juveniles being reared at water temperatures ranging from ≈ 8 to $\approx 14^{\circ}\text{C}$, the margin of safety exceeds 50 mg/L chloramine-T—but is, for practical purposes, less than 60 mg/L chloramine-T—when the drug is administered three times as a static-bath treatment. Such a margin of safety appears to be the same regardless of whether chloramine-T is administered three times on alternate days or three times on consecutive days; and

5. Rainbow trout juveniles are probably most susceptible to the toxic effects of relatively high concentrations (≥ 60 mg/L) of chloramine-T the first time that they are exposed to it.

Introduction

Bacterial gill disease (BGD) is one of the most common external bacterial diseases of hatchery-reared salmonids (Bullock 1990; Ferguson et al. 1991). Moreover, BGD causes more losses of fish at salmonid hatcheries than any other bacterial disease (Bills et al. 1988). Factors that predispose hatchery-reared salmonids to BGD include overcrowding, low dissolved oxygen levels, high ammonia concentrations, excessive particulate matter in the water, and other environmental stressors associated with intensive fish culture practices (Warren

1991; Lasee 1995). Although *Flavobacterium branchiophilum* (a yellow-pigmented, gram-negative bacterium) is generally recognized as the primary etiological agent of BGD (Wakabayashi et al. 1989; Ferguson et al. 1991), other gram-negative bacteria, including *Flavobacterium* spp., *Aeromonas* spp., and *Pseudomonas* spp., can also cause BGD. Generally, verification of the specific etiological agent is not required before drug therapy or other BGD-control measures are implemented (Lasee 1995).

Characteristic symptoms of BGD include acute onset, equidistant spacing of fish, decreased fright response, reduced food consumption, increased branchial rate, flared opercula, and "whitish" gill tips (Post 1987; Lumsden et al. 1994; Lasee 1995). Such symptoms, coupled with the microscopic identification of filamentous bacteria on gill filaments (without gill necrosis), are used to confirm the presence of BGD (Lasee 1995). Fish infected with BGD often produce excess mucus at the gills, and such fish also exhibit proliferation of gill epithelial tissue and clubbing and fusing of gill lamellae (Bullock 1990). Bacterial gill disease may not kill fish directly; rather, mortality probably results from asphyxiation because of inadequate oxygen uptake at the gills (Wakabayashi and Iwado 1985). Bacterial gill disease is horizontally transmitted, and if it is not diagnosed and treated during the early stages of an outbreak, an epizootic can occur within a 24-h period (Bullock et al. 1991).

Reducing the number and severity of environmental stressors can often alleviate mild outbreaks of BGD (Lasee 1995). However, chemotherapeutic treatment is usually needed to curtail severe BGD outbreaks. Several chemicals, including benzalkonium chloride (available as Hyamine 1622 and 3500), diquat, and chloramine-T, have historically been used for the control of mortality in hatchery-reared salmonids diagnosed with BGD (Bullock et al. 1991). However, these chemicals have not been approved for such use by the U.S. Food and Drug Administration (FDA). Chloramine-T has emerged as the chemotherapeutant of choice with respect to efforts to gain FDA approval because it appears to be the most effective drug to control BGD in fishes (From 1980; Bullock et al. 1991; Thorburn and Moccia 1993). 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.

Currently, a compassionate Investigational New Animal Drug (INAD) exemption granted by the FDA is required for the use of chloramine-T to control mortality in hatchery-reared fishes diagnosed with BGD. A compassionate INAD exemption allows for the large-scale treatment of fish and other aquatic animals while dose-response efficacy data and other data required to support a New Animal Drug Approval (NADA) are being generated. An important requirement for a NADA is the conduct of studies and the submission of resultant data (to FDA) that

demonstrate the safety of a drug or therapeutant with respect to the species for which the treatment is intended (i.e., the target species). Such studies are termed target animal safety (TAS) studies and are used to determine the potential toxicity of a drug or therapeutant to one or more target species. Target animal safety studies must follow rigorous protocols and comply with Good Laboratory Practice (GLP) standards for nonclinical laboratory studies (see 21 CFR, Part 58; CFR 1999).

To provide data needed to support a NADA for the use of chloramine-T to control mortality in hatchery-reared salmonids diagnosed with BGD, the United States Fish and Wildlife Service's (USFWS) National Investigational New Animal Drug Office (NIO) designed and conducted a TAS study (Study Protocol Number BFTC-99-CHLT-TAS) on rainbow trout *Oncorhynchus mykiss*. Rainbow trout was selected as the test species because it is commonly reared at hatcheries, is an important recreational and commercial species, and is often used as a surrogate test species for other salmonids (Mayer and Ellersieck 1986). Specifically, the NIO study was conducted to generate TAS data that would support the following proposed label claim for chloramine-T:

For use in the control of mortality in freshwater-reared salmonid fishes diagnosed with bacterial gill disease.

Treat fish three times at 12-20 mg/L for 1 h in a static-bath or flow-through treatment system. Fish may be treated on either consecutive or alternate days.

Study Overview and Report Objective

Study Protocol Number BFTC-99-CHLT-TAS consisted of 10 biologically and statistically independent experiments (Experiments 01 - 10) conducted at the USFWS Aquaculture Drug Research Laboratory (ADRL), Bozeman Fish Technology Center (BFTC), Bozeman, MT, between April 12, 1999, and September 13, 2000. All of the experiments were conducted under a written study protocol (Bowker 1999) and FDA regulations for GLPs for nonclinical laboratory studies (see 21 CFR, Part 58; CFR 1999). An independent Quality Assurance Officer monitored compliance with the study protocol, standard operating procedures, and GLPs.

In each experiment, groups of healthy rainbow trout fry, fingerlings, or juveniles (i.e., the test fish) were acclimated to a water temperature of either approximately 8 or 14°C and then exposed three times to chloramine-T (i.e., the test article) at concentrations ranging from 0 to 100 mg/L (i.e., at concentrations

up to five times greater than the proposed maximum therapeutic concentration of 20 mg/L). Chloramine-T exposures were administered three times on either alternate days (Experiments 01 - 09) or consecutive days (Experiment 10) as static-bath treatments that lasted 3 h each (i.e., three times longer than the standard therapeutic treatment duration of 1 h). Nine of the experiments (Experiments 01 - 08 and 10) were conducted to evaluate mortality of test fish resulting from exposure to chloramine-T, and one of the experiments (Experiment 09) was conducted to evaluate histological effects on test fish resulting from exposure to chloramine-T (Bowker et al. In preparation).

The objective of this report is to summarize data generated during the nine experiments conducted to evaluate mortality of test fish resulting from exposure to chloramine-T (Table 1). Two of the experiments were conducted on rainbow trout fry (Experiment 01 at 8°C; Experiment 02 at 14°C); in each, the chloramine-T concentrations tested were 0, 20, 60, and 100 mg/L. Two of the experiments were conducted on rainbow trout fingerlings (Experiment 05 at 8°C; Experiment 06 at 14°C); combined, the chloramine-T concentrations tested were 0, 20, 30, 40, 50, and 60 mg/L. Five of the experiments were conducted on rainbow trout juveniles (Experiment 03 at 8°C; Experiments 04, 07, 08, and 10 at 14°C); collectively, the chloramine-T concentrations tested were 0, 20, 30, 40, 50, 60, 70, 80, and 100 mg/L.

Materials and Methods

Test Article (chloramine-T)

The test article used in the experiments was the chemical chloramine-T (trade name: Halamid; CAS #127-65-1). Chloramine-T is a white, crystalline powder with a weak chlorine odor, and it is a pure compound with no inactive ingredients. The chemical name of chloramine-T is "benzene sulfonamide, N-chloro-4-methyl, sodium salt" (synonym, "sodium p-toluenesulphonchloramide"). The chemical formula of chloramine-T is $C_7H_7ClNNaO_2S \cdot 3H_2O$. The test article was manufactured by Akzo Chemicals B.V. (Nieuwendammerkade 1-3, P.O. Box 26223, 1002 GE Amsterdam, The Netherlands) and obtained from Akzo Chemical, Inc. (300 South Riverside Plaza, Chicago, IL 60606). A "certificate of analysis" obtained from Akzo Chemical, Inc., identified the test article as chloramine-T lot number 0299303520272 and listed the "strength and purity" of the test article as being approximately 100%.

Test Fish (rainbow trout)

The fish species used in the experiments was rainbow trout (Order: Salmoniformes; Family: Salmonidae; Genus and species: *Oncorhynchus mykiss*).

"Eyed" rainbow trout eggs from four different egg lots (Table 2) were obtained from the USFWS, Ennis National Fish Hatchery, Ennis, MT. Each group of "eyed" eggs obtained was incubated and hatched at the BFTC. Resultant fish were positively identified as rainbow trout and were reared at the BFTC until the start of the experiment in which they were used.

In each experiment, mean total length and length range of test fish were estimated by measuring a sample of 40 - 60 fish drawn from a reference population for pre-exposure fish health examinations (Table 2). For the two experiments conducted on rainbow trout fry (Experiments 01 and 02), estimated mean total lengths of test fish were 3.0 and 3.3 cm, respectively. For the two experiments conducted on rainbow trout fingerlings (Experiments 05 and 06), estimated mean total lengths of test fish were 7.8 and 7.7 cm, respectively. For the five experiments conducted on rainbow trout juveniles (Experiments 03, 04, 07, 08, and 10), estimated mean total lengths of test fish ranged from 14.6 to 16.0 cm. In all of the experiments, sex of test fish was neither determined nor considered; however, it was assumed that male and female test fish were present in roughly equal proportions.

Procedures

Blinding techniques

Blinding techniques were used to minimize the potential for bias in the conduct of the experiments. Non-blinded study participants were aware of which test tanks received which exposure concentrations of chloramine-T, whereas blinded study participants were not aware of which test tanks received which exposure concentrations of chloramine-T. Non-blinded study participants were responsible for the (1) random assignment of chloramine-T exposure concentrations to test tanks, (2) placement of tank labels on bottles of chloramine-T solutions before each exposure period, and (3) collection, dilution (if necessary), and labeling of water samples used for chloramine-T dose verification. Blinded study participants were responsible for collecting and recording all other data generated during the experiment, including the (1) collection and preparation of fish used for pre-exposure fish health examinations and pre-exposure histology samples, (2) collection and preparation of fish used for post-exposure fish health examinations and post-exposure histology samples, (3) random allocation and transfer of fish from holding tanks to test tanks, (4) daily care, feeding, and maintenance of test fish, (5) administration of chloramine-T exposures to test tanks, (6) analysis of water samples collected for chloramine-T dose verification, (7) collection of

mortality and water quality data, and (8) colorimetric testing of the strength and purity of the chloramine-T used in the experiment. In all of the experiments, blinded study participants were unblinded only after the experiment ended.

Test tanks and source water

Test tanks used in the experiments were rectangular, constructed of aluminum, and had total tank volumes of approximately 3.89 ft³ (48 in long x 14 in wide x 10 in deep; Figure 1). Each test tank was fitted with a tail screen to help prevent fish escapement and an outflow standpipe to regulate water depth. Outflow standpipes were cut so that each test tank had a water depth of approximately 7.6 in (Experiments 01 - 07), 7.5 in (Experiment 10), or 3.5 in (Experiment 08). With tail screen and standpipe in place, the approximate total-water and rearing-water volumes of each test tank were 2.96 ft³ and 2.56 ft³ (Experiments 01 - 07), 2.92 ft³ and 2.52 ft³ (Experiment 10), or 1.36 ft³ and 1.18 ft³ (Experiment 08). Removable covers made of polyvinyl chloride (PVC) pipe and 9-mm plastic-mech screen were placed on top of each tank to help prevent fish escapement.

Cold-spring and warm-spring water were plumbed into the ADRL and routed to a head box, where water flow and water temperature were adjusted (Figures 2

and 3). From the head box, water was gravity-fed to the test tanks. Adjustable spigots (one per test tank) were used to regulate water inflow to the test tanks. During the static-bath, chloramine-T exposures, a Hagen Model 80 electric air pump (capacity, 75 L/min; Rolf C. Hagen, Inc., Mansfield, MA) was used to supply air-supplementation to the test tanks. The pump forced ambient air through a 1.3-cm diameter PVC pipe tapped with brass nozzles, each of which was connected with flexible plastic tubing to one Sweetwater® medium-pore, air-diffuser stone (Aquatic Ecosystems, Inc., Apopka, FL) suspended just below the surface of the water in the head end of a test tank.

Allocation and transfer of test fish from holding tanks to test tanks

In all of the experiments, completely randomized design procedures were used to allocate and transfer fish from tanks holding a reference population to the test tanks. In Experiments 01 - 03, 05 - 08, and 10, test fish were transferred to test tanks on experiment day 1. However, logistic constraints (i.e., the availability of a sufficient number of study personnel) necessitated transferring test fish from holding tanks to test tanks 3 d before the start of Experiment 04.

The number of test tanks used in each experiment was a function of the number of chloramine-T exposure concentrations tested and the fact that each

exposure concentration tested was administered in triplicate (Table 2). In experiments where four chloramine-T exposure concentrations were tested (Experiments 01 - 05), 12 test tanks of test fish were used (Figure 2). In experiments where six chloramine-T exposure concentrations were tested (Experiments 06 - 08 and 10), 18 test tanks of test fish were used (Figure 3).

The number of test fish used per test tank was a function of test-fish life stage and test-tank rearing volume. Essentially, the number of test fish used per test tank was sufficient to minimize the potentially confounding effects of incidental mortality and ensure that test fish were not overcrowded. Therefore, in experiments conducted on rainbow trout fry (Experiments 01 - 02), 100 test fish were placed in each test tank; in experiments conducted on rainbow trout fingerlings (Experiments 05 - 06), 50 test fish were placed in each test tank; and in experiments conducted on rainbow trout juveniles, either 40 test fish (Experiment 03), 30 test fish (Experiments 04, 07, and 10) or 15 test fish (Experiment 08) were placed in each test tank.

Maintenance, care, feeding, and fate of test fish

During the experiments, test fish were maintained in test tanks located in rooms without windows; consequently, virtually all light was provided by overhead,

fluorescent lights. Each day, lights were turned on when study personnel arrived for work (range, 0600 - 0855 hours) and were turned off when study personnel left work for the day (range, 1345 - 1745 hours). Water inflow to the head end of each test tank was adjusted to either approximately 3.8 L/min (2.7 water exchanges/h; Experiments 01 - 07 and 10) or 2.8 L/min (4.4 water exchanges/h; Experiment 08). Water temperature at the start of each experiment was either approximately 8°C (Experiments 01, 03, and 05) or 14°C (Experiments 02, 04, 06 - 08, and 10).

Density Index (DI) and Flow Index (FI) values, which are indicators of whether or not hatchery-reared fish are being maintained within the carrying capacity of a given rearing unit (Piper et al. 1982), were calculated for each experiment (Table 2). The DI value relates total weight of fish per tank to rearing-water volume and mean length of fish; the FI value relates total weight of fish per tank to water inflow and mean length of fish. To maintain healthy trout in a hatchery environment, Piper et al. (1982) recommended that the DI value not exceed 0.5 (regardless of water temperature or elevation of the rearing facility). Density Index values calculated for the experiments ranged, respectively, from 0.02 to 0.22 (Table 2), which indicated that the test fish were maintained well within the carrying capacity of the test tanks used in the experiments.

Flow Index values recommended by Piper et al. (1984) vary with water temperature and elevation of the rearing facility. The BFTC lies at an elevation of approximately 1,463 m; therefore, for the three mortality experiments conducted at approximately 8°C (Experiments 01, 03, and 05), the maximum recommended FI value was 1.8. For the six mortality experiments conducted at approximately 14°C (Experiments 02, 04, 06 - 08, and 10), the maximum recommended FI value was 1.2. Flow index values calculated for the experiments ranged, respectively, from 0.06 to 0.57 (Table 2), which indicated that the test fish were maintained well within the carrying capacity of water flows used in the experiments.

In each experiment, care and feeding of test fish were consistent among test tanks; however, care and feeding routines differed between days when chloramine-T exposures were administered and days when chloramine-T exposures were not administered. On days when chloramine-T exposures were administered, the test fish were not fed, and test tanks were cleaned twice in the morning before the exposure period started. On days when chloramine-T exposures were not administered, test tanks were cleaned once in the morning and once in the afternoon, and each test tank of fish was hand-fed with an appropriate amount and size of Rangen Custom Trout Grower (manufactured by Rangen Inc., Buhl, ID). Rainbow trout fry were fed four times daily; rainbow trout fingerlings and juveniles were fed twice daily. During the exposure and post-exposure phases of each

experiment, the daily feed ration for a test tank was adjusted if and when dead fish were discovered and removed from the tank.

At the end of experiments 01 - 08, all but a maximum of two of the live fish remaining in each test tank that still contained live fish were euthanized in a solution of tricaine methanesulfonate (Tricaine-S; Western Chemical, Inc., Ferndale, WA). Euthanized fish were counted, recorded, and stored in a freezer before disposal in a local landfill. For each test tank that contained one or two live fish, the fish were collected, counted, recorded, and used for post-exposure histology samples. In Experiments 06 - 08, fish collected for post-exposure histology samples were also used for post-exposure fish health examinations. In Experiment 10, no post-exposure histology samples were collected and no post-exposure fish health examinations were conducted; therefore, at the end of this experiment, all live fish remaining in each test tank were euthanized in a solution of Tricaine S, counted, recorded, and stored in a freezer before disposal in a local landfill.

Fish health examinations

Pre-exposure fish health examinations — During the pre-exposure phase of each experiment, a sample of 40 - 60 fish was drawn from a reference population and sacrificed for pre-exposure fish health examinations (Table 2). Fish were

collected in groups of five, anesthetized in a solution of Tricaine S, and measured to the nearest 0.1 cm total length. Because rainbow trout fry were small (about 3 cm long), they were only examined for viral fish pathogens. However, rainbow trout fingerlings and juveniles were examined in the following manner: (1) selected external and internal tissues and organs were visually examined for signs of gross pathology; (2) tissue samples from the posterior kidney were streaked on Brain Heart Infusion Agar (BHIA), cultured at room temperature, and evaluated 2 - 7 d later for growth of gram-negative bacteria; and (3) skin-scrape and gill-squash slides were prepared from 20% of the fish examined (i.e., one of every five fish examined). Each slide was examined under a light microscope (set at 40 - 200X) for the presence of bacteria and parasites.

During pre-exposure fish health examinations, no pathogens were observed during virology testing of fry. For fingerling and juvenile fish, no severe external or internal gross pathologies were noted, no bacteria known to be fish pathogens were grown on BHIA media, and, with the exception of Experiment 10, no bacteria or parasites were observed during microscopic evaluation of skin-scrape and gill-squash slides. In Experiment 10, the parasite *Salmincola* sp. was found on 11 (1.9%) of 591 fish examined (55 fish drawn from the reference population for pre-exposure fish health examinations plus 536 test fish examined during the experiment). One or more eroded or frayed fins were found on nearly all juvenile

fish sampled (Experiments 03, 04, 07, 08, and 10) and on some of the fingerling fish sampled (Experiments 05 and 06). A few of the juvenile fish sampled also had slightly shortened opercula, exhibited mild exophthalmia, or had other minor abnormalities.

It was inferred from the results of the pre-exposure fish health examinations that (a) fish in all of the reference populations were "healthy" and that (b) "healthy" test fish were used in all of the experiments. In Experiment 10, *Salmincola* sp. was found on so few fish that it was concluded that its presence did not adversely affect the outcome of this experiment. Minor abnormalities, such as eroded or frayed fins, slightly shortened opercula, and mild exophthalmia (existing either singly or in combination) can be common in hatchery-reared fish and usually do not compromise their performance in a hatchery environment. Therefore, these minor abnormalities (and other minor abnormalities noted) were not considered to have adversely affected the outcomes of any of the experiments.

Post-exposure fish health examinations — At the end of Experiments 06 - 08 (test fish, juvenile rainbow trout), a maximum of two live fish were collected from each test tank that still contained live fish; these fish were sacrificed for post-exposure fish health examinations. Each fish collected was anesthetized in a solution of Tricaine S, measured to the nearest 0.1 cm total length, and examined

for signs of gross external and internal pathologies. In all three of these experiments, no severe external or internal pathologies were noted in any of the fish examined. The most common minor abnormalities noted were eroded or frayed fins (most fish), slightly shortened opercula (few fish), and mild exophthalmia (few fish). Such minor abnormalities (existing either singly or in combination) can be common in hatchery-reared fish and usually do not compromise their performance in a hatchery environment. Therefore, these minor abnormalities were not considered to have adversely affected the outcomes of these three experiments.

Collection and preparation of histology samples for long-term storage

Pre-exposure histology samples — In Experiments 01 - 08, fish were collected from a reference population and used for pre-exposure histology samples. In each of the experiments conducted on rainbow trout fry (Experiments 01 and 02), a sample of 60 fish (in addition to the 60 fish collected for pre-exposure fish health examinations) was collected from a reference population and used for pre-exposure histology samples. In each of the experiments conducted on rainbow trout fingerlings (Experiments 05 and 06) and juveniles (Experiments 03, 04, 07, 08, and 10), the sample of 40 - 60 fish collected from a reference population for pre-exposure fish health examinations was also used for pre-exposure histology samples. Euthanizing, fixing, embedding, and long-term storage procedures used

for fish sampled for pre-exposure histology are described in the final reports for each experiment. All pre-exposure histology samples collected and prepared for long-term storage are archived at the BFTC.

Post-exposure histology samples — On the last day of Experiments 01 - 08, a maximum of two live fish were collected from each test tank that still contained live fish and were sampled for histology. Euthanizing, fixing, embedding, and long-term storage procedures used for fish sampled for post-exposure histology were identical to such procedures used for fish collected and sampled for pre-exposure histology. All post-exposure histology samples collected and prepared for long-term storage are archived at the BFTC.

Assignment and administration of test article exposure concentrations to test tanks

Either completely randomized design procedures (Experiments 01 - 05; 12 test tanks per experiment; Figure 2) or randomized block design procedures (Experiments 06 - 08, and 10; 18 test tanks per experiment; Figure 3) were used to assign chloramine-T exposure concentrations to test tanks. In both the 12-tank and 18-tank experiments, all test tanks were supplied with water from a single head box. However, in the 18-tank experiments, the test tanks were physically

separated into one group of 12 tanks and one group of 6 tanks. Usually, there was a small difference in water temperature (less than 0.5°C) between the two groups of tanks. Therefore, for the purpose of randomly assigning chloramine-T exposure concentrations to test tanks, the group of 12 test tanks was divided into Blocks 1 and 2, and the group of 6 test tanks was designated as Block 3 (Figure 3).

In Experiments 01 - 03, 05 - 08, and 10, test fish were allowed to acclimate to conditions in the test tanks for 7 d (i.e., experiment days 1 - 7) before the first chloramine-T exposures were administered. However, logistic constraints (i.e., the availability of a sufficient number of study personnel) necessitated transferring test fish from holding tanks to test tanks 3 d before the start of Experiment 04. Therefore, in Experiment 04, test fish were allowed to acclimate to the conditions in the test tanks for 10 d before the first chloramine-T exposures were administered.

During the exposure phase of each experiment, chloramine-T exposures were administered three times on either alternate days (experiment days 8, 10, and 12; Experiments 01 - 08) or consecutive days (experiment days 8, 9, and 10; Experiment 10). To determine the chloramine-T "margin of safety" for each of the three rainbow trout life stages used in the experiments, the exposure concentrations tested varied among the nine experiments (Table 1). In each

experiment, the amount of chloramine-T required to achieve each target concentration was calculated. These amounts of chloramine-T were then weighed out on either a Sartorius Model AC 121S analytical balance (Sartorius Corp., Edgewood, NY; Experiments 01 - 06) or a Sartorius Model BP 211D analytical balance (Experiments 07, 08, and 10) and dissolved in distilled water. Immediately before chloramine-T and control solutions were added to test tanks, water inflows to all test tanks were turned off, and supplemental air to all test tanks was turned on. Each 3-h static-bath exposure period then began when chloramine-T and control solutions were added and mixed into the test tanks. Approximately 1 - 2.5 h into each exposure period, water samples were collected from each test tank for chloramine-T dose-verification and quality control purposes. At the end of each 3-h exposure period, water inflows to test tanks were turned on and adjusted, supplemental air to test tanks was turned off, and approximately half of the water was drained from each test tank to expedite flushing of tank contents. Test-tank contents were drained to the waste-water treatment facility at the BFTC.

Data Collection and Analysis

Mortality

During each experiment, mortality of test fish that occurred during the exposure and post-exposure phases was the primary response variable. For mortality results to be creditable, it was necessary to ensure that the correct number of test fish was present in each test tank at the start of the exposure phase. Therefore, during the pre-exposure phase, test fish found dead or moribund in test tanks and test fish known to have escaped from test tanks were replaced with live fish drawn from the reference population.

At the end of each experiment, "total mortality" for each test tank was calculated by adding the number of dead fish removed from the tank during the exposure and post-exposure phases to the number of fish missing and unaccounted for when test fish were counted out of the tank at the end of the experiment. For each test tank, "percent total mortality" was calculated by dividing "total mortality" by the number of live fish in the tank at the beginning of the exposure phase of the experiment and multiplying the result by 100. To facilitate statistical analyses of mortality data, "percent total mortality" for each test tank was re-expressed as a proportion, which was then transformed (to radians) using a modified form of the

Freeman-Tukey transformation (Zar 1984; Robison-Cox 1999). For all exposure concentrations tested in each experiment, transformed proportions were used in statistical analyses.

In each experiment, the null hypothesis was that there was no difference in mean total mortality among exposure groups ($H_0: \mu_1 = \mu_2 = \mu_3 = \dots \mu_n$). Initially, it had been decided to use parametric analysis of variance (ANOVA; $\alpha = 0.10$) and the Tukey multiple-means comparison test ($\alpha = 0.10$; Zar 1984; SPSS 1997; SPSS 1998; Robison-Cox 1999) to statistically compare mean total mortality among exposure groups. However, such an approach was only possible in two experiments (Experiments 02 and 08). In six experiments (Experiments 01, 03, 05 - 07, and 10), it was only possible to statistically compare mean total mortality between two of the exposure groups tested because mean total mortality in the remaining groups was either extreme (0% or 100%) or, if between 0 and 100%, was lacking within-group variation. In such cases, a two-sample, two sided t-test ($\alpha = 0.10$; Zar 1984) or its nonparametric equivalent (Mann-Whitney test; $\alpha = 0.10$; Zar 1984) was used to statistically compare mortality between two exposure groups. In one experiment (Experiment 04), statistical comparisons of mean total mortality among exposure groups was not warranted because mortality in three of the four exposure groups tested was extreme (0% or 100%). Regardless of whether or not mortality data were statistically analyzed, in each experiment, mean

total mortality for each exposure group was subjectively ranked (by the study director and investigator) as being "low," "moderate," "moderately high," or "high."

Strength and purity of test article

Strength and purity of the test article was evaluated by high-pressure liquid-chromatography (HPLC) and colorimetrically. The USFWS NIO sent a 5-g sample of the test article to the U.S. Geological Survey, Upper Midwest Environmental Sciences Center (UMESC), La Crosse, WI, where the "strength and purity" of the test article was evaluated by HPLC. Colorimetric evaluation of the "strength and purity" of the test article was conducted by the USFWS NIO during the pre-exposure and post-exposure phases of each experiment.

Colorimetric evaluation of the "strength and purity" of the test article involved preparing three reference solutions of chloramine-T (each with a calculated concentration of 7.5 mg/L) and then measuring the chloramine-T concentration of each reference solution to the nearest 0.1 mg/L with a HACH Chlorine Pocket Colorimeter (HACH Company, Loveland, CO). Each reference solution was measured once, and a mean measured chloramine-T concentration (based on $N = 3$ measurements) was calculated. The mean measured chloramine-T concentration of the reference solutions was statistically compared to the calculated concentration

of the reference solutions with a one-sample t-test ($H_0: \mu_{\text{measured}} = 7.5 \text{ mg/L}$ vs. $H_a: \mu_{\text{measured}} \neq 7.5 \text{ mg/L}$; $\alpha = 0.05$; Zar 1984; SPSS 1998). Percent "strength and purity" of the chloramine-T was calculated by dividing the mean measured chloramine-T concentration of the reference solutions by 7.5 mg/L and multiplying the result by 100.

Dose verification of test article concentrations in test tanks

During each 3-h static bath exposure period of each experiment, the concentration of chloramine-T in each test tank was measured to compare "actual" and "target" chloramine-T concentrations. Approximately 1 - 2.5 h into each exposure period, one water sample was collected from each test tank for chloramine-T dose-verification purposes. In addition, for quality control purposes, two additional water samples were collected from one test tank in which the target chloramine-T concentration was $\geq 20 \text{ mg/L}$. Water samples collected for chloramine-T dose-verification and quality control purposes were (if necessary) diluted, and the chloramine-T concentration of each sample was measured to the nearest 0.1 mg/L with a HACH Chlorine Pocket Colorimeter.

In each experiment, chloramine-T dose-verification measurements (i.e., those measurements used to compare "actual" and "target" chloramine-T concentrations)

were partitioned into three subsets of data that, although not mutually exclusive, allowed for "actual vs. target" comparisons to be made for (a) each exposure group for the overall experiment, (b) each exposure group on each exposure day, and (c) each test tank across all three exposure days. Because such partitioning generated a large amount of data, only overall-experiment data for each exposure group are presented and discussed in this summary report. For each experiment, overall-experiment mean chloramine-T concentrations calculated for all exposure groups that had target concentrations ≥ 20 mg/L were statistically compared to their corresponding target concentrations with one-sample t-tests ($H_0: \mu_{\text{calculated}} = \text{target}$ vs. $H_a: \mu_{\text{calculated}} \neq \text{target}$; $\alpha = 0.05$; Zar 1984; SPSS 1998). For the 0-mg/L exposure groups, such statistical comparisons were not warranted because mean measured chloramine-T concentrations that differed from 0.0 mg/L were considered to be either artifacts of the measurement process or the result of "color memory" in glassware and glassware caps from processing previous samples (personal communication, L. Schmidt, USGS, La Crosse, WI).

Measured chloramine-T concentrations of the quality control water samples were not statistically compared to the measured chloramine-T concentrations of the nine water samples collected for dose-verification. Instead, "quality" of the chloramine-T dose-verification process was considered to be satisfactory if

measured chloramine-T concentrations of the quality control samples were similar to the measured chloramine-T concentrations of the dose-verification samples.

Water quality parameters

To characterize the chemistry of the source water, hardness, alkalinity, and pH of water samples collected from the head box were measured during each experiment. In all of the experiments, hardness and alkalinity were measured to the nearest 1 mg/L (as CaCO₃) with a HACH Model 16900 digital titrator and HACH reagents. In Experiments 01 - 08, pH was measured to the nearest 0.01 pH unit with a HACH EC10 pH meter. In Experiment 10, pH was measured to the nearest 0.01 pH unit with an Orion Model 920A Electrochemistry meter (Orion Research, Inc., Beverly, MA). Hardness, alkalinity, and pH were usually measured twice during each experiment—once during the pre-exposure phase and once during the post-exposure phase. However, hardness, alkalinity, and pH of source water were measured three times in Experiment 01, and pH of source water was measured only once in Experiment 10.

In each experiment, either a hand-held YSI Model 55 or YSI Model 95 Dissolved Oxygen and Temperature meter (YSI, Inc., Yellow Springs, OH) was used to measure water temperature and dissolved oxygen (DO) concentration twice daily

() (morning and afternoon) in each test tank. During each 3-h static-bath exposure period, one or the other of these meters was also used to monitor changes in water temperature and DO concentration in all test tanks. Regardless of which meter was used, water temperature was measured to the nearest 0.1°C, and DO concentration was measured to the nearest 0.1 mg/L.

Results

Mortality

Rainbow trout fry

() Two mortality experiments were conducted on rainbow trout fry (Experiment 01 at 8°C; Experiment 02 at 14°C; Table 1). In each, chloramine-T exposures were administered three times on alternate days. Also, in each of these experiments, the chloramine-T concentrations tested were 0, 20, 60, and 100 mg/L.

() Based on mortality data summarized for Experiments 01 and 02 (Table 3), mean total mortality was 0.3 and 2.3% in the 0-mg/L exposure groups and 0.0% in the 20 mg/L exposure groups. In the 60-mg/L exposure groups, mean total mortality was 0.0 and 0.3%. In the 100-mg/L exposure groups, mean total

mortality was 2.7 and 3.3%. Guided by the results of statistical comparisons made in both experiments, mean total mortality of rainbow trout fry was ranked as "low" in all four of the exposure groups tested (0, 20, 60, and 100 mg/L chloramine-T).

Rainbow trout fingerlings

Two experiments were conducted on rainbow trout fingerlings (Experiment 05 at 8°C; Experiment 06 at 14°C; Table 1). In each, chloramine-T exposures were administered three times on alternate days. Between these two experiments, the chloramine-T concentrations tested were 0, 20, 30, 40, 50, and 60 mg/L.

Based on mortality data summarized for both experiments (Table 3), mean total mortality in the 0-mg/L exposure groups was 0.0 and 2.7% and in the 20-mg/L exposure groups was 0.0 and 0.7%. Mean total mortality in the 30-mg/L exposure group was 2.7%. In the 40-mg/L exposure groups, mean total mortality was 0.0 and 0.7%. Mean total mortality was 0.0% in the 50-mg/L exposure group and 0.0% in the 60-mg/L exposure groups. Guided by the results of statistical comparisons made in both experiments, mean total mortality of rainbow trout fingerlings was ranked as "low" in all six of the exposure groups tested (0, 20, 30, 40, 50, and 60 mg/L).

Rainbow trout juveniles

Five experiments were conducted on rainbow trout juveniles (Experiment 03 at 8°C; Experiments 04, 07, 08, and 10 at 14°C; Table 1). In Experiments 03, 04, 07, and 08, chloramine-T exposures were administered three times on alternate days. In Experiment 10, chloramine-T exposures were administered three times on consecutive days. Among these five experiments, the chloramine-T concentrations tested were 0, 20, 30, 40, 50, 60, 70, 80, and 100 mg/L.

Based on mortality data summarized for all five experiments (Table 3), mean total mortality ranged from 0.0 to 4.4% in the 0-mg/L exposure groups, from 0.0 to 4.2% in the 20-mg/L exposure groups, and was 0.0% in the 30-mg/L exposure group. In the 40-mg/L exposure groups, mean total mortality was 0.0 and 1.1%; in the 50-mg/L exposure groups, mean total mortality was 0.0 and 2.2%. In the 60-mg/L exposure groups, mean total mortality ranged from 0.0 to 23.3%. In the 70-mg/L exposure group, mean total mortality was 13.3%. In the 80-mg/L exposure groups, mean total mortality was 34.4 and 37.8%. Finally, in the 100-mg/L exposure groups, mean total mortality ranged from 90.0 to 100%.

Guided by results of statistical comparisons made in all five experiments, mean total mortality of rainbow trout juveniles was ranked as "low" in the 0-, 20-,

30-, 40-, and 50-mg/L exposure groups. Although mean total mortality of rainbow trout juveniles varied widely (0 - 23.3%) in experiments in which groups of these fish were exposed to 60 mg/L chloramine-T, such mortality was ultimately ranked as "moderate" for this exposure group. Mean total mortality of rainbow trout juveniles was ranked as "moderate" in the 70-mg/L exposure group. Finally, mean total mortality of rainbow trout juveniles was ranked as "moderately high" in the 80-mg/L exposure groups and "high" in the 100-mg/L exposure groups.

Strength and Purity of Test Article

The "strength and purity" of the test article was, as claimed by the manufacturer, approximately 100%. The HPLC tests conducted by UMESC indicated that the test article was 98.6 - 100% pure, depending on the wavelength with which the samples were measured. In colorimetric tests conducted by the USFWS NIO, mean measured chloramine-T concentrations of the reference solutions determined for each experiment (range, 7.0 - 7.7 mg/L; Table 4a) never differed significantly (P -values > 0.05) from the calculated concentration of the reference solutions (7.5 mg/L). In addition, percent "strength and purity" of the test article calculated for each experiment ranged from 93.3 to 102.7% (Table 4a). Mean "strength and purity" of the test article, calculated from data generated during all nine experiments, was 98.7% (Table 4b).

Dose-verification of Test Article Concentrations in Test Tanks

For each experiment, overall mean chloramine-T concentrations calculated for all exposure groups are listed in Table 5. For the 0-mg/L exposure groups, overall mean chloramine-T concentrations ranged from 0.1 to 0.3 mg/L. For the 20- and 30-mg/L exposure groups, overall mean chloramine-T concentrations ranged, respectively, from 19.0 to 21.8 mg/L and from 30.8 to 31.3 mg/L. For the 40-, 50-, and 60-mg/L exposure groups, overall mean chloramine-T concentrations ranged, respectively, from 41.0 to 42.6 mg/L, from 49.8 to 52.0 mg/L, and from 57.6 to 63.3 mg/L. For 70-mg/L exposure group, overall mean chloramine-T concentration was 72.4 mg/L. For the 80- and 100-mg/L exposure groups, overall mean chloramine-T concentrations ranged, respectively, from 82.0 to 86.0 mg/L and from 97.1 to 105.0 mg/L.

For the 20-, 30-, 40-, 50-, 60-, 70-, 80-, and 100-mg/L exposure groups, one-sample t-tests revealed that 12 (34.3%) of 35 overall mean chloramine-T concentrations differed slightly, but significantly (P -values < 0.05), from their respective target concentrations (Table 5). In the 11 cases where overall mean chloramine-T concentrations were significantly greater than their respective target concentrations, the differences ranged from 3.4 to 9.0%. In the one case where the overall-experiment mean chloramine-T concentration was significantly less than

its respective target concentration, the difference was -4.0%. For the 0-mg/L exposure groups, such statistical and percent-difference comparisons were not warranted because, in these groups, measured chloramine-T concentrations that differed from 0.0 mg/L were considered to be either artifacts of the measurement process or the result of "color memory" in glassware and glassware caps from processing previous samples (personal communication, L. Schmidt, USGS, La Crosse, WI).

Quality Control of Measurements of Test Article Concentrations in Test Tanks

For all nine experiments, mean chloramine-T concentrations calculated for quality control samples are listed in Table 6. For each exposure period of each of the nine experiments, the mean, range, and coefficient of variation of the chloramine-T quality control samples were similar to the mean, range, and coefficient of variation of the corresponding chloramine-T dose-verification samples. Based on such results, it was concluded that chloramine-T exposures had been administered with reasonable precision and accuracy during the course of each experiment.

Water Quality Parameters

Hardness, alkalinity, and pH of source water

Mean hardness and alkalinity of the source water were calculated for each experiment, and with the exception of Experiment 10, mean pH of source water was also calculated for each experiment (Table 7). In two experiments (Experiments 01 and 05), either individual measurements of water hardness or mean water hardness were slightly outside limits specified in the study protocol. However, in Experiments 01 and 05, individual measurements and mean levels of water hardness were within limits specified by Piper et al. (1982) as being suitable for rearing healthy salmonids. In all nine experiments, individual measurements of alkalinity and mean alkalinity were within limits specified in the study protocol and within limits specified by Piper et al. (1982) as being suitable for rearing healthy salmonids. In one experiment (Experiment 07), one individual pH measurement was slightly outside the limits specified in the study protocol; however, in all nine experiments, mean pH levels were within the limits specified in the study protocol and within limits specified by Piper et al. (1982) as being suitable for rearing healthy salmonids.

Water temperature and dissolved oxygen concentration

Mean water temperatures and DO concentrations under which all nine mortality experiments were conducted were within limits specified in the study protocol (Table 8). In the three experiments conducted at approximately 8°C (Experiments 01, 03, and 05), overall-experiment mean water temperatures and DO concentrations ranged, respectively, from 7.6 to 8.0°C and from 9.5 to 10.9 mg/L. In the six experiments conducted at approximately 14°C (Experiments 02, 04, 06 - 08, and 10), overall-experiment mean water temperatures ranged from 13.6 to 14.4°C, and overall-experiment mean DO concentrations ranged from 7.5 to 9.2 mg/L.

Water temperature and dissolved oxygen concentration during chloramine-T exposures

During each 3-h static-bath exposure period (when water flow to the test tanks was "off" and air supplementation to the test tanks was "on"), mean water temperatures observed in the test tanks were usually slightly higher than mean water temperatures observed in the test tanks before and after each exposure period (Table 9). Although water temperature tended to increase slightly during each exposure period, mean water temperatures calculated for each exposure

period always remained within limits specified in the study protocol ($8 \pm 2^{\circ}\text{C}$ or $14 \pm 2.5^{\circ}\text{C}$).

During each 3-h static-bath exposure period, mean DO concentrations observed in the test tanks were usually slightly lower than mean DO concentrations observed in the test tanks before and after each exposure period (Table 10). With two exceptions, mean DO concentrations observed during each exposure period remained within limits specified in the study protocol (9.5 ± 2 mg/L for experiments conducted at 8°C ; 8.5 ± 2 mg/L for experiments conducted at 14°C). The first exception occurred during the first exposure period of Experiment 03 (8°C), when the lowest mean DO concentration observed in the test tanks (7.4 mg/L) was 0.1 mg/L below the lower limit specified in the study protocol. The second exception occurred during the first exposure period of Experiment 08 (14°C), when the lowest mean DO concentration measured in the test tanks (6.4 mg/L) was 0.1 mg/L below the lower limit specified in the study protocol. Although, in these two cases, mean DO concentrations were slightly below limits specified in the study protocol, they were well above the 5-mg/L minimum DO concentration level specified by Piper et al. (1982) as being suitable for rearing healthy salmonids.

Discussion

All nine mortality experiments were conducted on "healthy" rainbow trout fry, fingerlings, and juveniles that were maintained under near-optimal rearing conditions. Results of the pre-exposure and post-exposure fish health examinations, coupled with mortality data collected during the pre-exposure, exposure, and post-exposure phases of each experiment, indicated that the test fish used in all experiments were healthy. For example, during pre-exposure fish health examinations, no pathogens were detected during virology testing (of fry) or microscopic evaluation of skin-scrape and gill-squash slides, and no bacteria known to be fish pathogens were grown on BHIA media. In addition, during pre- and post-exposure fish health examinations, no severe external or internal gross pathologies were noted. The few minor external and internal abnormalities noted during pre- and post-exposure fish health examinations were not considered to have adversely affected the outcomes of any of the experiments. The few parasites (*Salmincola* sp.) found on a few of the fish sampled during Experiment 10 were not considered to have adversely affected the outcome of this experiment. Moreover, during the pre-exposure phase of each experiment, few moribund or dead fish were found in any of the test tanks. Finally, during the exposure and post-exposure phases of each experiment, there was very little mortality in the 0-mg/L test tanks.

Data collected on seven environmental variables (density index, flow index, and water hardness, alkalinity, pH, temperature, and DO concentration) during all nine experiments indicated that test fish were maintained under near-optimal rearing conditions. At nearly all times during each experiment, all seven of these variables were maintained within limits specified in the study protocol and within ranges described by Piper et al. (1982) as being suitable for rearing healthy salmonids. In addition, at nearly all times during each 3-h static bath exposure period, water temperature and DO concentration were maintained within ranges specified in the study protocol and within ranges specified by Piper et al. (1982) as being suitable for rearing "healthy" salmonids.

In all nine experiments, the strength and purity of the test article used was, as claimed by the manufacturer, approximately 100%. The results of the HPLC analysis conducted by the UMESC laboratory at La Crosse, WI, indicated that the chloramine-T used was 98.6 - 100% pure. Colorimetric analysis conducted by the USFWS NIO indicated that the "strength and purity" of the chloramine-T used was about 98.7%.

In all nine experiments, chloramine-T quality control results indicated that chloramine-T exposures had been administered with reasonable precision and accuracy. With respect to the 20-, 30-, 40-, 50-, 60-, 70-, 80-, and 100-mg/L

exposure groups, one-sample t-tests used to compare "actual" chloramine-T concentrations with "target" concentrations revealed that 12 (34.3%) of 35 overall-experiment mean chloramine-T concentrations differed slightly, but significantly, from their respective target concentrations. However, in these 12 cases, the differences were small (n = 11 cases, range of percent differences = 3.4 to 9.0%; n = 1 case, percent difference = -4.0%) and were not considered to have adversely affected the outcomes of any of the experiments.

Mortality results observed in all nine experiments were considered to be creditable. As such, it was evident that rainbow trout juveniles were more sensitive than either rainbow trout fry or rainbow trout fingerlings to the toxic effects of chloramine-T. For example, at a chloramine-T exposure concentration of 100 mg/L, the highest level of mean total mortality observed for juvenile fish was 100%, while for fry it was 3.3%. Moreover, at a chloramine-T exposure concentration of 60 mg/L, the highest level of mean total mortality observed for juvenile fish was 23.3%, whereas for fry and fingerlings it was, respectively, 0.3 and 0.0%. Rainbow trout fry and fingerlings both appeared to be little affected by chloramine-T at exposures up to 60 mg/L.

It was also evident from the mortality results obtained in this study that the "margin of safety" for chloramine-T exposure extends to at least 100 mg/L for

rainbow trout fry, to at least 60 mg/L for rainbow trout fingerlings, and is between 50 and 60 mg/L for rainbow trout juveniles. For experiments in which rainbow trout fry were tested at 100 mg/L chloramine-T, mean total mortality was only 2.7 - 3.3%. For experiments in which rainbow trout fingerlings were tested at 60 mg/L, mean total mortality was 0.0%. For experiments in which rainbow trout juveniles were tested at 50 or 60 mg/L, mean total mortality at 50 mg/L ranged from 0.0 to 2.2% but at 60 mg/L ranged from 0.0 to 23.3%.

Mortality results also showed that the toxicity of chloramine-T to juvenile rainbow trout was similar regardless of whether the drug was administered three times on alternate days or three times on consecutive days. For example, in Experiment 08, chloramine-T was administered to juvenile rainbow trout three times on alternate days; in experiment 10, chloramine-T was administered to juvenile rainbow trout three times on consecutive days. In both experiments, mean total mortality was ranked as "low" in groups of fish exposed to chloramine-T at concentrations ranging from 20 to 60 mg/L. Mean total mortality in groups exposed to 80- and 100-mg/L chloramine-T was 37.8% and 97.8% in experiment 08, and 34.4% and 90.0% in experiment 10.

From observations made on rainbow trout juveniles, it appeared that these fish were most susceptible to the toxic effects of relatively high concentrations (\geq

60 mg/L) of chloramine-T the first time that they were exposed to it. In experiments in which groups of rainbow trout juveniles were tested at chloramine-T concentrations of 60 or 70 mg/L, 22 (50%) of 44 fish found dead in 60-mg/L test tanks and 6 (100%) of 6 fish found dead in 70-mg/L test tanks died during or within approximately 20 h of the end of the first chloramine-T exposure period. In experiments in which groups of rainbow trout juveniles were tested at a chloramine-T concentration of 80 mg/L, 39 (81.3%) of 48 fish found dead in test tanks died during or within approximately 20 h of the end of first chloramine-T exposure period. In experiments in which groups of rainbow trout juveniles were tested at a chloramine-T concentration of 100 mg/L, 331 (98.5%) of 336 fish found dead in test tanks died during or within approximately 20 h of the end of first chloramine-T exposure period. Because mortality of fry and fingerlings was so low at the range of concentrations tested in this study, it was not possible to determine if these two life stages of rainbow trout would exhibit such "first-time" susceptibility if exposed to chloramine-T concentrations > 100 mg/L.

Finally, based on the original study design, it was hypothesized that, at a given chloramine-T concentration, the toxicity of chloramine-T to rainbow trout would increase with increasing water temperature. In a comparison of two experiments, mean total mortality (23.3%) of juvenile test fish held at $\approx 14^{\circ}\text{C}$ and exposed to 60 mg/L chloramine-T (Experiment 04) was greater than that (16.2%)

for juvenile test fish held at $\approx 8^{\circ}\text{C}$ and exposed to 60-mg/L (Experiment 03). However, this one comparison did not provide enough evidence to definitively support the hypothesis.

Conclusions

For several reasons, the nine mortality experiments conducted under Study Protocol Number BFTC-99-CHLT-TAS (Experiments 01 - 08 and 10) were considered to be valid tests of the safety of chloramine-T to rainbow trout fry, fingerlings, and juveniles. First, the test fish were healthy and maintained under near-optimal rearing conditions. Second, the strength and purity of the test article was, as claimed by the manufacturer, approximately 100%. Third, chloramine-T exposures were administered with reasonable precision and accuracy during the course of each experiment. Fourth, although overall-experiment mean measured (i.e., "actual") chloramine-T concentrations sometimes differed slightly, but significantly, from "target" concentrations, such differences were not large enough to adversely affect the outcomes of the experiments. Therefore, results from the nine experiments indicated that:

1. The proposed maximum therapeutic treatment concentration of 20 mg/L chloramine-T, when administered as a static-bath treatment three

times on alternate or consecutive days, is safe for use on rainbow trout fry, fingerlings, and juveniles being reared at water temperatures ranging from ≈ 8 to $\approx 14^{\circ}\text{C}$;

2. For rainbow trout fry being reared at water temperatures ranging from ≈ 8 to $\approx 14^{\circ}\text{C}$, the margin of safety extends to nearly 100 mg/L chloramine-T when the drug is administered three times as a static-bath treatment;

3. For rainbow trout fingerlings being reared at water temperatures ranging from ≈ 8 to $\approx 14^{\circ}\text{C}$, the margin of safety extends to at least 60 mg/L chloramine-T when the drug is administered three times as a static-bath treatment;

4. For rainbow trout juveniles being reared at water temperatures ranging from ≈ 8 to $\approx 14^{\circ}\text{C}$, the margin of safety exceeds 50 mg/L chloramine-T—but is, for practical purposes, less than 60 mg/L chloramine-T—when the drug is administered three times as static-bath treatment. Such a margin of safety appears to be the same regardless of whether chloramine-T is administered three times on alternate days or three times on consecutive days; and

5. Rainbow trout juveniles are probably most susceptible to the toxic effects of relatively high concentrations (≥ 60 mg/L) of chloramine-T the first time that they are exposed to it.

BFTC-99-CHLT-TAS-Summary
for Experiments 01 - 08 and 10

Table 1. Overview information for Experiments 01 - 08 and 10.

Experiment number	Dates experiment conducted	Life stage of test fish (rainbow trout)	Approximate water temperature (°C)	Test article (chloramine-T)		Experiment days for each phase of experiment			
				Concentrations tested (mg/L)	Exposure regimen ^a	Pre-exposure phase	Exposure phase ^a	Post-exposure phase	Total number of days
01	Apr 12 - May 7, 1999	Fry	8	0, 20, 60, 100	alternate days	1 - 7	8 - 12	13 - 26	26
02	May 10 - Jun 4, 1999	Fry	14	0, 20, 60, 100	alternate days	1 - 7	8 - 12	13 - 26	26
03	Jun 14 - Jul 9, 1999	Juvenile	8	0, 20, 60, 100	alternate days	1 - 7	8 - 12	13 - 26	26
04	Jul 5 - Jul 30, 1999	Juvenile	14	0, 20, 60, 100	alternate days	1 - 7	8 - 12	13 - 26	26
05	Aug 9 - Sep 3, 1999	Fingerling	8	0, 20, 40, 60	alternate days	1 - 7	8 - 12	13 - 26	26
06	Aug 23 - Sep 17, 1999	Fingerling	14	0, 20, 30, 40, 50, 60	alternate days	1 - 7	8 - 12	13 - 26	26
07	Jan 3 - Jan 21, 2000	Juvenile	14	0, 20, 30, 40, 50, 60	alternate days	1 - 7	8 - 12	13 - 19	19
08	Jan 24 - Feb 18, 2000	Juvenile	14	0, 50, 60, 70, 80, 100	alternate days	1 - 7	8 - 12	13 - 26	26
10	Aug 21 - Sep 13, 2000	Juvenile	14	0, 20, 40, 60, 80, 100	consecutive days	1 - 7	8 - 10	11 - 24	24

^a During Experiments 01 - 08, chloramine-T was administered on experiment days 8, 10, and 12 (alternate-day exposures). During Experiment 10, chloramine-T was administered on experiment days 8, 9, and 10 (consecutive-day exposures).

Table 2. Summary information for test fish used and fish sampled from the reference populations for pre-exposure fish health examinations.

Experiment number	Test fish used						Fish sampled from reference populations for pre-exposure fish health examinations ^a		
	Egg lot number	Life stage	Number of test tanks	Number of test fish per test tank	Density index	Flow index	Mean total length (cm)	Total length range (cm)	Sample size (n)
01	RBT-SSD-99-ENN	Fry	12	100	0.02	0.06	3.0	2.4 - 3.7	60
02	RBT-FLD-99-ENN	Fry	12	100	0.03	0.06	3.3	2.5 - 4.1	60
03	RBT-ERD-98-ENN	Juvenile	12	40	0.22	0.57	15.1	12.1 - 17.5	60
04	RBT-ERD-98-ENN	Juvenile	12	30	0.17	0.45	15.5	11.8 - 18.5	60
05	RBT-SSD-99-ENN	Fingerling	12	50	0.07	0.19	7.8	5.0 - 10.6	60
06	RBT-FLD-99-ENN	Fingerling	18	50	0.07	0.19	7.7	5.1 - 10.0	60
07	RBT-SSD-99-ENN	Juvenile	18	30	0.16	0.40	14.6	11.4 - 17.6	60
08	RBT-SSD-99-ENN	Juvenile	18	15	0.20	0.32	16.0	12.9 - 19.5	40
10	RBT-ERD-99-ENN	Juvenile	18	30	0.18	0.44	15.3	13.8 - 18.0	55

^a Fish sampled from the reference populations for pre-exposure fish health examinations were used to estimate the mean total length and total length range of test fish.

Table 3. Mean total mortality of rainbow trout fry, fingerlings, and juveniles exposed to various chloramine-T concentrations while held at a water temperature of either 8°C or 14°C.^{a, b}

Experiment number	Life stage of test fish	Approximate water temperature (°C)	Mean total mortality (%) observed at each chloramine-T exposure concentration											
			0 mg/L	20 mg/L	30 mg/L	40 mg/L	50 mg/L	60 mg/L	70 mg/L	80 mg/L	100 mg/L			
01	Fry	8	0.3	0.0	ND	ND	ND	0.0	ND	ND	2.7	ND	ND	2.7
02	Fry	14	2.3	0.0	ND	ND	0.3	ND	ND	ND	3.3	ND	ND	3.3
03	Juvenile	8	0.0	4.2	ND	ND	16.2	ND	ND	ND	100	ND	ND	100
04	Juvenile	14	0.0	0.0	ND	ND	23.3	ND	ND	ND	100	ND	ND	100
05	Fingerling	8	2.7	0.7	ND	0.0	0.0	ND	ND	0.0	ND	ND	ND	ND
06	Fingerling	14	0.0	0.0	2.7	0.7	0.0	0.0	0.0	0.0	ND	ND	ND	ND
07	Juvenile	14	0.0	0.0	0.0	1.1	1.1	0.0	0.0	1.1	ND	ND	ND	ND
08	Juvenile	14	4.4	ND	ND	ND	2.2	8.9	13.3	37.8	97.8	ND	ND	97.8
10	Juvenile	14	0.0	0.0	ND	0.0	0.0	0.0	0.0	0.0	34.4	90.0	90.0	90.0

^a ND = No data collected because this exposure concentration was not tested.

^b With two exceptions, mean total mortalities are based on a sample size of n = 3 test tanks. In Experiment 10, the mean chloramine-T concentrations calculated for the 40- and 60-mg/L exposure groups are each based on a sample size of n = 2 test tanks.

Table 4a. Summary of data generated during colorimetric tests conducted to verify the strength and purity of the test article.

Experiment number	Overall-experiment mean chloramine-T concentration of reference solutions (mg/L) ^a	Calculated concentration of reference solutions (mg/L)	Percent strength and purity of test article ^b
01	7.0	7.5	93.3
02	7.2	7.5	96.0
03	7.1	7.5	94.7
04	7.3	7.5	97.3
05	7.6	7.5	101.3
06	7.7	7.5	102.7
07	7.4	7.5	98.7
08	7.7	7.5	102.7
10	7.4	7.5	98.7

^a Except for Experiment 06 (where n = 5 measurements), each mean is based on a sample size of n = 6 measurements.

^b Percent strength and purity = (overall-experiment mean chloramine-T concentration ÷ 7.5) x 100

Table 4b. Overall percent strength and purity of test article as calculated from all data generated during colorimetric tests conducted to verify the strength and purity of the test article.^a

Overall mean chloramine-T concentration of reference solutions (n = 53)	Overall percent strength and purity
7.4 mg/L	98.7

^a Overall percent strength and purity = (7.4 ÷ 7.5) x 100

Table 5. Comparison of target and mean chloramine-T concentrations calculated for each exposure group for each experiment.^{a, b, c}

Experiment number	Life stage of test fish	Approximate water temperature (°C)	Mean chloramine-T concentration											
			0 mg/L	20 mg/L	30 mg/L	40 mg/L	50 mg/L	60 mg/L	70 mg/L	80 mg/L	100 mg/L			
01	Fry	8	0.3 ^{NA}	21.7 ^S	ND	ND	ND	63.3 ^S	ND	ND	ND	ND	ND	105.0 ^S
02	Fry	14	0.1 ^{NA}	20.9 ^{NS}	ND	ND	ND	59.8 ^{NS}	ND	ND	ND	ND	ND	97.1 ^{NS}
03	Juvenile	8	0.3 ^{NA}	19.4 ^{NS}	ND	ND	ND	62.7 ^{NS}	ND	ND	ND	ND	ND	99.7 ^{NS}
04	Juvenile	14	0.1 ^{NA}	19.0 ^{NS}	ND	ND	ND	57.6 ^S	ND	ND	ND	ND	ND	97.1 ^{NS}
05	Fingerling	8	0.3 ^{NA}	21.8 ^S	ND	ND	42.6 ^S	ND						
06	Fingerling	14	0.2 ^{NA}	21.2 ^S	30.8 ^{NS}	ND	41.5 ^{NS}	52.0 ^{NS}	63.3 ^S	ND	ND	ND	ND	ND
07	Juvenile	14	0.1 ^{NA}	20.7 ^{NS}	31.3 ^{NS}	ND	41.2 ^{NS}	49.8 ^{NS}	60.0 ^{NS}	ND	ND	ND	ND	ND
08	Juvenile	14	0.1 ^{NA}	ND	ND	ND	ND	51.6 ^{NS}	57.8 ^{NS}	72.4 ^S	86.0 ^S	97.1 ^{NS}	97.1 ^{NS}	97.1 ^{NS}
10	Juvenile	14	0.1 ^{NA}	20.5 ^{NS}	ND	ND	41.0 ^{NS}	62.5 ^S	82.0 ^{NS}	97.9 ^{NS}				

^a ND = No data collected because this exposure concentration was not tested.

^b With three exceptions, each mean chloramine-T concentration is based on a sample size of n = 9 measurements. In Experiment 07, the mean chloramine-T concentration for the 40-mg/L exposure group is based on a sample size of n = 8 measurements. In Experiment 10, the mean chloramine-T concentrations for the 40- and 60-mg/L exposure groups are each based on a sample size of n = 8 measurements.

^c "S" indicates that a mean was significantly different (P-value < 0.05) from its respective target concentration; "NS" indicates that a mean was not significantly different (P-value > 0.05) from its respective target concentration; and "NA" indicates that a mean was not statistically compared to its respective target concentration. For the 0-mg/L exposure group, mean measured and target chloramine-T concentrations were not statistically compared because, in this group, measured chloramine-T concentrations that differed from 0.0 mg/L were considered to be either artifacts of the measurement process or "color memory" in the glassware from processing previous samples.

Table 6. Mean chloramine-T concentrations of quality control water samples collected during exposure periods.^{a, b}

Experiment number	Target chloramine-T concentration (mg/L)	Mean chloramine-T concentration (mg/L)		
		Exposure period 1	Exposure period 2	Exposure period 3
01	100	105.2	99.3	101.3
02	60	55.6	64.5	63.5
03	60	54.6	56.6	58.6
04	100	87.4	85.4	95.3
05	60	61.5	61.6	63.5
06	50	52.6	56.6	48.6
07	40	41.7	39.7	41.7
08	80	83.4	83.4	79.4
10	60	60.6	NA	63.5

^a Each quality control mean chloramine-T concentration in the table is based on a sample size of n = 2 measurements.

^b NA = Not applicable (Quality control water samples collected and measured were not used because the test tanks from which the samples were taken had been mis-dosed).

Table 7. Summary of measurements of the hardness, alkalinity, and pH of source water.^a

Experiment number	Life stage of test fish	Approximate water temperature (°C)	Hardness (mg/L CaCO ₃) mean (range) ^b	Alkalinity (mg/L CaCO ₃) mean (range) ^c	pH mean (range) ^d
01	Fry	8	197 (184 - 206)	181 (179 - 182)	7.84 (7.79 - 7.87)
02	Fry	14	201 (200 - 202)	171 (166 - 175)	7.73 (7.72 - 7.73)
03	Juvenile	8	182 (178 - 186)	169 (161 - 176)	7.52 (7.51 - 7.52)
04	Juvenile	14	205 (200 - 210)	160 (159 - 161)	7.77 (7.70 - 7.84)
05	Fingerling	8	204 (202 - 206)	180 (178 - 181)	7.54 (7.39 - 7.69)
06	Fingerling	14	207 (196 - 218)	167 (165 - 168)	7.73 (7.73 - 7.73)
07	Juvenile	14	213 (212 - 214)	160 (159 - 160)	7.34 (7.13 - 7.54)
08	Juvenile	14	216 (214 - 218)	165 (160 - 169)	7.66 (7.57 - 7.74)
10	Juvenile	14	209 (208 - 210)	166 (162 - 170)	7.93 ^e

^a The sample size for hardness, alkalinity, and pH of source water for Experiment 01 was n = 3 and for Experiments 02 - 08 was n = 2.

^b Hardness limits as listed in Study Protocol: 180±20 (8°C); 210±20 (14°C)

^c Alkalinity limits as listed in Study Protocol: 170±20 (8°C); 160±20 (14°C)

^d pH limits as listed in Study Protocol: 7.8±0.5 (8°C); 7.7±0.5 (14°C)

^e pH was measured only once during Experiment 10.

Table 8. Overall-experiment mean water temperatures and dissolved oxygen concentrations.^a

Experiment number	Target means and ranges as specified in the study protocol		Mean water temperature calculated from data collected (°C)	Mean dissolved oxygen concentration calculated from data collected (mg/L)
	Water temperature (°C)	Dissolved oxygen concentration (mg/L)		
01	8 ± 2	9.5 ± 2	7.6	10.9
02	14 ± 2.5	8.5 ± 2	13.6	9.2
03	8 ± 2	9.5 ± 2	8.0	9.5
04	14 ± 2.5	8.5 ± 2	14.3	8.1
05	8 ± 2	9.5 ± 2	8.0	10.1
06	14 ± 2.5	8.5 ± 2	14.2	8.4
07	14 ± 2.5	8.5 ± 2	14.3	7.5
08	14 ± 2.5	8.5 ± 2	14.1	8.0
10	14 ± 2.5	8.5 ± 2	14.4	7.6

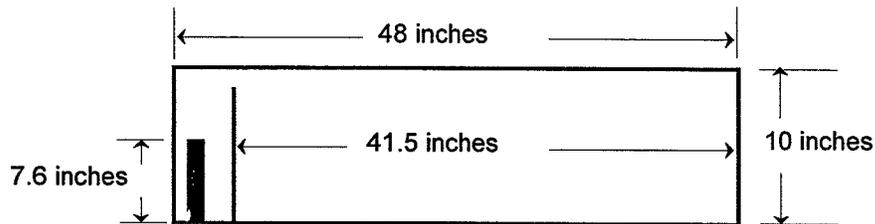
^a For each experiment, overall mean water temperature and dissolved oxygen concentration were calculated by using water temperature and dissolved oxygen concentration data collected on each morning and each afternoon of each experiment day (i.e., water temperature and dissolved oxygen concentration data collected during each exposure period of each experiment were not used to generate this summary table).

Table 9. Mean water temperature (°C) observed in the test tanks before, during, and after each 3-h exposure period.

Experiment number and target water temperature	Exposure day	Mean morning temperature (°C)	Highest mean temperature (°C) recorded during exposure period	Mean afternoon temperature (°C)
01 (8 ± 2°C)	1	7.9	9.0	7.9
	2	7.5	8.6	7.5
	3	7.6	8.5	7.7
02 (14 ± 2.5°C)	1	13.6	14.2	14.4
	2	13.5	14.0	13.7
	3	13.5	13.8	13.4
03 (8 ± 2°C)	1	8.0	9.5	8.2
	2	8.1	9.2	8.2
	3	7.2	8.4	7.2
04 (14 ± 2.5°C)	1	13.9	14.6	14.3
	2	14.2	14.6	14.2
	3	14.2	14.4	14.3
05 (8 ± 2°C)	1	7.9	9.0	8.0
	2	7.9	9.1	8.1
	3	8.0	9.2	8.1
06 (14 ± 2.5°C)	1	13.8	14.9	14.2
	2	14.1	14.4	14.4
	3	14.4	14.5	14.4
07 (14 ± 2.5°C)	1	14.2	14.4	14.3
	2	14.2	14.4	14.3
	3	14.4	14.7	14.4
08 (14 ± 2.5°C)	1	14.0	14.3	14.2
	2	14.3	15.1	14.2
	3	13.9	14.7	14.1
10 (14 ± 2.5°C)	1	14.2	14.5	14.5
	2	14.4	14.5	14.4
	3	14.2	14.4	14.3

Table 10. Mean dissolved oxygen (DO) concentration (mg/L) observed in the test tanks before, during, and after each 3-h exposure period.

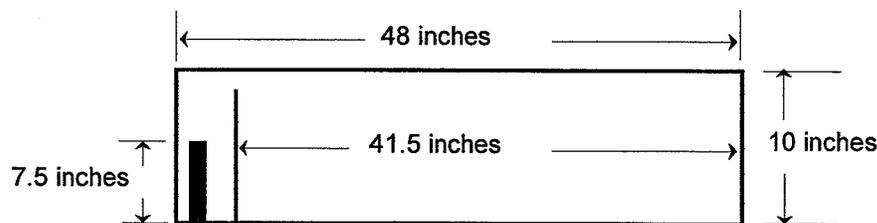
Experiment number and target DO concentration	Exposure day	Mean morning DO concentration (mg/L)	Lowest mean DO concentration (mg/L) recorded during exposure period	Mean afternoon DO concentration (mg/l)
01 (9.5 ± 2 mg/L)	1	11.3	11.2	11.4
	2	11.1	10.9	11.2
	3	11.0	10.7	10.9
02 (8.5 ± 2 mg/L)	1	9.3	9.0	9.1
	2	9.5	9.1	9.4
	3	9.5	9.0	9.0
03 (9.5 ± 2 mg/L)	1	9.7	7.4	9.6
	2	9.8	8.2	9.6
	3	10.2	8.6	9.8
04 (8.5 ± 2 mg/L)	1	7.9	6.8	8.2
	2	7.9	7.0	7.7
	3	9.3	8.5	9.4
05 (9.5 ± 2 mg/L)	1	9.6	9.3	9.6
	2	10.3	9.8	10.2
	3	10.2	9.7	10.1
06 (8.5 ± 2 mg/L)	1	8.7	8.2	8.6
	2	9.4	9.1	9.5
	3	8.1	7.7	8.3
07 (8.5 ± 2 mg/L)	1	7.4	6.5	7.7
	2	7.3	6.8	7.7
	3	7.4	7.1	7.7
08 (8.5 ± 2 mg/L)	1	7.6	6.4	8.0
	2	8.1	7.2	8.2
	3	8.5	7.6	8.2
10 (8.5 ± 2 mg/L)	1	7.6	7.4	7.8
	2	8.0	7.7	7.8
	3	8.1	8.0	8.0



Experiments 01 - 07

Water in-flow 3.8 L/min
(2.7 water exchanges/h)

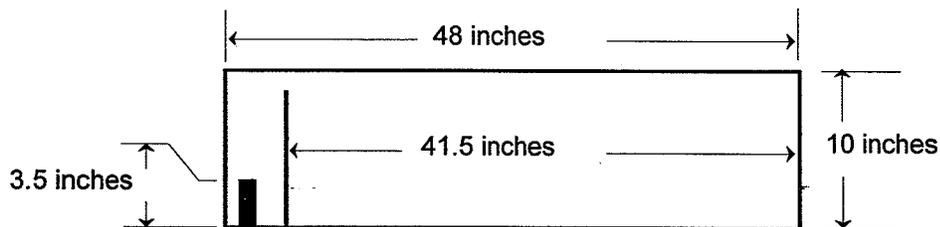
Total tank volume 3.89 ft³
 Total water volume (standpipe in place) 2.96 ft³
 Tank rearing volume (tail screen and standpipe in place) 2.56 ft³



Experiment 10

Water in-flow 3.8 L/min
(2.7 water exchanges/h)

Total tank volume 3.89 ft³
 Total water volume (standpipe in place) 2.92 ft³
 Tank rearing volume (tail screen and standpipe in place) 2.52 ft³



Experiment 08

Water in-flow 2.8 L/min
(4.4 water exchanges/h)

Total tank volume 3.89 ft³
 Total water volume (standpipe in place) 1.36 ft³
 Tank rearing volume (tail screen and standpipe in place) 1.18 ft³

Figure 1. Side views of test tanks used in Experiments 01 - 08 and 10.

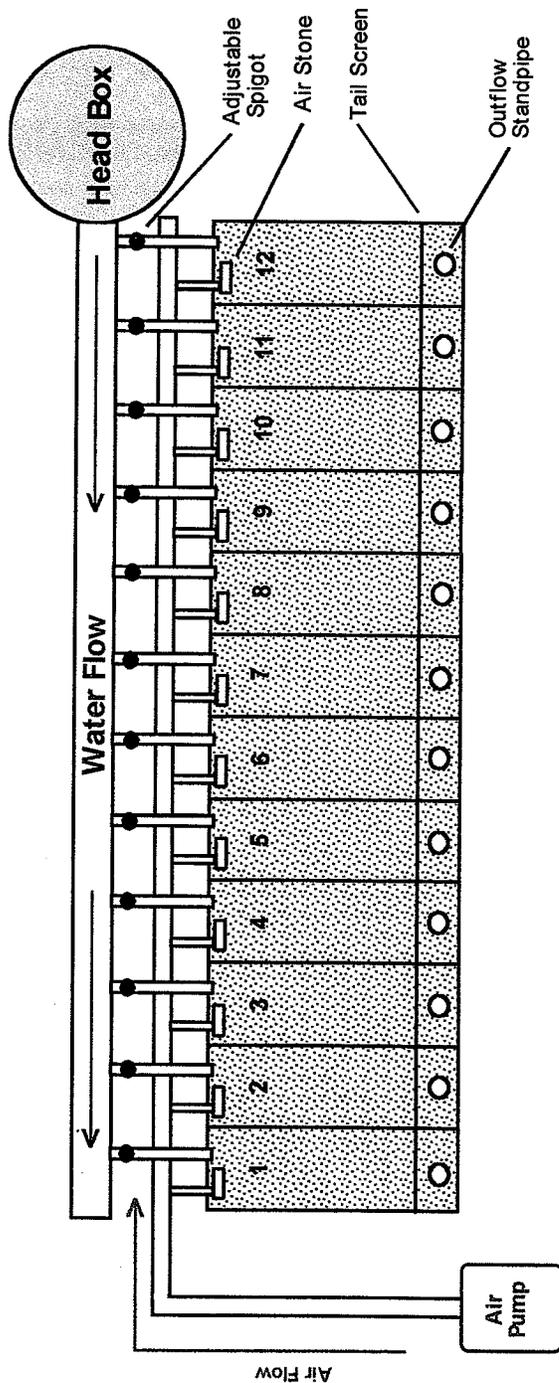


Figure 2. Test tanks 1 - 12, used in Experiments 01 - 05, showing water inflow system, tail screens, outflow standpipes, and air supplementation system.

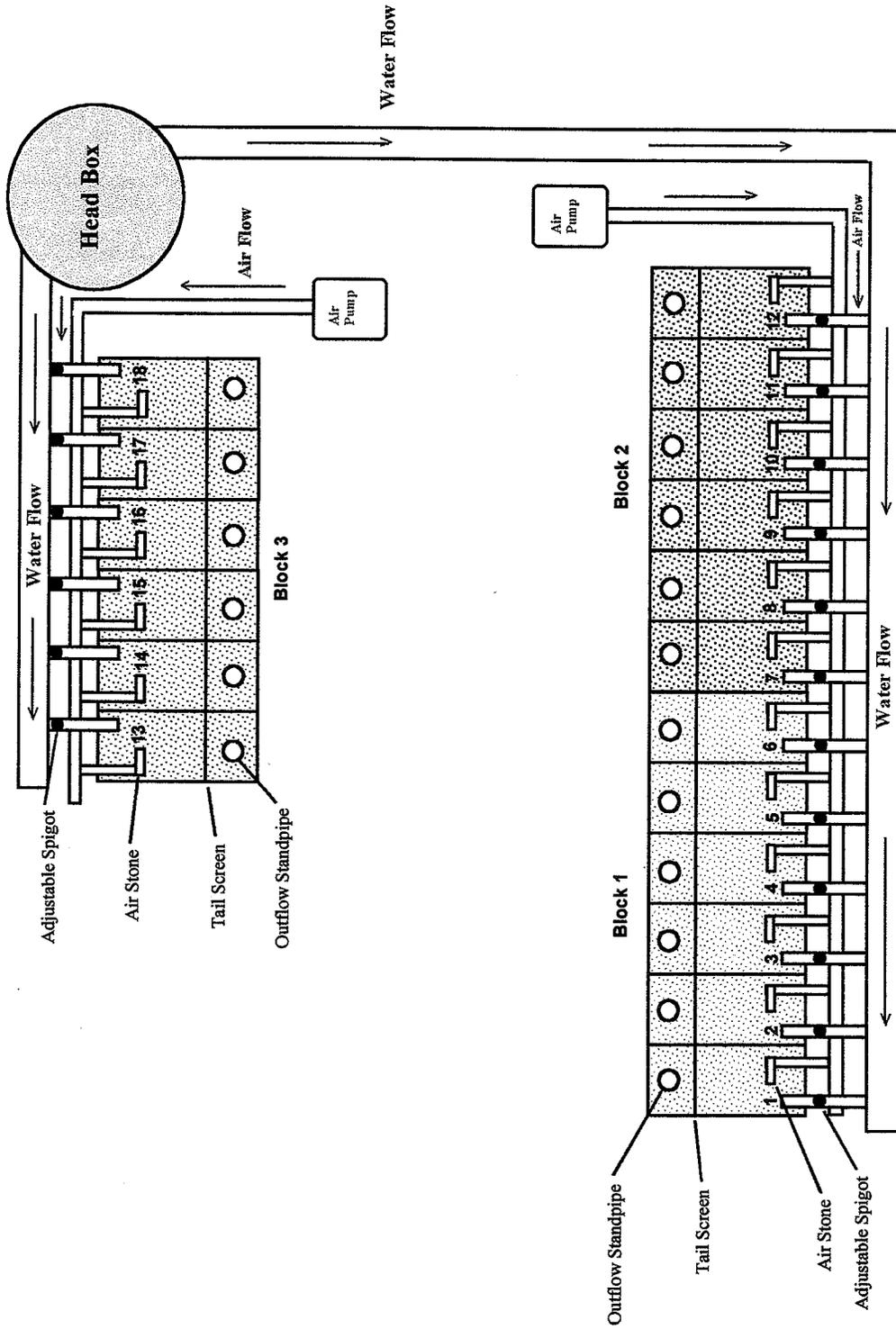


Figure 3. Test tanks 1 - 18, used in Experiments 06 - 08 and 10, showing water inflow system, tail screens, outflow standpipes, air supplementation system, and blocks.

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