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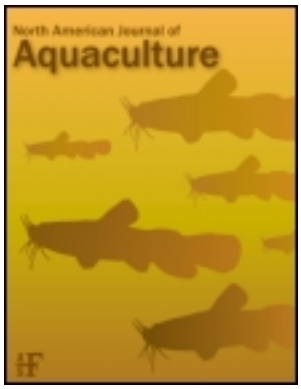

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Chloramine-T Margin-of-Safety Estimates for Fry, Fingerling, and Juvenile Rainbow Trout

James D. Bowker [a], Daniel Carty [a], Charlie E. Smith [b c] & Silas R. Bergen [d e]

[a] U.S. Fish and Wildlife Service, Aquatic Animal Drug Approval Partnership Program, 4050 Bridger Canyon Road, Bozeman, Montana, 59715, USA

[b] U.S. Fish and Wildlife Service, 715 Southeast 5th Street, College Place, Washington, 99324, USA

[c] Retired

[d] Winona State University, 102 East 8th Street, Winona, Minnesota, 55987, USA

[e] University of Washington, 7101 6th Avenue Northeast, Seattle, Washington, 98115, USA

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ARTICLE

Chloramine-T Margin-of-Safety Estimates for Fry, Fingerling, and Juvenile Rainbow Trout

James D. Bowker* and Daniel Carty
U.S. Fish and Wildlife Service, Aquatic Animal Drug Approval Partnership Program, 4050 Bridger Canyon Road, Bozeman, Montana 59715, USA

Charlie E. Smith[1]
U.S. Fish and Wildlife Service, 715 Southeast 5th Street, College Place, Washington 99324, USA

Silas R. Bergen[2]
Winona State University, 102 East 8th Street, Winona, Minnesota 55987, USA

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Abstract

Chloramine-T (CLT) is a candidate for approval for use in U.S. aquaculture to control mortality in freshwater-reared salmonids caused by bacterial gill disease (causative agent, *Flavobacterium branchiophilum*). The proposed treatment regimen is to administer CLT at 12–20 mg/L in a static or flow-through bath for 60 min/d on three alternate or consecutive days. To estimate a CLT margin of safety, defined as the highest dosing regimen above the proposed maximum therapeutic regimen at which no adverse effects are observed, we conducted seven experiments with fry, fingerling, and juvenile rainbow trout *Oncorhynchus mykiss* that examined mortality and an eighth experiment that examined mortality, gross pathology, and histopathology after CLT exposure. In each experiment, triplicate groups of fish were exposed to a range of CLT concentrations representing 0, 1, 1.5, 2, 2.5, 3, 3.5, 4, or 5× the highest proposed dose (20 mg/L) for 3× the proposed treatment duration (60 min) on three alternate or consecutive days at 8°C or 14°C. The survival of fry and fingerlings was unaffected by exposure to CLT concentrations as high as 100 and 60 mg/L, respectively (survival = 97.3–100%). Although the survival of juvenile fish was unaffected by exposure to 20 mg/L, exposure to higher CLT concentrations significantly reduced survival (≤10.0% at 100 mg/L). Across experiments, 92% of all mortalities occurred within 20 h of the first exposure to CLT. The histopathological changes of most concern were associated with gill tissues, but these were evident only in moribund fish exposed to doses of 60 mg/L or higher. Based on analysis of the survival data, the margin-of-safety estimates were approximately 100 mg/L for rainbow trout fry, at least 60 mg/L for fingerlings, and 50–60 mg/L for juveniles. Tissue responses to CLT at these concentrations were minor and did not warrant decreasing these estimates.

Achieving U.S. Food and Drug Administration (FDA) approval of a New Animal Drug Application (NADA) for the initial or expanded use of a therapeutic drug or other chemical (product) on food animals (including food fishes) is a complex and data-intensive process (Greenlees 1997). To meet requirements of the Federal Food, Drug, and Cosmetic Act, a product sponsor (e.g., pharmaceutical or chemical company) must provide the following technical information to FDA: (1) product chemistry, manufacturing, and controls (consistency in product chemistry and quality); (2) human food safety for food-producing animals (analytical method validation, short- and long-term toxicology, metabolism, tissue residues and depletion, and antimicrobial resistance); (3) environmental assessment (fate and effects of the product in the environment); (4) effectiveness for proposed

*Corresponding author: jim_bowker@fws.gov
[1]Retired.
[2]Present address: University of Washington, 7101 6th Avenue Northeast, Seattle, Washington 98115, USA.
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uses (dose titration, dose confirmation, and field efficacy); and (5) target animal safety (TAS). The TAS section summarizes data generated to identify toxic syndrome(s) associated with exposure to the product and margin(s) of safety of product use in or on treated animals (USOFR 1999a). Margin-of-safety estimates are required by FDA because there is little difference between therapeutic and toxic doses for products with narrow safety margins.

In the United States, TAS testing is conducted under federal Good Laboratory Practice (GLP) guidelines for nonclinical laboratory studies (USOFR 1999b) and designed to estimate margin(s) of safety associated with administering a product to a target animal under a specified treatment regimen (dose, delivery method, duration, and frequency). Typically, TAS testing is done on the most sensitive life stage of a target species. A product is administered to healthy animals at up to 10× the proposed maximum treatment dose for up to 3× the proposed treatment duration and up to 3× the proposed treatment frequency (e.g., Darwish et al. 2005; Gaikowski et al. 2008, 2009). Margin-of-safety estimates are made from data generated (e.g., survival, gross lesions, histopathological changes, behavior, and dose verification) and submitted to FDA for review (Greenlees 1997). In this paper, we define margin of safety as the highest dosing regimen above the proposed maximum therapeutic regimen at which no adverse effects are observed.

Chloramine-T (CLT; $C_7H_7ClNNaO_2S{\cdot}3H_2O$) is a biocide used worldwide as a disinfectant and antiseptic. Chloramine-T is a white, crystalline powder with a weak chlorine odor and is a pure compound with no inactive ingredients. Hypochlorite ions form when CLT is dissolved in water (Booth and McDonald 1988). The hypochlorite ion is a strong oxidizer, which can quickly destroy cellular material and disrupt essential cell processes (Powell and Clark 2003). Microbes do not develop resistance to oxidation over time; thus, antimicrobial resistance is not an issue for CLT. Consequently, efforts have been underway for many years to obtain a NADA for CLT for use in U.S. aquaculture to control mortality in freshwater-reared finfish caused by bacterial gill disease (BGD; causative agent, *Flavobacterium branchiophilum*) and other external flavobacterial infections. Chloramine-T effectively controls BGD-related mortality in salmonids (From 1980; Speare and Ferguson 1989a; Bullock et al. 1991; Thorburn and Moccia 1993; Ostland et al. 1995; Bowker and Erdahl 1998; Bowker et al. 2008). The toxicity of CLT to fishes has also been investigated (Bills et al. 1988; Powell et al. 1994, 1995; Powell and Perry 1996, 1998; Sanchez et al. 1996, 1997; King and Farrell 2002; Powell and Harris 2004); however, these studies were not designed to meet current FDA TAS testing requirements (USFDA Center for Veterinary Medicine 1989, 1999). Recently, Gaikowski et al. (2008, 2009) conducted studies designed, in part, to meet FDA requirements for evaluating the safety of CLT to representative freshwater-reared, cool- and warmwater finfishes. At present, the safety of CLT has not been evaluated on marine fish species, baitfish, or ornamental fish. As such, additional studies are needed to demonstrate the safety of CLT to all cultured finfishes.

For salmonids, the proposed CLT treatment regimen is to administer the drug at 12–20 mg/L in a static or flow-through bath for 60 min/d on three alternate or consecutive days (Bowker et al. 2008). To evaluate the safety of this proposed treatment regimen, we conducted eight TAS experiments with CLT on a representative salmonid, rainbow trout *Oncorhynchus mykiss*. We used rainbow trout as a test species because it is commonly reared at fish culture facilities, important recreationally and commercially, and often used in laboratory experiments as a surrogate for other salmonids (Mayer and Ellersieck 1986). In seven experiments, we evaluated survival of rainbow trout fry, fingerlings, and juveniles exposed to a range of CLT concentrations (up to 5× the maximum proposed therapeutic dose of 20 mg/L) for 180 min (3× the standard therapeutic duration of 60 min) on three alternative or consecutive days. In the eighth experiment, we evaluated survival and gross lesions and histopathological changes associated with exposing rainbow trout juveniles (most sensitive life stage tested in first seven experiments) to a similar spectrum of CLT treatment regimens. Our objective was to use data generated in all eight TAS experiments to estimate a CLT margin of safety for each of fry, fingerling, and juvenile rainbow trout stages.

METHODS

Testing facility, test article, and test fish.—Experiments were conducted from 1999 to 2000 at the U.S. Fish and Wildlife Service (USFWS), Bozeman Fish Technology Center (BFTC), Bozeman, Montana. The TAS laboratory housed flow-through tank systems supplied with warm and cold source waters. Each system was equipped with head boxes for mixing source waters to desired temperatures and gravity-feeding reference population tanks (fiberglass, 1.21 m diameter × 0.61 m deep) and test tanks (aluminum, 1.2 m long × 0.36 m wide × 0.25 m deep). Individual tanks were equipped with air stones for supplemental aeration during static-bath exposures. All light in the laboratory was provided by overhead fluorescent lights set on an 8 h light: 16 h dark photoperiod.

Chloramine-T used in the study (Halamid; lot no. 0299303520272) was manufactured by Akzo Chemicals B.V. (Amsterdam, The Netherlands) and obtained from Akzo Chemical, Inc. (Chicago, Illinois). Before TAS testing began, we confirmed spectrophotometrically (Dawson et al. 2003) that "strength and purity" of the CLT used was 100%.

Rainbow trout used in the study were obtained as eyed eggs from the USFWS Ennis National Fish Hatchery (Ennis, Montana). Each group of eggs was incubated, hatched, and reared to required size at the BFTC. During the 2-year testing period, four rainbow trout egg lots were available for the experiments. Each experiment used fish from a single egg lot (Table 1), and we assumed sensitivity to CLT was similar among rainbow trout strains or lots.

TABLE 1. Nominal water temperatures and analytically verified chloramine-T doses administered in eight target animal safety experiments conducted on rainbow trout at the U.S. Fish and Wildlife Service's Bozeman Fish Technology Center, Bozeman, Montana, 1999–2000.

	Rainbow trout[a]					Chloramine-T[b, c] Mean (SD) dose (mg/L) administered								
Experiment number and test water temperature (°C)		Life stage	Egg lot	Mean (SD) total length	Number of fish per test tank	Exposure regimen	0	20	30	40	50	60	70	80	100
1	8	Fry	SSD-99	3.3 (0.3)	100	3 alt	0.3	21.7 (0.2)				63.3(0.9)			105.0 (1.9)
2	14	Fry	FLD-99	3.3 (0.4)	94–100	3 alt	0.1	20.9 (0.5)				59.8 (1.4)			97.1 (2.7)
3	8	Fng	SSD-99	7.8 (1.2)	48–50	3 alt	0.3	21.8 (0.4)		42.6 (0.7)		62.4 (0.8)			
4	14	Fng	FLD-99	7.7 (1.1)	46–50	3 alt	0.2	21.2 (0.3)	30.8 (0.5)	41.5 (1.1)	52.0 (1.1)	63.3 (0.4)			
5	8	Juv	ERD-98	15.1 (1.4)	35–40	3 alt	0.3	19.4 (0.4)				62.7 (1.7)			99.7 (3.8)
6	14	Juv	SSD-99	16.0 (1.8)	15	3 alt	0.1				51.6 (1.4)	57.8 (1.9)	72.4 (0.9)	86.0 (0.9)	97.1 (1.9)
7	14	Juv	ERD-98	15.3 (0.8)	30	3 con	0.1	20.5 (0.4)		41.0 (1.0)		62.5 (0.9)		82.0 (2.1)	97.9 (1.5)
8	14	Juv	FLD-99	15.6 (1.7)	30	3 alt	0.1	20.4 (0.4)		40.4 (0.6)		60.2 (0.9)		80.7 (1.6)	100.2 (2.3)
Overall mean chloramine-T dose administered							na	20.8 (0.1)	30.80.5)	41.4 (0.4)	51.8 (0.9)	61.5 (0.4)	72.4 (0.9)	82.9 (0.9)	99.5 (1.0)
Overall percent difference from target chloramine-T dose							na	+4.0	+2.7	+3.5	+3.6	+2.5	+3.4	+3.6	–0.5

[a]Fry = fry, Fng = fingerling, and Juv = juvenile. The abridged egg lot numbers listed correspond to the strains of rainbow trout tested.

[b]3 alt = three alternate-day exposures, and 3 con = three consecutive-day exposures. Except in experiment 7, there were three test tanks per chloramine-T dose tested; in experiment 7, dosing errors resulted there being two test tanks each at 40 and 60 mg/L CLT.

[c]A blank cell indicates that a given chloramine-T dose was not tested in a given experiment. At 0 mg/L, the chloramine-T doses listed were considered artifacts; thus, the overall results are listed as not applicable (na).

Study design and procedures.—Eight independent experiments were conducted with different size-classes of rainbow trout: fry (total length, 3.3 ± 0.4 cm [mean ± SD]; experiments 1 and 2), fingerlings (7.8 ± 1.2 cm; experiments 3 and 4), or juveniles (15.5 ± 1.7 cm; experiments 5–8). In all cases, fish were assumed to be sexually immature and present in a 50:50 sex ratio. Each experiment included an acclimation period (7 d), an exposure period (3 or 5 d, for consecutive and alternate-day exposure regimens, respectively), and a postexposure period (14 d). In each experiment, triplicate groups of fish were exposed to a range of CLT concentrations representing 0, 1, 1.5, 2, 2.5, 3, 3.5, 4, or 5× the highest proposed dose (20 mg/L) for 3× the proposed treatment duration (60 min) on three alternate or consecutive days at 8°C or 14°C (Table 1). In each experiment, test fish were collected from a reference population and allocated to test tanks (15–100 fish per tank, depending on size) that followed a completely randomized design. Chloramine-T doses were allocated to test tanks that followed completely randomized designs (12-tank experiments) or randomized complete block designs (18-tank experiments). The number of test fish stocked into each test tank minimized potential confounding effects of incidental mortality or escapement. Test fish that died or were known to have escaped during acclimation were replaced with fish from the reference population before the first CLT exposure was administered. All experiments were "single-masked" such that researchers involved in administering exposures or collecting data did not know which CLT doses were applied to which test tanks. An independent quality assurance officer monitored compliance with GLP guidelines.

Before initiating the exposure period in any experiment, 40–60 fish were collected from that experiment's reference population and evaluated for fish health. Fry were evaluated following standardized virology testing procedures; fingerlings and juveniles were examined for external parasites, gill bacteria, and systemic bacteria following standardized bacteriology procedures (AFS-FHS 2005). In experiment 8, 10 reference population fish were also evaluated histologically before the exposure period. All preexposure evaluations indicated the reference population fish were healthy and pathogen-free, except for a few fish sampled in experiment 7. In this experiment, the copepod parasite *Salmincola* spp. was found in low numbers (1–2 organisms per fish) in 2% of the fish collected from the reference population and in some test fish sampled during the experiment. Given the low infestation prevalence and intensity observed, we concluded these copepods would not adversely affect experiment 7 and proceeded with the CLT exposures as planned.

During each study, fish were hand-fed daily (2–4 feedings per day; Rangen Custom Trout Grower, Rangen, Buhl, Idaho), except on CLT exposure days when fish were not fed. Feeding rates were based on initial fish size and water temperature (Piper et al. 1982) and adjusted for each tank as needed to compensate for mortality occurring during the experiment. Tanks were cleaned daily before CLT was administered.

Chloramine-T stock solutions were prepared by dissolving preweighed amounts of CLT in 100 mL of hatchery source water (control solutions were 100 mL of distilled water). To administer CLT, water inflow was turned off, air supplementation was turned on, and tanks were dosed with their assigned CLT or control solutions. At approximately 120–150 min into each 180-min exposure, one 250-mL water sample was collected from each test tank for CLT dose-verification purposes. At 180 min, water inflow was turned on, air supplementation turned off, and tanks were drained halfway to flush out CLT and control

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TABLE 2. Number of juvenile rainbow trout collected for fish health and histology and number of fish evaluated histologically at each of the sample collection periods in the target animal safety study (experiment 8) conducted at the Bozeman Fish Technology Center, 1999–2000. The abbreviation "na" indicates that no fish were available to collect.

Chloramine-T exposure (mg/L)	Day 8 (1st exposure)	Day 10 (2nd exposure)	Day 12 (3rd exposure)	Day 19 (7 d postexposure)	Day 26 (14 d postexposure)
0	0/0	0/0	15/15	15/6	15/6
20	0/0	0/0	15/15	15/6	15/6
40	0/0	0/0	15/15	15/6	15/6
60	0/0	3/3	15/15	15/6	15/6
80	15/15	1/1	15/15	15/6	6/5
100	15/15	0/0	1/1	na	na

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solutions. The CLT concentration of each 250-mL water sample was measured with a Hach Chlorine Pocket Colorimeter (Hach, Loveland, Colorado: Dawson et al. 2003). In control tanks, measured concentrations that differed from 0 mg/L were considered artifacts.

Experiments were conducted at water temperatures of 8°C or 14°C. In 8°C experiments (experiments 1, 3, and 5), mean water temperature and mean dissolved oxygen (DO) concentration ranged from 7.6°C to 8.0°C and from 9.5 to 10.9 mg/L, respectively, and mean water hardness, alkalinity, and pH ranged from 182 to 204 mg/L ($CaCO_3$), from 169 to 180 mg/L ($CaCO_3$), and from 7.5 to 7.8, respectively. In 14°C experiments (experiments 2, 4, and 6–8), mean water temperature and mean DO concentration ranged from 13.6 to 14.4°C and from 8.0 to 9.2 mg/L, respectively, and mean water hardness, alkalinity, and pH ranged from 201 to 216 mg/L ($CaCO_3$), from 165 to 171 mg/L ($CaCO_3$), and from 7.7 to 7.9, respectively. In experiments 1–5, 7, and 8, inflow water to each test tank was set at 3.8 L/min (2.7 tank water exchanges/h). Fewer fish were available for use in experiment 6; thus, stocking densities were maintained by reducing tank volumes and reducing inflow rates to 2.8 L/min (4.4 tank water exchanges/h).

Data collection.—In experiments 1–7, primary response criteria were time (to nearest day) until first mortality after initiation of exposure and overall survival. All tanks were inspected twice daily during exposure and postexposure periods to remove dead and moribund fish. In experiment 8 (Table 1), mortality data were collected as before, and gross lesions and histopathological changes detected in individual fish were described. Test fish were collected and evaluated for gross lesions and histopathological changes on five occasions: during or immediately after the three CLT or control exposures, and on days 7 and 14 of the postexposure period. Depending upon morbidity and mortality observed among the exposures, the number of fish collected and evaluated varied (Table 2). On the first two occasions, we collected moribund fish to document immediate effects of CLT exposure. No moribund fish were found on the last three occasions; consequently, we collected live fish to document delayed and reversible effects of CLT exposure. Approximately equal numbers of test fish were collected from each test tank at each dose; however, the total number and condition (live versus moribund) of test fish collected differed somewhat by dose. We assumed that all live test fish collected would have survived until the end of the experiment.

Fish collected and evaluated for gross lesions and histopathological changes were euthanized in a solution of tricaine methanesulfonate (Tricaine-S, Western Chemical, Ferndale, Washington) and initially examined for external and internal gross lesions. Eye, skin, liver, gill, and kidney (excretory and hematopoietic) tissue samples were collected, fixed in Davidson's solution for ~48 h, and stored in 70% ethyl alcohol. Tissues were placed in cassettes, processed, and embedded in paraffin. Embedded tissues were sectioned at 5 μm, placed on microscope slides, and stained with hematoxylin and eosin for histological evaluation (Sheehan and Hrapchek 1983). Cover slips were affixed to each slide, and slides were examined by a trained histopathologist to identify type, severity (1 = none, 2 = mild, 3 = moderate, and 4 = severe), and prevalence of histopathological changes. Histopathological change scores of 1 or 2 were considered nonpathological, and scores of 3 or 4 were considered pathological.

Data analysis.—To help us estimate CLT exposure margins of safety, we used Cox proportional hazards analysis (Cox 1972) via the SAS PROC TPHREG procedure (SAS 1997) to model probability of survival as affected by CLT dose (20, 30, 40, 50, 60, 70, 80, or 100 mg/L), water temperature (8°C or 14°C), life stage (fry, fingerling, juvenile), and exposure regimen (consecutive-day versus alternate-day exposures). The SAS TPHREG procedure, which allows input of continuous and classification (categorical) predictor variables, was used to fit three analysis models. Model effects were evaluated with Wald chi-square tests and considered significant if $P < 0.05$.

Initially, we analyzed experiments 1–8 separately and modeled probability of survival as affected by CLT dose alone (TPHREG model 1),

$$h_i(t) = \lambda_0(t)\exp(\beta_1 C_{i1}),$$

where $h_i(t)$ is the hazard for individual i during time interval t, and $\exp(\beta_1 C_{i1})$ is the proportional change in risk for each

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one-unit change in dose C. For each experiment, CLT dose was entered into the analysis as a continuous variable. If there was an overall significant ($P < 0.05$) adverse effect of CLT dose on probability of survival, a second analysis was conducted in which the CLT dose was entered as a classification (categorical) variable so that pairwise comparisons of probability of survival could be made between all CLT doses. In this latter analysis, it was not possible to compare CLT doses at which there was 100% survival to CLT doses at which there was less than 100% survival. Hence, in such cases, either all pairwise comparisons (experiments 1 and 2) or some pairwise comparisons (experiments 5, 7, and 8) were made by plotting mean ± 95% confidence interval (CI) survival probability functions and visually inspecting the graphs for presence (no significant difference) or absence (significant difference) of CI overlap.

Next, we pooled survival data from experiments 1–6 and 8 (all seven alternate-day exposure experiments) and modeled probability of survival as affected by CLT dose, life stage, and nominal water temperature (TPHREG model 2),

$$h_i(t) = \lambda_0(t)\exp(\alpha_{j1}+\alpha_{k2}+\beta_1 C_{i1}),$$

where $h_i(t)$ is the hazard for individual i during time interval t, $\exp(\alpha_{j1})$ is the hazard ratio of 14–8°C, $\exp(\alpha_{k2})$ is the hazard ratio of age k to the baseline age, and $\exp(\beta_1 C_{i1})$ is the proportional change in risk for each one-unit change in concentration C. This model was initially fit with a life stage × temperature interaction term, which was not significant at $\alpha = 0.05$. Therefore, the interaction term was deleted to provide more straightforward comparisons of model effects. We excluded experiment 7 from this analysis because it was the only experiment in which CLT exposures were administered on consecutive days.

Finally, data from experiments 6 and 8 were pooled and results compared with experiment 7 to model probability of survival as affected by exposure regimen and CLT dose (TPHREG model 3),

$$h_i(t) = \lambda_0(t)\exp(\alpha_{m1} + \beta_1 C_{i1}),$$

where $h_i(t)$ is the hazard for individual i during time interval t, $\exp(\alpha_{m1})$ is the hazard ratio of consecutive-day treatment to alternate-day treatment, and $\exp(\beta_1 C_{i1})$ is the proportional change in risk for each one-unit change in concentration C. We used the method of Efron (1977) to adjust for equal survival times.

RESULTS

Survival

Across experiments, there was 100% survival in groups of rainbow trout fry, fingerlings, or juveniles exposed at the proposed maximum therapeutic dose of 20 mg/L CLT (Table 3). Within experiments, survival was 97–100% among fry (up to 100 mg/L exposure), 99–100% among fingerlings (up to 60 mg/L exposure), and ranged from 100% at 20 mg/L to 10% or less at 100 mg/L in groups of juveniles. Across experiments, 91% of the 488 mortalities recorded in CLT-treated tanks and 0% of the four mortalities recorded in control tanks occurred during or within 20 h of the first 180-min exposure, indicating that fish that survived initial exposure to CLT were likely to survive two additional exposures. All fish that survived to the end of experiments appeared healthy (i.e., fed and behaved normally).

In experiments 1 and 2 (fry) and 5–8 (juveniles), probability of survival decreased significantly as CLT dose increased from 0 to 100 mg/L (Table 3). In experiments 1 and 2, survival probability was adversely affected at 100 mg/L CLT compared with 0, 20, and 60 mg/L, although observed survival in 100 mg/L treatment was still quite high (97%). In experiments 5–8, survival probability was always adversely affected at 100 and 80 mg/L CLT and sometimes adversely affected at 60 mg/L CLT. For

TABLE 3. Mean percent survival of rainbow trout in eight chloramine-T target animal safety experiments conducted at the Bozeman Fish Technology Center, 1999–2000. A blank cell indicates that a given chloramine-T dose was not tested in a given experiment. Within experiments, a Wald chi-square result with an asterisk is significant at $P < 0.05$. In experiments 1, 2, and 5–8, means with the same lowercase letter are not significantly ($P \geq 0.05$) different from each other; pairwise comparisons were not performed in experiments 3 and 4 because Wald chi-square results were not significant.

Experiment number and test water temperature (°C)		Life stage[a]	Expo-sure regi-men[b]	Chloramine-T dose (mg/L) administered									Wald χ^2 (P-value)
				0	20	30	40	50	60	70	80	100	
1	8	Fry	3 alt	99 z	100 z				100 zy			97 y	7.23*(0.0072)
2	14	Fry	3 alt	100 z	100 z				100 z			97 y	6.20* (0.0127)
3	8	Fng	3 alt	99	100		100		100				0.0000 (0.9970)
4	14	Fng	3 alt	100	100	100	100	100	100				Not applicable
5	8	Juv	3 alt	100 z	100 z				84 y			0 x	87.31* (<0.0001)
6	14	Juv	3 alt	96 z				98 z	91 z	87 z	62 y	2 x	93.41* (<0.0001)
7	14	Juv	3 con	100 z	100 z		100 z		100 z		66 y	10 x	115.69* (<0.0001)
8	14	Juv	3 alt	100 z	100 z		100 z		96 y		40 y	1 w	187.57* (<0.0001)

[a]Fry = fry, Fng = fingerling, and Juv = juvenile.
[b]3 alt = three alternate-day exposures, and 3 con = three consecutive-day exposures.

TABLE 4. Prevalence (%) of gross lesions detected in juvenile rainbow trout exposed three times to chloramine-T in experiment 8. The abbreviation "na" indicates that no moribund fish were available.

Exposure concentration (mg/L)	Total number evaluated	Live fish		Moribund fish	
		Number	Percent with gross lesions	Number	Percent with gross lesions[a]
Reference population	10	10	0	0	na
0 (control)	45	45	0	0	na
20	45	45	0	0	na
40	45	45	0	0	na
60	48	45	0	3	33
80	52	36	0	16	81
100	16	1	0	15	47

[a]Pale gills was the only gross lesion detected.

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example, in experiments 5–8, survival never exceeded 10% at 100 mg/L or 66% at 80 mg/L. However, in these four experiments, observed survival at 60 mg/L CLT averaged between 84% and 100%. In experiments 3 and 4 (fingerlings), the relation between CLT dose and survival probability was not significant, probably because 60 mg/L was the highest dose administered in each of these experiments (Table 3).

When results from experiments 1–6 and 8 were pooled, probability of survival was adversely affected by CLT dose (Wald $\chi^2 = 468.45$, $P < 0.0001$) and life stage (Wald $\chi^2 = 429.18$, $P < 0.0001$) but not by water temperature (Wald $\chi^2 = 2.53$, $P = 0.1119$). Pairwise comparisons across life stages revealed that survival probability of juveniles was reduced compared with fry (Wald $\chi^2 = 412.57$, $P < 0.0001$) or fingerlings (Wald $\chi^2 = 15.58$, $P < 0.0001$) but survival probability did not differ between fry and fingerlings (Wald $\chi^2 = 1.42$, $P = 0.2333$).

When results from experiments 6 and 8 were pooled and compared with experiment 7, probability of survival was adversely affected by CLT dose (Wald $\chi^2 = 292.46$, $P < 0.0001$) and by exposure regimen (Wald $\chi^2 = 5.33$, $P = 0.0210$). This latter result indicated that probability of survival under a three-consecutive-day exposure regimen was likely to be greater than that under a three-alternate-day exposure regimen.

Gross Lesions and Histopathological Changes in Juvenile Fish (Experiment 8 Only)

No gross lesions were detected in reference population fish, in 0- (control), 20-, or 40-mg/L test fish, or in live fish collected from the 60-, 80-, and 100-mg/L test tanks (Table 4). However, pale gills were evident in 33% of moribund fish collected from 60-mg/L test tanks, 88% of moribund fish collected from 80-mg/L test tanks, and 44% of moribund fish collected from 100-mg/L test tanks. Pale gills were characterized as a gross lesion, often associated with severe histopathological changes later detected in gill tissue, and considered an indicator of deteriorating fish health.

No reference population fish or 0-, 20-, 40-, 60-, 80-, or 100-mg/L CLT test fish sampled for histology exhibited histopathological changes in eye or skin tissues. Although some histological changes (corneal separation of the eye and separation of the epidermis from the dermis) were observed in a few fish, such changes were considered processing artifacts (Grizzle and Rogers 1976).

Nonpathological (mild) histological changes were commonly observed in gill tissue taken from reference population fish and test fish that survived all three exposure periods (Table 5). However, of the seven gill tissue characteristics evaluated (necrotic red blood cells, aneurysms in capillaries, scatter fusion of gill lamellae, epithelial separation from basement membrane, basal hyperplasia of gill epithelium, necrosis of gill epithelium, and hypertrophy of gill epithelium), a dose–response trend was evident only in the percentage of live fish sampled that exhibited mild numbers of necrotic red blood cells. Mild numbers of necrotic red blood cells were not found in live fish sampled from the reference population or the 0- and 20-mg/L exposure groups but were found in 4, 30, and 27% of the live fish sampled from the 40-, 60-, and 80-mg/L exposure groups, respectively. The one 100-mg/L fish that survived all three exposures did not provide sufficient histological data to be included in any comparisons.

There was no morbidity or mortality in the 0-, 20-, and 40-mg/L exposure groups; consequently, only moribund 60-, 80-, and 100-mg/L CLT-treated fish were sampled for histology. Moreover, moribund 60-, 80-, and 100-mg/L treated fish were sampled only during or immediately after the first and second exposures because no morbidity or mortality was observed during or immediately after the third exposure. In the 60-, 80-, and 100-mg/L exposure groups, histopathological (moderate or severe) changes were observed in the gill tissue taken from all fish sampled (Table 6). Among these three groups, prevalence of moderate or severe hypertrophy and epithelial separation was virtually 100%. Although epithelial separation has been, in some cases, considered to be a fixation artifact (Speare and Ferguson 1989b; Ferguson 2006), it was observed in virtually

TABLE 5. Mild (histological score = 2) gill changes, chloramine-T exposure concentrations (mg/L), and percentages of live fish sampled in which changes were detected.

		Chloramine-T exposure (*n*)					
Histological change	Reference population (10)	0 (27)	20 (26)	40 (27)	60 (27)	80 (26)	100 (1)
NRBC[a]	0	0	0	4	30	27	0
A[b]	10	15	0	7	4	8	0
SF[c]	50	89	73	44	59	42	0
ES[d]	10	11	19	7	15	23	0
BH[e]	10	4	12	11	7	23	100
EN[f]	0	4	12	7	7	35	100
H[g]	30	56	54	52	48	65	100

[a]Necrotic red blood cells in capillaries.
[b]Aneurysms in capillaries.
[c]Scattered fusion of gill lamellae.
[d]Epithelial separation from basement membrane.
[e]Basal hyperplasia of gill epithelium.
[f]Necrosis of gill epithelium.
[g]Hypertrophy of gill epithelium.

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all moribund fish but in virtually no fish sampled live immediately after the third and final CLT exposure (Table 5), and was therefore considered a histopathological change. Prevalence of moderate or severe necrotic red blood cells and scattered fusion was greater in the 60-mg/L test group than in the 80- or 100-mg/L test groups. In contrast, prevalence of moderate or severe epithelial necrosis and basal hyperplasia was greater in the 80- and 100-mg/L treatment groups than in the 60-mg/L group. Often, moderate or severe changes in the gill tissue left only bloody capillaries, many of which were congested and contained degenerate erythrocytes (Figures 1, 2).

Prevalence of histopathological (moderate or severe) changes observed in gill tissue taken from live fish sampled immediately after the third exposure was reduced compared with that observed in moribund fish sampled during or immediately after the first and second exposures. Prevalence of necrotic red blood cells or scattered fusion increased with increasing CLT exposure concentration; however, no such trend was observed in the other five gill tissue characteristics. Few fish sampled from the 20–60 mg/L exposure groups at this time had moderate or severe aneurysms, epithelial necrosis, hypertrophy, or basal hyperplasia, but no fish had a combination of any two these changes. Aneurysms and basal hyperplasia were observed in one fish sampled from the 80-mg/L exposure group, and gill hypertrophy was observed in two other 80-mg/L treated fish.

At 7 and 14 d after the third exposure, no histopathological (moderate or severe) changes were detected in gill tissue taken from live fish sampled from the 0-, 20-, 40-, or 60-mg/L exposure groups. However, 5 of the 11 fish sampled from the 80-mg/L exposure group on these two days exhibited moderate or severe scattered fusion, basal hyperplasia, and/or hypertrophy. By 7 d after the third exposure, no 100-mg/L treated fish were left to sample.

Abnormal histopathological changes in livers and kidneys were rare. Only one test fish (a moribund fish from the 80-mg/L treatment collected during the second exposure) exhibited focal

TABLE 6. Gill histopathological changes (moderate or severe), chloramine-T exposure concentrations (mg/L), and percentages of fish sampled in which histopathological changes were detected. See Table 5 for additional details.

		Chloramine-T exposure (*n*)								
		0	20	40	60		80		100	
Histopathological change	Reference population (10)	Live fish (27)	Live fish (26)	Live fish (27)	Live fish (27)	Moribund fish (3)	Live fish (26)	Moribund fish (16)	Live fish (1)	Moribund fish (15)
NRBC	0	0	0	0	4	100	23	6	100	7
A	0	0	0	0	0	0	4	6	0	0
SF	0	0	0	4	7	100	50	6	100	7
ES	0	0	4	0	0	100	0	94	0	93
BH	0	0	0	0	0	33	8	100	0	87
EN	0	0	0	0	0	0	0	38	0	60
H	0	0	0	15	4	100	15	100	0	100

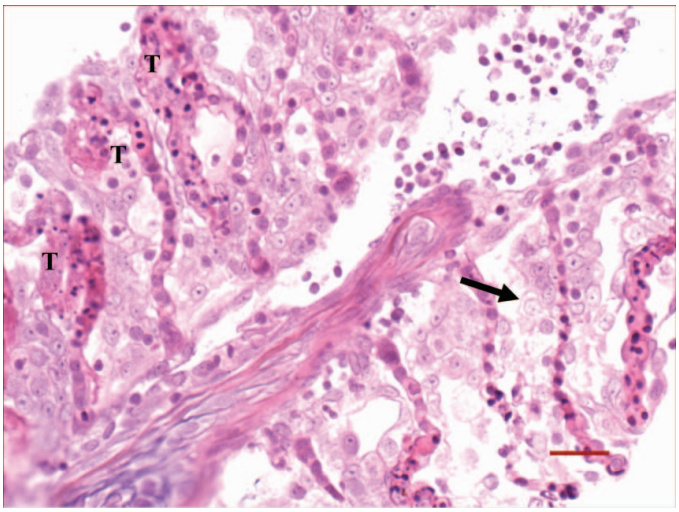

FIGURE 1. Gill tissue from a rainbow trout exposed to 80 mg/L of chloramine-T and sampled on day 10 (second exposure). Note the hypertrophy, degeneration, and sloughing of the lamellar epithelium (arrow). The lamellae in upper left have fused, and thrombotic lesions (indicated by "T") containing degenerate erythrocytes are seen in the lamellar capillaries. Scale bar = 25 μm. [Figure available in color online.]

necrosis of hepatocytes. Hydropic degeneration and necrosis of renal tubule epithelium were seen in this same fish. One test fish exposed at 80 mg/L and one test fish exposed at 100 mg/L exhibited glomerular fibrosis in kidney tissue. Other histopathological changes observed in kidney tissue consisted primarily of hematopoietic hyperplasia and erythrophagia. Hematopoietic hyperplasia was detected in only live fish sampled 7 or 14 d postexposure, and erythrophagia was detected in some live or moribund fish sampled during each sampling event. Erythrophagia increased over time, suggesting a delayed response by the kidneys to remove damaged erythrocytes. Hyaline droplet degeneration of renal tubule epithelium was observed infrequently after the final exposure and returned to normal in all cases examined 14 d postexposure.

Prevalence of gill and kidney lesions increased with dose but decreased postexposure, suggesting resolution and repair. For example, live fish sampled immediately after the final 60- or 80-mg/L exposure showed a higher incidence of scattered fusion of lamellae, hypertrophy, basal hyperplasia, and thrombotic lesions containing necrotic erythrocytes in lamellar capillaries than live fish sampled 7 or 14 d postexposure (Figures 3, 4).

Dose Verification

Within experiments, mean CLT doses administered were 96–109% of targets; across experiments, mean CLT doses administered were 99–104% of targets (Table 1).

Discussion

In the United States, 20 mg/L CLT is the maximum therapeutic dose (1× dose) proposed for use to control mortality in freshwater-reared finfish due to BGD and other external flavobacterial gill infections. Based on the analysis of effects of CLT dose on survival, our margin-of-safety estimates were ~100 mg/L (~5× dose) for fry, at least 60 mg/L (≥3× dose) for fingerlings, and 50–60 mg/L (2.5–3× dose) for juveniles. Our estimate for fry seemed reasonable because fry survival at 100 mg/L, although high (mean, 97% in experiments 1 and 2), was significantly reduced compared with that at 0, 20, and 60 mg/L. Our estimate for juveniles also seemed reasonable because juvenile survival at 50 mg/L was high (mean, 98% in experiment 6) and did not differ significantly from that at 0 mg/L. However,

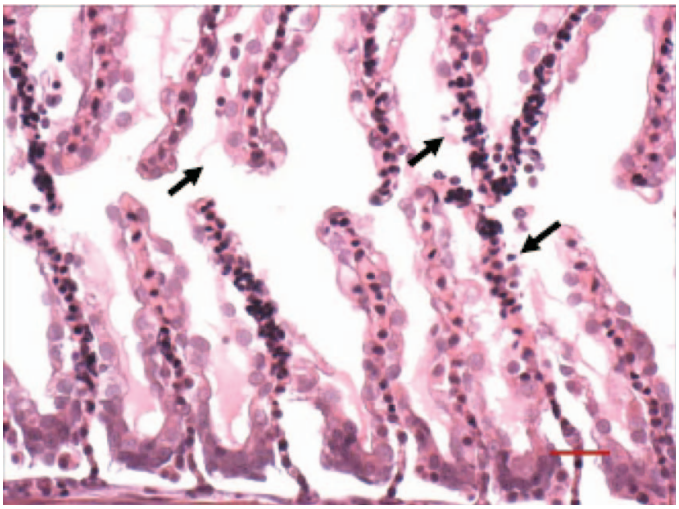

FIGURE 2. Gill tissue from a rainbow trout exposed to 100 mg/L of chloramine-T and sampled on day 8 (first exposure). Note the hypertrophy (left arrow) and sloughed lamellar epithelial cells (two arrows on the right). Capillaries are congested and contain degenerate erythrocytes. Bar = 25 μm. [Figure available in color online.]

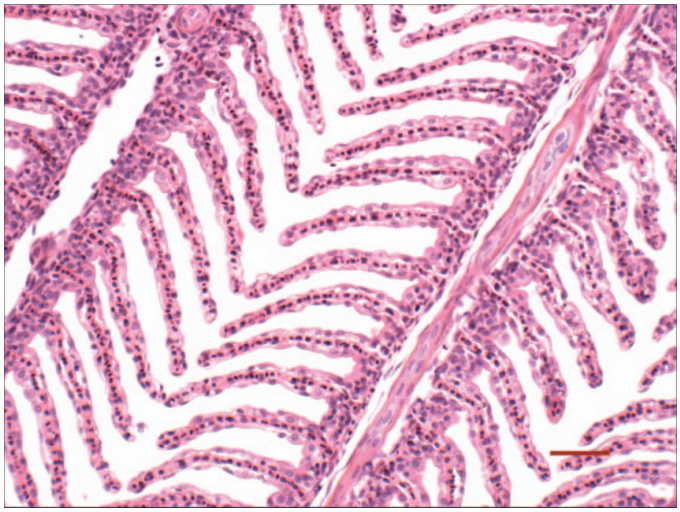

FIGURE 3. Gill tissue of a rainbow trout exposed to 80 mg/L of chloramine-T and sampled 7 d postexposure. While there is some mild hypertrophy of the lamellar epithelium, it is apparent that repair has taken place. Bar = 50 μm. [Figure available in color online.]

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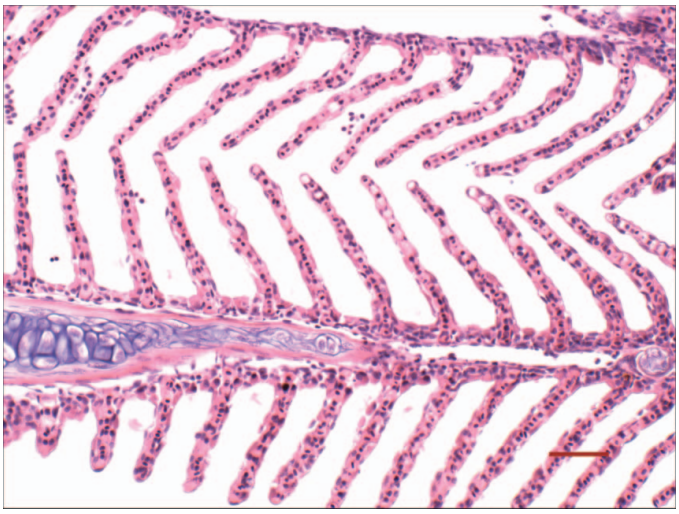

FIGURE 4. Repair of the gill tissue of a rainbow trout exposed to 80 mg/L of chloramine-T and sampled 14 d postexposure is apparent in normal gill lamellae. Bar = 50 μm. [Figure available in color online.]

juvenile survival at 60 mg/L was variable (range, 83.8–100% in experiments 5–8) and in experiments 5 and 8 was significantly reduced compared with that at 0 and 20 mg/L. Our margin-of-safety estimate for fingerlings was probably conservative because fingerlings were only tested at 60 mg/L or less. However, based on the analysis of effects of CLT dose, life stage, and water temperature on survival, we speculate that the actual margin of safety for fingerlings is more similar to that for fry than for juveniles. Also, the gross lesions and histopathological changes detected in juvenile test fish did not warrant decreasing the fry, fingerling, and juvenile margin-of-safety estimates made from survival data. No gross lesions were detected in any juvenile test fish exposed at 0, 20, or 40 mg/L CLT, and no gross lesions were found in live juvenile test fish collected from the 60-, 80-, and 100-mg/L test tanks. Moreover, histopathological changes detected in live juvenile test fish collected from test tanks were generally mild, of low prevalence, and autorepairable. Analysis revealed no significant effect of water temperature on survival and indicated that consecutive-day exposures were likely to cause less mortality than alternate-day exposures.

Our survival results were comparable with those of Bills et al. (1988), who reported a 3-h LC50 of greater than 60 mg/L for 4-cm rainbow trout tested at 12°C and pH 8.1 in very hard water (280–320 mg/L as $CaCO_3$). Those authors also found that CLT was more toxic to rainbow trout in warmer water (17°C) than in colder water (12°C or 7°C) during exposures lasting less than 24 h, that CLT was six times more toxic to rainbow trout and channel catfish *Ictalurus punctatus* at pH 6.5 than at pH 9.5, and that CLT-induced mortality of rainbow trout was less in flow-through systems than in static-bath systems. It is likely that rainbow trout—and most other salmonids—are more sensitive to the toxic effects of CLT than are cool- or warmwater finfishes. Bills et al. (1988) reported 96-h LC50s of 2.8 mg/L for rainbow trout (mean weight, 0.7 g), 3.8 mg/L for channel catfish (mean weight, 1.5 g), and 7.3 mg/L for fathead minnow *Pimephales promelas* when fish were tested in a static bath at 12°C and in soft water. King and Farrell (2002) conducted a static test to determine acute toxicity of CLT to 2.1-g (juvenile) Atlantic sturgeon *Acipenser oxyrinchus* at 17°C and reported a 96-h LC50 of 7.7 mg/L CLT. Gaikowski et al. (2008) reported that a variety of freshwater finfish fry could tolerate no more than100 mg/L CLT exposures administered for 60 min/d in a static bath on four consecutive days and that fingerling walleyes *Sander vitreus* and channel catfish could tolerate no more than 60 mg/L CLT exposures administered for 60 min/d in a static bath on four consecutive days with almost no mortality. Those authors also observed increased mortality of test fish at warmer water temperatures and found that larger fish (walleye fingerlings) were more sensitive to the toxic effects of CLT than smaller fish (walleye fry).

In our study, pale gills were present in many moribund test fish collected during or immediately after their first exposure at 80 or 100 mg/L CLT. Given that this condition was not detected in all moribund test fish and was not dose-dependent, it may not be a reliable indicator of CLT toxicity. However, there appeared to be a positive association between pale gills and severe histopathological changes observed in these fish. The most severe histopathological changes associated with CLT toxicity were observed in gills. Gill lesions developed rapidly following CLT exposure and were considered the main cause of mortality. The incidence of mild gill lesions observed in fish exposed at 20 mg/L were similar to those observed in fish exposed at 0 or 40 mg/L—except for the relatively few mild thrombotic lesions seen in one fish exposed to 40 mg/L.

Histopathological changes observed in gill tissue of fish from experiment 8 were consistent with those described by Mallatt (1985) in his review of fish gill structural changes induced by toxicants and other irritants. In Mallatt's (1985) review, gill alternations such as epithelial lifting, necrosis, fusion, hyperplasia, and hypertrophy were some of the most common alterations reported in lethal studies (acute plus chronic lethal) and sublethal studies (chronic sublethal) studies. Mallatt (1985) also noted that, in general, gill lesions occurred more frequently when fish were exposed to lethal doses than to nonlethal doses of a toxicant. In our study, epithelial necrosis was most severe in moribund fish exposed at 100 mg/L CLT, and the severity of necrosis decreased as exposure dose decreased to 60 mg/L. Degenerative changes in gills of live fish sampled after the last 80-mg/L exposure and at 7 d postexposure were more severe than those observed in fish sampled 14 d postexposure. The severity of almost all degenerative changes was substantially greater in fish exposed to acutely toxic doses of CLT than in fish exposed to safe or marginally safe doses.

The oxidative mechanism of CLT that makes it so effective as a biocide also results in the disruption of rainbow trout gill transcellular ionic effluxes, a consequence of cellular membrane damage by the hypochloride ion that leads to ionoregulatory compromise (Powell and Perry 1998). The severe CLT-induced lesions that we observed in gill tissue of fish from high CLT

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exposure concentration groups were considered the main cause of death. Acutely toxic doses of CLT caused significant damage to branchial epithelium, which likely affected gas exchange across gill epithelium, ion permeability, and ionoregulatory function. This resulted in the inability of fish to maintain osmotic balance. Similar changes were observed by Powell and Harris (2004) for Atlantic salmon *Salmo salar* smolts exposed to acutely toxic concentrations of CLT and by Altinok (2004) for goldfish *Carassius auratus* fingerlings exposed at 25 or 30 mg/L CLT for 24 h. The degeneration of red blood cells, occurrence of thrombotic lesions in gill capillaries, and erythrophagia observed in hematopoietic tissue in the kidneys of trout in our study confirm the findings of Powell and Harris (2004) for Atlantic salmon that, in freshwater, hypoosmotic plasma results in hemolysis and ultimately hemostasis because of the coagulation of the proteins from the degenerating erythrocytes. Such changes no doubt result in the production and recruitment of new erythrocytes, as evidenced by the hematopoietic hyperplasia that we observed in fish exposed at 40, 60, 80, and 100 mg/L CLT. In contrast, in our study, erythrophagia observed in hematopoietic tissue of the kidney appeared to be a delayed response associated with severe gill lesions. Also, mortality of test fish was very low, and severity and prevalence of gill damage decreased after the third CLT exposure. These results suggest that fish that survive initial exposure to CLT are likely to be less affected by additional exposures.

Two other oxidizing biocides are being used (or being tested for use) as external therapeutants in U.S. aquaculture. One is hydrogen peroxide (H_2O_2), which is used worldwide as a disinfectant and antiseptic and which vigorously decomposes to oxygen and water in the presence of organic matter or alkaline conditions. Recently, 35% PEROX-AID (35% H_2O_2; Eka Chemicals, Marietta, Georgia) was approved by FDA for use to control mortality in (1) freshwater-reared finfish eggs due to saprolegniasis, (2) freshwater-reared salmonids due to BGD, and (3) freshwater-reared coolwater finfish and channel catfish due to external columnaris. The other compound is potassium permanganate ($KMnO_4$), which can be effective for the treatment of numerous skin and gill diseases of cultured fish (Darwish et al. 2002). However, FDA approval of $KMnO_4$ for use in U.S. aquaculture has been deferred pending the outcome of recent research (Straus and Griffin 2001).

Rach et al. (1997) found that rainbow trout sensitivity to H_2O_2, as well as H_2O_2-induced mortality of rainbow trout increased with fish size and water temperature. These authors suspected that the relation between toxicity and fish size was due, in part, to the increased ratio of gill surface area to body weight of fish. Gaikowski et al. (1999) reported that when healthy individuals of various fish species were overdosed with and overexposed to H_2O_2, virtually all mortality occurred during or within 30 h of the first exposure. Darwish et al. (2002) reported that $KMnO_4$-induced mortalities were only observed in fish exposed to 3× and 5× the therapeutic dose of $KMnO_4$ and that most mortalities occurred from first exposure up to 2 d postexposure.

In our study, histopathological changes observed in rainbow trout exposed to CLT were similar to lesions reported by others in fish exposed to H_2O_2 or $KMnO_4$. Kiemer and Black (1997) noted that H_2O_2-induced gill lesions were not homogeneous; instead, severely damaged areas of gills were often adjacent to healthy areas. Tort et al. (2002) reported that microscopic alterations resulting from exposure to H_2O_2 were restricted to gills and that mortality in 90-d-old walleyes appeared to be due to these alterations. In our study, some effects detected in gill tissue that were characterized as safety concerns are typically repairable or reversible (Darwish et al. 2002). The ability of severely damaged gills to return to normal in about 6–8 d postexposure is remarkable but not surprising (Speare et al. 1999; Ferguson 2006), and is a finding similar to what we observed. Darwish et al. (2002) reported that fish exposed to a proposed therapeutic dose of $KMnO_4$ had mild gill lesions while fish exposed to acutely toxic doses of $KMnO_4$ had severe degenerative changes, including necrotizing branchitis. Thus, in addition to agreeing with the previously reported effects of CLT exposure in fishes, our results are largely consistent with evaluations of other oxidizing biocides.

In conclusion, our study demonstrated that rainbow trout—and probably all freshwater-reared salmonids—can be safely exposed to the proposed maximum therapeutic CLT dose of 20 mg/L in a static or flow-through bath for 60 min/d on three alternate or consecutive days. Moreover, based on the results of our study, there appear to be reasonable CLT margins of safety for fry, fingerling, and juvenile rainbow trout should fish be inadvertently overdosed or overexposed during therapeutic treatment.

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