Relative sensitivity of brown and rainbow trout to pulsed exposures of an acutely lethal mixture of metals typical of the Clark Fork River, Montana[1]

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Abstract: Brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) fry and juveniles were episodically or continuously exposed to a metals mixture (Zn, Cu, Pb, Cd): the concentrations and ratios of the metals, and variations in water quality (pH, hardness), were selected to represent conditions measured during episodic storm events in the Clark Fork River, Montana. Brown trout fry were more sensitive (lower LC_{50}) than rainbow trout fry to the metals in 8-h exposures with constant hardness and pH, but less sensitive to elevated metal concentrations in conjunction with depressed hardness and pH. Fry were more sensitive than juveniles when exposure was continuous, but neither life stage was clearly more sensitive when exposure was pulsed. Whole-body concentrations of K^+ and Ca^{2+} but not Na^+ were significantly depressed in fry exposed to metals. Results support the hypotheses that changes in water quality during thunderstorms are lethal to fry and juvenile life stages of brown and rainbow trouts and that the relative sensitivity of the species to the metals mixture may explain their distributions in the Clark Fork River. Low-frequency extreme conditions may effectively act as a bottleneck on the viability of populations whose relative sensitivities to such extremes may control distributions of species in a system.

Résumé : Des alevins et des juvéniles de truite brune (*Salmo trutta*) et de truite arc-en-ciel (*Oncorhynchus mykiss*) ont été exposés de façon épisodique ou continue à un mélange de métaux (Zn, Cu, Pb, Cd) : les concentrations et les proportions relatives des métaux, ainsi que les variations de la qualité de l'eau (pH, dureté), ont été sélectionnées de façon à reproduire les conditions mesurées pendant des épisodes orageux dans la rivière Clark Fork, au Montana. Les alevins de truite brune étaient plus sensibles (CL_{50} plus faible) aux métaux que les alevins de truite arc-en-ciel pendant des expositions de 8 h, la dureté et le pH étant constants, mais moins sensibles à des concentrations élevées de métaux coïncidant avec une baisse de la dureté et du pH. Les alevins étaient plus sensibles que les juvéniles quand l'exposition était continue, mais ni aucun de ces stades n'était nettement plus sensible que l'autre quand l'exposition était épisodique. Les concentrations corporelles de K^+ et de Ca^{2+}, mais non de Na^+, étaient nettement abaissées chez les alevins exposés aux métaux. Les résultats confirment l'hypothèse selon laquelle la qualité de l'eau pendant les orages est mortelle pour les stades de l'alevin et du juvénile chez les truites brune et arc-en-ciel, et la sensibilité relative des espèces au mélange de métaux peut expliquer leur répartition dans la rivière Clark Fork. Les conditions extrêmes, de faible fréquence, peuvent jouer efficacement un rôle de goulot d'étranglement sur la viabilité des populations dont les sensibilités relatives à de tels extrêmes peut ainsi régir la répartition des espèces dans un système.
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Introduction

Episodic fish kills observed in the upper Clark Fork River, Montana, have been associated with the release of elevated concentrations of toxic metals from tailings deposits and contaminated sediments (Nimick and Moore 1991; Lipton et al. 1995). Zinc, copper, lead, and cadmium are the metals most frequently elevated above water quality criteria in the upper Clark Fork River, a river in west-central Montana affected by mining (U.S. Geological Survey 1989; Moore and Luoma 1990; Lambing 1991). These metals, classified as "very toxic and relatively accessible," are among the metals most toxic to fish (Förstner and Wittmann 1983; Heath 1987). Aquatic biota in the Clark Fork River are subject to three distinct metals exposure regimes: (*i*) chronic exposures to relatively elevated concentrations during spring runoff, (*ii*) chronic exposures to lower concentrations during winter low-flow conditions, and (*iii*) episodic spikes, or pulses of extremely elevated concentrations coupled with concurrent reductions in pH and hardness. These pulses generally have been associated with thunderstorms that mobilize metals from streamside mine wastes (Brooks and Moore 1989). In addition, physical conditions such as ice breakup can resuspend contaminated bed sediments, causing metals pulses in surface water (Johns and Moore 1985). Finally, runoff from streamside acidic bank and floodplain sediments enhances the toxicity of metals by increasing the bioavailable forms, especially for Zn, Cu, and Cd (Ray 1985).

Although concentrations of metals in aquatic systems generally vary temporally, most laboratory toxicity tests are conducted using relatively constant exposures. Water quality criteria developed from continuous exposures may not be adequately protective in natural systems, where episodic or intermittent events can cause changes in both concentrations and speciation of metals (Ingersoll and Winner 1982; Seim et al. 1984; Pascoe and Shazili 1986).

For both fish and invertebrates, pulsed, episodic, or intermittent exposures to toxicants may have a more severe impact than continuous exposure (Ingersoll and Winner 1982; Seim et al. 1984; Pascoe and Shazili 1986; Siddens et al. 1986). For instance, developing steelhead trout intermittently exposed to Cu (4.5 h daily) had lower survival and growth rates and accumulated more Cu than did fish continuously exposed to unchanging equivalent levels of Cu (Seim et al. 1984). A laboratory experiment that measured effects on rainbow trout from exposure to intermittent or continuous Cd concentrations showed that although continuous exposure may reinforce toxicity and accelerate mortality, the initial toxic effects from brief Cd exposures were irreversible and the lethal effect was inevitable (Pascoe and Shazili 1986). In other words, low-frequency extreme conditions may effectively act as a bottleneck on the viability of populations of aquatic organisms. The relative sensitivity of responses to such extremes may therefore control the distribution of species in a system. The objective of this study was to determine the sensitivity of two species of trout to toxic metals when challenged by pulsed and continuous exposures.

Methods

Experimental animals

Hatchery brown (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) were obtained as eyed embryos and as juveniles from hatcheries operated by the Wyoming Game and Fish Department (Dubois Fish Hatchery and Dan Speas Fish Hatchery). Hatchery embryos and juveniles were acclimated to laboratory culture conditions at the Red Buttes Environmental Biology Laboratory, University of Wyoming (see Experimental procedures, below). Following the swim-up stage and throughout pre-exposure acclimation to control water, fry of brown trout (mean ± SE; weight 0.20 ± 0.01 g, length 29.0 ± 0.3 mm) and rainbow trout (weight 0.18 ± 0.01 g, length 28.8 ± 0.2 mm) were fed 5% body weight/d of combined Silver Cup Starter Chow, brine shrimp, and Romet 30 treated chow, and eventually solely Biodiet chow. Juveniles of brown trout (mean ± SE; weight 24.66 ± 0.62 g, length 131.5 ± 1.1 mm) and rainbow trout (mean ± SE; weight 17.80 ± 0.48 g, length 121.5 ± 1.1 mm) were fed 2% body weight/d of a vitamin-fortified commercial trout diet. Fish were acclimated to control water for a minimum of 1 month before testing. Fish condition and health were monitored daily. Thirty-eight hours before each experiment, fish were transferred to exposure chambers and deprived of food. Photoperiod simulated natural light cycles throughout the study period.

Exposure water and system

Exposure and control waters were formulated by continuously mixing well water (hardness, 220 mg/L as $CaCO_3$; alkalinity, 180–210 mg/L as $CaCO_3$; pH, 7.2–7.8) and deionized water (well water treated with sediment filtration, reverse osmosis, and separate-bed deionization) to simulate spring conditions in the Clark Fork River, Montana (hardness, 100 or 200 mg/L as $CaCO_3$; alkalinity, 80–110 or 180–210 mg/L as $CaCO_3$; pH, 7.2–7.8; temperature, 10°C). Hardness levels exceeding 400 mg/L as $CaCO_3$ (in pulsed exposures) were achieved by metering a $CaSO_4$ stock solution into formulated waters. Fiberglass headtanks (190 L; Frigid Units, Inc.) continuously received the formulated waters, which were adjusted to the desired pH by automatic pH analyzer–controllers (Leeds and Northrup model 7083) using dilute H_2SO_4 (these waters were continuously aerated to reduce the concentration of CO_2). Temperature and pH were monitored and recorded continuously using a Hewlett-Packard (model 3497A) data-acquisition and alarm system. Exposure and control waters were analyzed daily to ensure that hardness, alkalinity, pH, dissolved oxygen, and temperature were within 10% of the desired levels.

For the pulsed experiments involving only fry, water was delivered to 1-L glass aquaria (filled to 0.5 L) at 25 mL/min to provide an average flow rate of 1500 mL/h to each aquarium and a 90% volume replacement time of <1 h (Sprague 1969). For the pulse experiment involving both juveniles and fry, water was delivered to 340-L circular fiberglass tanks (Figid Unit, Inc.) filled to 56.8 L at 1.9 L/min to provide an average flow rate of 114 L/h to each tank and a 90% volume replacement time of 1.1 h

(Sprague 1969). For the continuous-exposure experiment involving juveniles and fry, water was delivered to 340-L tanks (total volume) at 1.5 L/min to provide an average flow rate of 90 L/h to each tank and a 90% volume replacement time of 8 h (Sprague 1969). For experiments using both life stages combined in the 340-L tanks, fry were placed in suspended enclosures (20.3 × 9.2 × 9.2 cm) made of nitex polypropylene mesh (4 mm diameter mesh) to prevent interactions with juveniles.

Stock solutions of combined metals were prepared from reagent-grade chloride salts ($ZnCl_2$, $CuCl_2$, $PbCl_2$, $CdCl_2$) dissolved and continuously mixed in deionized water. Exposure concentrations of the metals were achieved by metering the stock solution via a Mariotte bottle into continuous-flow diluters. Exposure and control waters were delivered by proportional diluters that provided replicates for each exposure dilution and control.

Exposure waters were identical to the control water, but contained one of four or five geometric dilutions of an 8P or 5P reference mixture during the exposures (P units represent the notation for observed metals concentrations during a documented fish kill in the Clark Fork River), where nominal concentrations for the 1P mixture were 230 μg/L Zn, 120 μg/L Cu, 3.2 μg/L Pb, and 2.0 μg/L Cd. The pulsed exposures used four dilutions (8P, 4P, 2P, and 1P) and the continuous exposure used five dilutions (5P, 2.5P, 1.2P, 0.6P, and 0.3P). The metals concentrations (in μg/L) and ratios of Zn, Cu, and Cd in the 1P reference mixture represent dissolved concentrations measured in the Clark Fork River at Deer Lodge, Montana, during a fish kill on July 12, 1989 (Lambing 1991); because measured concentrations of dissolved Pb at the Deer Lodge site were below detection limits on that date, the Pb concentration for the 1P reference mixture was set at 3.2 μg/L, which is the U.S. Environmental Protection Agency's chronic water quality criterion at a water hardness of 100 mg/L $CaCO_3$ (U.S. Environmental Protection Agency 1987).

Experimental procedures

Fish were acclimated for a minimum of 1 month to the control water used during a given experiment (e.g., hardness, 100 mg/L as $CaCO_3$; alkalinity, 80–110 mg/L as $CaCO_3$; pH, 7.2–8.0; temperature, 10°C). Positions were randomly assigned for the species or life stage to the exposure and control chambers. Individuals of each species and life stage were sequentially transferred in groups of two from holding chambers to exposure or control chambers, thus randomizing the allocation of individuals to the chambers. Ten or 15 individuals were used per exposure dilution and control within each replicate. The initial loading of fish in exposure or control chambers was less than 5.5 g/L.

Mortality, defined as cessation of opercular movement, was monitored every 20–40 min during the pulsed exposure tests and twice daily for 96 h after the pulse; during continuous exposure tests, mortality was monitored every 2 h for the first 12 h and every 6 h subsequently. Exposure and control waters were sampled for metals, temperature, dissolved oxygen, pH, alkalinity, and hardness every 20–40 min before and during pulsed exposures and twice each day after the pulse, or once each day during continuous exposures. Water samples (25 mL) for metals analysis were taken directly from each exposure or control chamber and immediately acidified with 250 μL of 70% HNO_3 to preserve the sample until atomic absorption spectroscopy (AAS) analysis was performed. Water samples (50 mL) for pH, alkalinity, and hardness were also taken directly from the exposure or control chamber and analyzed immediately.

For fry receiving pulsed exposure (tests I–V), metals were increased linearly over a 1-h period from control to maximum pulse concentrations, held constant for 6 h, and decreased linearly over a 1-h period to control concentrations (1,6,1-h pulse); the water chemistry was then held constant at control concentrations for the remainder of the experiment for monitoring postpulse mortality. For juveniles and fry receiving pulsed exposure (tests VI and VII), metals were increased linearly over a 2-h period from control to maximum pulse concentrations, held constant for 4 h, and decreased linearly over a 2-h period to control concentrations (2,4,2-h pulse); again, the water chemistry was then held constant at control concentrations for postpulse monitoring. Additionally, during each pulse, the hardness, alkalinity, and pH either remained constant, were depressed, or were increased; changes were linear and occurred over the same time as did changes in metals concentrations. In the text that follows, we use the following abbreviations for exposure conditions according to directions of change and parameters changed: constant hardness and constant pH (CHCp), depressed hardness and constant pH (DHCp), depressed hardness plus depressed pH (DHDp), depressed high hardness plus depressed pH (DHHDp), and elevated hardness and depressed pH (EHDp). Water chemistry was held constant during the continuous exposure. The experimental design for this sequence of tests is shown in Table 1, which includes nominal hardness, alkalinity, and pH during baseline (pre- and post-pulse) and during pulse conditions.

Fish sampled for whole-body Na^+, K^+, and Ca^{2+} concentrations were frozen in acid-cleaned vials until they were processed for analysis. Individuals were collected at the time of death or at the end of the experiment.

Analysis of water and tissue chemistry

Concentrations of Zn, Cu, Pb, and Cd dissolved in water were determined by AAS using a graphite furnace or flame (Perkin-Elmer models 2380 and 372). Whole-body ion concentrations (Na^+, K^+, and Ca^{2+}) in fish were determined on a wet-weight basis (Shearer 1984) with AAS using techniques described by Woodward et al. (1989). Blank, spike, standard, and replicate analyses of the same samples used to evaluate quality control were also used to verify instrument calibration and accuracy. Blanks always had element concentrations below instrument detection limits. Spikes introduced at the beginning of sample preparation and spikes added to digestates at the instrument showed an average recovery of >90%, standards generally within 10% of the theoretical values, and values for the second replicate analysis generally within 10% of the first value. Analyses were rejected and samples rerun if values for standards were not within 20% of the theoretical values.

Table 1. Nominal and measured water quality conditions from the pulsed and continuous exposures for hardness (ppm $CaCO_3$), alkalinity (ppm $CaCO_3$), and pH (units).

			Baseline conditions				Mid-pulse conditions			
Test	Life stage		Nominal	Average	Range	*n*	Nominal	Average	Range	*n*
I (CHCp)	Fry	Hard.	100	104.4	100.0–112.0	11	100	108.8	104.0–112.0	5
		Alk.	80–110	98.5	92.0–104.0	8	80–110	105.0	102.0–108.0	5
		pH	7.2–8.0	7.45	7.30–7.60	8	7.2–8.0	7.58	7.40–7.70	5
I (DHCp)	Fry	Hard.	100	105.9	96.0–120.0	30	50	64.5	44.0–122.0	27
		Alk.	80–110	102.2	94.0–120.0	24	40–60	50.3	46.0–56.0	6
		pH	7.2–8.0	7.61	7.30–7.80	30	7.2–8.0	7.30	7.20–7.40	6
III (DHDp)	Fry	Hard.	100	102.2	93.4–109.6	30	50	47.1	40.6–55.0	24
		Alk.	80–110	94.5	90.0–102.0	15	0–10	1.4	0.9–2.0	4
		pH	7.2–8.0	7.71	7.52–7.85	30	4.5	4.84	4.45–5.90	25
IV (DHHDp)	Fry	Hard.	200	203.4	190.8–212.0	30	100	119.4	108.0–176.0	25
		Alk.	180–200	186.5	182.0–192.0	15	0–10	2.3	0.4–8.0	14
		pH	7.2–8.0	7.90	7.68–8.09	30	4.5	5.12	4.38–6.38	25
V (EHDp)	Fry	Hard.	200	207.2	196.3–220.3	30	400	423.9	403.7–452.9	25
		Alk.	180–200	185.6	176.0–192.0	15	0–10	4.2	0.9–10.0	10
		pH	7.2–8.0	7.86	7.62–8.10	30	4.5	4.96	4.61–5..65	25
VI (DHHDp)	Fry and juvenile	Hard.	200	204.5	195.8–216.2	25	100	110.6	106.1–118.3	20
		Alk.	180–200	185.5	177.4–192.0	13	0–10	2.2	0.97–5.85	8
		pH	7.2–8.0	7.87	7.66–8.20	25	4.5	4.83	4.25–5.46	20
VII (DHHDp)	Fry and juvenile	Hard.	200	207.3	199.7–220.5	30	100	134.4	108.2–199.7	19
		Alk.	180–200	185.3	179.4–191.1	18	0–10	2.9	1.95–3.98	6
		pH	7.2–7.8	7.86	7.69–8.11	30	4.5	5.05	4.48–5.58	20
Continuous exposure (CHCp)	Fry and juvenile	Hard.	100	101.5	95.9–111.3	42	na			
		Alk.	80–110	87.3	79.9–91.6	14	na			
		pH	7.2–7.8	7.66	7.52–7.78	42	na			

Note: Conditions of exposure are abbreviated according to directions of change and parameters changed: constant hardness and constant pH (CHCp), depressed hardness and constant pH (DHCp), depressed hardness plus depressed pH (DHDp), depressed high hardness plus depressed pH (DHHDp), and elevated hardness and depressed pH (EHDp). na, not applicable; Hard., hardness; Alk., alkalinity.

Water samples were analyzed for hardness and alkalinity by titrimetric methods (American Public Health Association, American Water Works Association, and Water Pollution Control Federation 1985), pH was measured using a calibrated Beckman Omega 12 pH/ISE meter and probes, and dissolved oxygen was measured using a calibrated YSI (model 54A) meter and probe.

Statistical analyses

To evaluate the sensitivity of fish to the metals mixture, LC_{50} values and 95% confidence intervals were computed using the Spearman–Karber method (Hamilton et al. 1977, 1978). Values for LC_{50} were computed on the basis of maximum average Zn concentrations measured during exposures. Zn data were used for these computations because the Zn concentration was higher than those of Cu, Pb, or Cd in the Clark Fork River and because it could be measured with greater precision in our experiments, again because Zn occurred in higher concentrations. Estimates of LC_{50} were deemed significantly different if 95% confidence intervals did not overlap. This is analogous to a one-tailed test of significant differences at $\alpha = 0.05$ with overall protection of $(0.95)^n$, where n is the number of species or life stages being compared.

In addition, for each species and for each test (experimental design for tests I–V was a completely randomized block; experimental design for tests VI and VII was a completely randomized block with blocks separated by time), relative sensitivity to exposure concentrations was evaluated using a two-way analysis of variance (ANOVA). Specifically, this analysis evaluated effects of metals concentrations and blocks (replicates) on the mean proportion surviving the pulsed exposures and the 96-h postpulse period. The response variable, the proportion of fish surviving, was arcsine square-root transformed (Gelber et al. 1985). Dunnett's multiple comparison procedure (Zar 1984) was conducted to compare survival in each exposure concentration with survival in the corresponding control. Dunnett's comparisons determined the highest concentration of metals (P units) having no statistical difference in survival compared with the control (NOEC, no observed effect concentration) and the lowest concentration of metals (P units) having a statistically reduced survival compared with the control (LOEC, lowest observed effect concentration) (Gelber et al. 1985).

The status of whole-body ions for fish exposed to metals was evaluated using a one-way ANOVA to investigate the effects of survival on the mean concentrations of Na^+, K^+,

Table 2. Measured metals concentrations in exposure dilution (P units) and control (C) waters during the pulsed and continuous exposures.

Test	Dilution (P units)	Maximum concentration (μg/L)			
		Zn[a]	Cu[b]	Pb[b]	Cd[b]
I	8	2537.6±48.7	1245.0±7.1	58.12±0.53	11.25±0.35
	4	1294.9±68.6	601.0[c]	23.30[c]	5.80[c]
	2	628.1±35.0	285.2±4.0	11.05±0.14	2.63±0.25
	1	321.6±21.8	141.0±2.4	5.30±0.21	1.28±0.04
	C	22.2±15.8	nd[c]	nd[c]	nd[c]
II	8	2294.5±149.1	1323.0±72.1	58.88±0.53	11.63±0.53
	4	1216.3±39.4	680.5±23.3	23.85±0.92	6.80±0.07
	2	577.0±24.2	314.7±14.1	10.60±0.78	3.48±0.04
	1	283.2±17.8	149.2±17.2	4.50±0.42	1.78±0.00
	C	23.6±15.0	nd[c]	nd[c]	nd[c]
III	8	2290.8±178.3	1478.8±158.4	60.63±10.08	12.00[c]
	4	1151.0±89.8	674.8±19.6	22.70±0.78	6.13±0.04
	2	566.0±20.6	404.0±22.6	12.68±0.60	3.75±0.14
	1	270.7±20.6	186.0[c]	7.00±0.42	1.90±0.55
	C	14.3±13.3	nd[c]	nd[c]	nd[c]
IV	8	2170.3±179.4	1286.6±132.1	55.50±3.54	10.75±0.35
	4	1186.3±120.2	708.2±6.4	25.05±2.62	6.35±0.07
	2	569.4±8.8	360.8±16.3	14.50±2.19	3.77±0.25
	1	300.4±10.5	200.7[c]	7.00±0.28	1.68±0.39
	C	8.6±7.5	nd[c]	nd[c]	nd[c]
V	8	2042.3±251.1	1473.2±9.2	63.62±2.30	12.63±0.18
	4	1040.4±50.2	789.0±142.2	25.38±3.50	6.73±0.18
	2	545.7±8.2	399.7±31.5	15.00±0.64	3.80±0.07
	1	302.6±27.4	213.0±8.6	8.30±0.00	2.18±0.04
	C	nd[c]	7.2±5.5	nd[c]	nd[c]
VI	8	1814.0±212.7	1146.7±23.8	31.50±0.71	8.80±0.14
	4	947.4±31.8	557.6±18.2	13.48±0.18	3.83±0.04
	2	468.1±42.9	291.3±8.1	7.25±0.64	2.05±0.07
	1	251.1±47.3	190.5±39.6	3.53±0.04	1.10±0.00
	C	12.7±20.6	6.0±0.3	nd[c]	nd[c]
VII	8	1898.8±75.3	1146.9±40.1	31.75±5.66	8.23±0.67
	4	958.3±21.8	578.2±16.3	14.30±0.07	4.08±0.11
	2	466.4±47.7	286.6±3.3	7.00±0.00	1.97±0.18
	1	231.2±5.0	145.7±0.1	2.98±0.32	0.95±0.00
	C	nd[c]	11.2±0.0	nd[c]	nd[c]
Continuous[d]	5	1187.7±78.4	753.0±3.6	16.68±0.35	13.05±0.35
	2.5	590.8±45.4	369.2±11.3	8.18±0.11	5.60±0.49
	1.2	297.7±19.6	180.4±3.9	4.05[c]	3.06±0.06
	0.6	146.5±9.9	92.0±3.2	nd[c]	1.57±0.04
	0.3	68.6±8.6	44.6±0.0	nd[c]	0.70±0.00
	C	nd[c]	nd[c]	nd[c]	nd[c]

Note: Values are given as the average ± SD, as determined by atomic absorption spectroscopy.
nd, values below the method detection limit of 4.9 μg/L Zn, 4.6 μg/L Cu, 1.7 μg/L Pb, and 0.4 μg/L cd.
[a] $7 \leq n \leq 16$.
[b] $n = 2$.
[c] $n = 1$.
[d] Average during continuous test.

or Ca^{2+}. Tukey's multiple means comparison test was used to detect differences between the mean ionic concentrations in fish that died during exposure and the mean concentrations in both fish that survived exposure and control fish. A type-I error of 0.05 (p) was used to judge significance in statistical tests.

Results

Water chemistry

Measured hardness, alkalinity, and pH did not deviate from the nominal values by more than 26% in all tests for all characteristics (Table 1). Dissolved oxygen was greater

Table 3. LC_{50} estimates and LOEC and NOEC estimates for brown and rainbow trout fry or juvenile life stages from pulsed exposures (8-h pulse + 96-h postpulse observation) and continuous exposure (96 h) to dilutions of a mixture of Zn, Cu, Pb, and Cd.

		Brown trout			Rainbow trout		
		LC_{50} (μg Zn/L)			LC_{50} (μg Zn/L)		
Test	Life stage	Estimate	95% CL	LOEC, NOEC (P units)	Estimate	95% CL	LOEC, NOEC (P units)
Pulsed exposures							
I (CHCp)	Fry	1000	903–1108*a*	4P,2P	1397	1222–1597*b*	4P,2P
II (DHCp)	Fry	454	426–484*c*	2P,1P	393	381–405*d*	1P,<1P
III (DHDp)	Fry	700	639–767*e*	2P,1P	386	367–407*d*	1P,<1P
IV (DHHDp)	Fry	1260	1142–1390*b*	4P,2P	490	440–546*c*	2P,1P
V (EHDp)	Fry	>2042	—	>8P,8P	>2042	—	8P,4P
VI–VII (DHHDp)	Fry	1041	816–1329*abf*	4P,2P	738	643–848*ef*	4P,2P
	Juvenile	>1,856	—	8P,4P	691	646–739*e*	4P,2P
Continuous exposure							
CHCp	Fry	100	93–109*g*	0.3P,<0.3P	97	87–108*g*	0.3P,<0.3P
	Juvenile	132	118–148*h*	0.6P,0.3P	182	147–226*h*	1.2P,0.6P

Note: LC_{50} values are based on the measured Zn concentrations (μg/L) in the metals mixture, where 1P nominal concentrations (μg/L) for the metals were as follows: 230 Zn, 120 Cu, 3.2 Pb, and 2.0 Cd. LC_{50} calculations were made using the Spearman–Karber method (Hamilton et al. 1977). For comparisons, LC_{50} values (μg Zn/L) followed by the same letter are not significantly different, on the basis of comparison of 95% confidence limits (95% CL) ($\alpha = 0.05$) with overall protection of $(0.95)^n$, where n is the number of species or life stages being compared. LOECs and NOECs were determined by Dunnett's comparisons of exposure survival means to control survival means (Gelber et al. 1985). Conditions of exposure are abbreviated according to directions of change and parameters changed: constant hardness and constant pH (CHCp), depressed hardness and constant pH (DHCp), depressed hardness plus depressed pH (DHDp), depressed high hardness plus depressed pH (DHHDp), and elevated hardness and depressed pH (EHDp).

than 75% saturation and temperature remained at 10.0 ± 1.5°C in all exposure chambers for all tests. Maximum average concentrations of Zn were 100–140% of nominal concentrations during the pulsed exposures and average concentrations of Zn were 95–104% of nominal concentrations during the continuous exposure. Concentrations (average maximum, average) and standard deviations measured for Zn, Cu, Pb, and Cd in different tests and dilutions are shown in Table 2.

Fry pulsed exposure: survival response

Survival was 90–100% for all control fry during the pulse and postpulse periods. Mortality occurred as early as 2 h into the pulse for the highest exposure concentration. Metals exposures as low as 1P and 2P significantly reduced survival in rainbow trout (8-h pulsed exposures) and brown trout (96-h postpulse) when hardness or hardness plus pH were depressed during exposure to metals (Table 3).

Rainbow trout were more tolerant than brown trout under CHCp (i.e., significantly higher LC_{50}), but the reverse was true under DHCp, DHDp, or DHHDp (i.e., the LC_{50} was significantly lower for rainbow trout) (Table 3). In all tests with depressed hardness or depressed hardness plus pH, rainbow trout had lower values for LC_{50} and LOEC than when exposed under CHCp (Table 3). Additionally, rainbow trout were less tolerant (lower LC_{50}) in tests where hardness was decreased from 100 to 50 ppm as $CaCO_3$ than in tests where the hardness was decreased from 200 to 100 ppm. A similar trend was found for brown trout. Tolerance was clearly greatest (higher LC_{50}s and LOECs) when either rainbow or brown trout were exposed under EHDp compared with other pulse conditions.

Juvenile and fry pulsed exposure: survival response

Survival was 100% for all control fish during the pulse and postpulse periods. Because tests VI and VII used the same exposure conditions and, for each species and life stage, there was no significant difference in survival, these tests were treated as replicates in calculations for LC_{50} and in the Dunnett's comparisons for survival analyses. For rainbow trout at the highest exposure concentration, mortalities occurred as early as 3 h into the pulse for both juveniles and fry, and cumulative survivals were reduced to 0% during the pulse or within 24 h postpulse. For brown trout at the highest exposure concentration, mortalities occurred by 7 h into the pulse for both juveniles and fry, and cumulative survivals were reduced to 60% (juveniles) and 15% (fry) at 96-h postpulse.

LC_{50}s for rainbow trout juveniles and fry were similar in the pulsed exposure (Table 3). Although brown trout juveniles had a higher LC_{50} than did fry, because confidence limits could not be calculated for juveniles, statistical significance was not assigned to this difference (Table 3). LOEC values were 4P for brown trout fry and rainbow trout fry and juveniles, and 8P for brown trout juveniles (Table 3). Thus, considering both estimates of LC_{50} and mean survival proportions, the relative sensitivity to pulsed exposures of the two species and two life stages was rainbow trout juveniles ≥ rainbow trout fry > brown trout fry > brown trout juveniles. Therefore, both life stages of the

Table 4. Mean whole-body Na^+, K^+, and Ca^{2+} concentrations (μequiv./g, wet weight) for brown and rainbow trout fry determined from pulsed exposure (test I) using dilutions of the metals mixture of Zn, Cu, Pb, and Cd. Control and exposure survivor groups were sampled at the end of the 8-h pulse + 96-h post-pulse observation and exposure mortality groups were sampled at the time of death.

	Whole-body ion concentration (μequiv./g)					
	Na^+		K^+		Ca^{2+}	
Species and group	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE	*n*
Brown trout						
Control	108.3±10.9	38	159.9±8.8	36	570.8±51.3	36
Survivor	103.6±7.5	88	165.1±7.2	87	554.8±23.2	87
Mortality	85.5±4.3	112	115.9±3.5[a,b]	110	385.1±10.4[a,b]	110
Rainbow trout						
Control	53.8±10.2	38	141.2±15.3	38	484.9±35.0	38
Survivor	88.1±10.2	41	127.0±7.5	49	443.1±35.1	49
Mortality	55.5±3.7	42	96.1±5.1[a,b]	41	293.0±13.1[a,b]	41

[a]Significantly different ($p < 0.005$) from control.
[b]Significantly different ($p \leq 0.01$) from survivor.

rainbow trout appeared to be more sensitive to pulsed exposures than either life stage of the brown trout.

Juvenile and fry continuous exposure: survival response

Survival was 100% for all control fish during the continuous exposure. In the highest exposure concentration, mortalities occurred as early as 8 h and survival was at or near 0% at 12 h for all species and life stages. At 96 h, LC_{50} values for both fry species were significantly lower than those for juveniles (Table 3). Whereas the values of LC_{50} and LOEC for brown and rainbow trout fry were nearly identical, the LC_{50} values for juveniles were not significantly different from one another but brown trout juveniles had notably lower LC_{50} and LOEC values than rainbow trout (Table 3).

Finally, LC_{50} and LOEC values from the continuous exposures were lower than for the pulsed exposures for both species and life stages, reflecting the larger time-integrated exposure of metals to fish.

Whole-body ions

Data for whole-body ion concentrations are restricted to the individual fish from the pulsed exposure of test I. Brown and rainbow trout fry that died during the pulsed exposures had significantly decreased concentrations of whole-body K^+ and Ca^{2+}, but not Na^+ (Table 4). Relative to controls, both brown and rainbow trout fry that died exhibited mean losses of K^+ and Ca^{2+} of ≥27 and ≥33%, respectively. When data from fish that survived exposure to metals were compared with data from control fish, no significant differences were found in whole-body concentrations for any of the measured ions.

Discussion

Episodic fish kills have been observed in the Clark Fork River coincident with spring and summer thunderstorms that release elevated concentrations of Zn, Cu, Pb, and Cd from contaminated floodplain, bank, and, potentially, bed sediments (Nimick and Moore 1991; Lipton et al. 1995). The set of experiments described in this study demonstrates that pulsed exposure to elevated Zn, Cu, Pb, and Cd concentrations, within the range of concentrations and ratios observed during fish kills in the Clark Fork River, adversely and significantly affected survival in both fry and juveniles of brown and rainbow trout. For experiments during which maximum concentrations of metals were present for 6 h or less (8-h exposures), metals concentrations as low as 1P for rainbow trout and 2P for brown trout adversely affected fry survival (see Table 2 for measured metals concentrations). Thus, results from both this laboratory simulation study and from the field support the conclusion that short-term pulsed exposures to metals cause mortality in brown and rainbow trout in the Clark Fork River, Montana.

Additional data emphasize the toxicity of the metals mixture and support the conclusion that episodic events in the Clark Fork produce toxic conditions; significant mortality occurred during or after pulsed exposures in all species and life stages tested. For example, although rainbow trout fry survived the 8-h pulse, none survived the 96-h postpulse observation in the 2P exposure concentration (test II). Pascoe and Shazili (1986) made similar observations; whereas there was no mortality in rainbow trout fry during brief pulsed exposures to Cd (1.0 mg/L for 32 min), mortality after transfer to control water was 50% by 8 d.

As described in Lipton et al. (1995), rainbow trout are uncommon in the more contaminated, upstream reaches of the Clark Fork River. This study and additional studies (Marr et al. 1995; Woodward et al. 1995) examined whether this observed species distribution is consistent with the relative sensitivities of the two trout species to metals. To evaluate the relative sensitivity of the trout species to a metals mixture typical of Clark Fork River

pulses, we compared LC_{50} values computed from the 8-h pulsed exposures (+96-h postpulse observation). Under conditions of acutely lethal metals pulses, the relative sensitivity of the two species was altered dramatically if the water quality involved in the exposure was changed. When water hardness and pH were held constant brown trout were more sensitive than rainbow trout, but under conditions more typical of a thunderstorm (i.e., depressed hardness and pH along with elevated metals concentrations) the relative sensitivity to the metals pulse was reversed, with rainbow trout being the most sensitive. As noted in Marr et al. (1995), this response of rainbow trout to complex stress was also observed in acclimation experiments in which brown trout demonstrated apparent enhanced physiological capability, relative to that of rainbow trout, to acclimate to sublethal metals exposures.

The magnitude, duration, and frequency of episodic events have all been found to be important in affecting response to toxicants, especially in more sensitive species. For example, the effect of pH on brook trout was related to the number and magnitude of the acidic episodes (Cleveland et al. 1991), and postpulse mortality in rainbow trout briefly exposed to 1–100 mg Cd/L depended upon the duration of exposure (Pascoe and Shazili 1986). We also found that rainbow trout were more sensitive to exposure duration. This is additional evidence suggesting that, in the Clark Fork, rainbow trout fry are more sensitive than brown trout fry to metals at any duration of pulsed exposure.

We also assessed the relative sensitivity of two life stages (juvenile and fry). Again, the results were dependent on conditions of exposure; for brown trout, juveniles appeared to be only slightly less sensitive (higher LC_{50}, no statistical analysis) to pulsed exposures than were fry, and no statistical differences were observed for rainbow trout. Under continuous exposures, juveniles of both brown and rainbow trout were significantly less sensitive (higher LC_{50}s) than fry. Previous studies also have reported such differences in susceptibility. For example, the sensitivity of rainbow trout to Cu varied according to fish size, with larger rainbow trout being more resistant (Howarth and Sprague 1978; Chakoumakos et al. 1979). Both chinook salmon and steelhead trout showed differences in sensitivity to Zn and Cd, but not to Cu (Chapman 1978). Alevins were the most resistant among the various life stages examined (newly hatched alevins, swim-up alevins, parr, and smolts).

During pulsed exposures, water quality (i.e., hardness, alkalinity, pH) dramatically influenced survival rates of hatchery brown and rainbow trout fry. Survival was reduced when hardness or hardness plus pH were reduced, and survival increased when hardness increased and pH decreased. Greatest sensitivity occurred for both species when hardness was depressed during the pulse (i.e., significantly lowest LC_{50} values in DHCp- or DHDp-pulsed exposures, tests II and III; Table 3). However, the influence of water hardness and (or) pH on sensitivity was different for brown and rainbow trout (compare values for LC_{50} and LOEC among the various conditions of hardness and pH in the pulsed exposures, Table 3).

The effects of pH reductions on toxicity (at least in the presence of reduced hardness) can be seen by comparing the results of tests II and III (in which the hardness regime was similar). For brown trout, reduced pH during the metals pulse was associated with an increased tolerance to the metals toxicity (higher LC_{50}). For rainbow trout, pH reduction had no observable effect on metals sensitivity.

Changes in hardness had a strong inverse effect on metals toxicity (i.e., lower hardness, higher sensitivity). For example, comparing the results of tests I and II demonstrates that brown and rainbow trout were significantly more sensitive to the metals mixture when hardness decreased. Similarly, comparing the results of tests IV and V demonstrates that both species were significantly less sensitive when hardness increased.

The relative effects of initial hardness versus hardness during the pulse are difficult to interpret. Comparing the results of tests I and II versus IV and V (comparisons in which pH was similar) suggests that the hardness during the pulse exposure is most relevant to mitigation of toxicity; in both comparisons, initial hardness was similar and the observed differences in sensitivity were explained by the hardness during the pulse. However, comparison of the results of tests I and IV presents a slightly confounded interpretation. In this comparison, the hardness during the pulse was similar (100 mg/L $CaCO_3$) whereas the initial hardness was different (100 vs. 200); in addition, the pH was lower in the test IV pulse. In this case, brown trout were less sensitive when hardness and pH decreased; this could be explained by the protective effect of reduced pH on brown trout fry. However, rainbow trout were substantially more sensitive when hardness and pH decreased. This increased sensitivity can be explained by changes in initial hardness (again, no effects of pH on rainbow trout sensitivity were observed).

In general, most studies show that metals toxicity is reduced as hardness, alkalinity, and pH increase (e.g., Alabaster and Lloyd 1980; Everall et al. 1989). However, reported effects of pH on metal toxicity are somewhat variable. For instance, in both soft and hard waters (Everall et al. 1989; Bradley and Sprague 1985), Zn was less toxic to juvenile rainbow and brown trout at lower rather than higher values of pH. Zn also was less toxic to steelhead trout fry in soft water (2.3 mg Ca/L) as pH decreased from 7.0 to 5.7 to 4.7 (Cusimano et al. 1986).

Two common mechanisms by which the toxicities of most metals are influenced by chemical properties (i.e., pH, alkalinity, hardness) of the exposure water are (*i*) control of chemical speciation of metals, which determines the relative proportion and availability of different metals, and (*ii*) alteration of metal adsorptivity to cell membranes, which in turn can influence biological responses. Campbell and Stokes (1985) suggested that reduced pH may affect the overall toxicity of metal cations via both mechanisms, i.e., influencing metal speciation by increasing bioavailability of the unbound-cationic species, and also influencing biological sensitivity to metals by decreasing cell membrane adsorption of metals. It should be noted, moreover, that pH – metals speciation relationships (and hence bioavailability) may be complicated by pH changes at the gill surface (gill microenvironment). Playle et al. (1992) found that the pH in the gill microenvironment can differ from that of ambient water by as much as two pH units. At ambient pH in the range of 7–8, the pH at the gill surface has been

found to be reduced by more than one pH unit, whereas at ambient pH in the range of 4–5, the gill surface pH was increased slightly by 0.5 of a pH unit (Playle and Wood 1989). Further, over the pH range 5.5–8 of ambient water, net H^+ excretion increased linearly at the gill surface in response to increased ambient pH (Lin and Randall 1993). It is possible that, in our study, the observed increase in metals toxicity for brown trout as ambient pH increased may be a function of both H^+ competition and decreased pH at the gill surface.

Alkalinity moderates copper toxicity externally; it influences copper speciation by controlling the equilibrium of toxic forms, Cu^{2+}, $CuOH^+$, and $CuOH_2^0$, and the less toxic carbonate forms, $CuHCO_3^+$, $CuCO_3^0$, and $Cu(CO_3)_3^{2-}$. The carbonate or less toxic form generally predominates in high-alkaline conditions, which is why alkalinity is considered to afford protection for fishes (Chakoumakos et al. 1979; Laurén and McDonald 1986). However, in some cases copper toxicity to rainbow trout depends on the total concentration of copper (that is, the concentration of cupric copper, Cu^{2+}, and copper carbonate, $CuCO_3^0$) rather than the concentration of either cupric copper or copper carbonate alone (Shaw and Brown 1974).

Calcium, which typically contributes to hardness, may moderate copper toxicity internally by controlling the permeability of branchial epithelium in gills. Thus, the presence of metals when hardness and alkalinity are low should increase electrolyte loss and water uptake (Eddy 1975; Laurén and McDonald 1986; Everall et al. 1989). Eddy (1975) found that, in the absence of calcium, the potentials of brown trout gill epithelia were more negative than in other species (e.g., goldfish), and that calcium additions immediately restored the normal potential. This suggests that in brown trout, calcium greatly reduces ion loss by reducing membrane permeability to Na^+. Everall et al. (1989) also found that high Ca^{2+} (i.e., high hardness) altered the membrane permeability of branchial epithelial cells to zinc in brown trout, so that influx was lowered and efflux was enhanced.

Our results showing loss of whole-body ions suggest that the mode by which metals caused toxicity involved ionoregulatory disturbance. For both brown and rainbow trout fry, significant losses of K^+ and Ca^{2+} occurred only in individuals that died from the pulsed exposure (Table 4). Acute toxicity of metals to fish often is attributed to cytological damage to gill epithelia (e.g., Mueller et al. 1991). This in turn may disrupt ionoregulation or specific gill functions (e.g., chloride cell function) that are particularly important to freshwater fishes (Eddy 1981, 1982). For example, sticklebacks exposed for 16 h to approximately 1.0 mg Zn/L demonstrated extensive gill damage 5 d after replacement in control water (Matthiessen and Brafield 1973). Laurén and McDonald (1985) showed that loss of K^+, Na^+, and Cl^- in rainbow trout was strongly dependent on the exposure concentration of Cu and was due to disruptions of branchial ionoregulatory mechanisms within 2 h of the Cu exposure.

Although significant losses of K^+ and Ca^{2+} but not Na^+ were attributed to the metals toxicity in the present study, some previous work suggests this might not be expected. For instance, Cu may cause loss of both K^+ and Na^+ by increasing the permeability of (*i*) apical cell membranes, thereby stimulating passive loss of intracellular K^+, and (*ii*) branchial tight junctions controlled by bound Ca^{2+}, thereby stimulating loss of Na^+ (Laurén and McDonald 1985). In the present experiments, the effects of metals on branchial tight junctions may have been repressed by high concentrations of Ca^{2+} in exposure waters. These waters may have supplied sufficient Ca^{2+} to binding sites and thus prevented passive Na^+ efflux.

Calcium protects against respiratory impairment from metal toxicity in several fish species. For example, the presence of calcium significantly reduced Zn toxicity in fathead minnows (Judy and Davies 1979). Conversely, lack of Ca^{2+} (low hardness) increased the acute toxicity of Cd to rainbow trout fry (Calamari et al. 1980). Calcium reduced the permeability of branchial epithelia to water in carp but did not reduce Cu uptake, so here apparently calcium provided protection by stabilizing cell membranes rather than reducing toxicant (Cu) uptake (Gregory and Macfarlane 1981). The effect of Ca^{2+} may involve increased stability of gill-epithelial membranes and cell junctions owing to maintenance of cross links between proteins and other cell-wall structures (McWilliams 1983).

In summary, our results support the conclusion that episodic pulses of metals in the Clark Fork River are lethal to both early and juvenile life stages of brown and rainbow trout. This suggests that episodic acute exposures reduce survival in the wild and lower recruitment of subadults into resident trout populations, thus affecting overall population viability.

Additionally, the results of this laboratory study demonstrated that rainbow trout were more sensitive than brown trout to the metals mixture during pulse events when hardness and pH were depressed. This relative sensitivity appeared, in part, to be (*i*) a function of the relatively smaller protective effect of pH reductions on rainbow trout survival relative to brown trout, and (*ii*) a function of the greater adverse effects of hardness reductions on rainbow trout relative to brown trout. In a companion study (Marr et al. 1995), we demonstrated the apparent increased physiological resilience of brown trout to metals toxicity: enhanced capacity for metallothionein induction and sublethal metals acclimation. The results of our pulsed exposure study suggest that similar physiological distinctions between trout species may play a role in relative sensitivity to acute exposures. Given that metal speciation was similar for brown and rainbow trout for the various exposures, the differential responses to hardness, alkalinity, and pH changes support the hypothesis that physiological distinctions between species (e.g., branchial permeability and ion transport) control toxicity. Further, the concept of bioavailability should be viewed as an operational definition that is controlled by both chemical conditions (e.g., speciation) and physiological conditions.

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