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# Acute Toxicity of Cadmium, Lead, Zinc, and their Mixtures to Stream-Resident Fish and Invertebrates

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## Abstract

We conducted 150 tests of the acute toxicity of resident fish and invertebrates to cadmium (Cd), lead (Pb), and zinc (Zn), separately and in mixtures, in waters from the South Fork Coeur d'Alene River watershed, Idaho, USA. Field collected shorthead sculpin (*Cottus confusus*), westslope cutthroat trout (WCT), *Oncorhynchus clarki lewisi*, two mayflies, *Baetis tricaudatus* and *Rhithrogena* sp., a stonefly, *Sweltsa* sp., a caddisfly *Arctopsyche* sp., a snail *Gyraulus* sp., and hatchery rainbow trout (RT), *O. mykiss*, were tested with all three metals. With Pb, the mayflies *Drunella* sp., *Epeorus* sp. and Leptophlebiidae, a Simuliidae sp. black fly, a Chironomidae sp. midge, a *Tipula* sp. crane fly, a Dytiscidae sp. beetle, and another snail *Physa* sp. were also tested. Adult WCT were captured to establish a broodstock to provide fry of known ages for testing. With Cd, the range of 96-h EC50s was 0.4 to >5329 µg/L and the relative resistances of taxa were WCT≈RT≈sculpin << other taxa; with Pb, EC50s ranged from 47 to 3323 µg/L with WCT<RT< other taxa; and with Zn, EC50s ranged from 21 to 3704 µg/L with RT<WCT≈sculpin<< other taxa. With swim-up trout fry, there was a pattern of decreasing resistance with increasing fish size. In metal mixtures, the toxicities of the three metals were less than additive on a concentration-addition basis.

**Keywords:** Site-specific criteria, Resident species procedure, Fish size, Soft water, Metals mixture toxicity

## Introduction

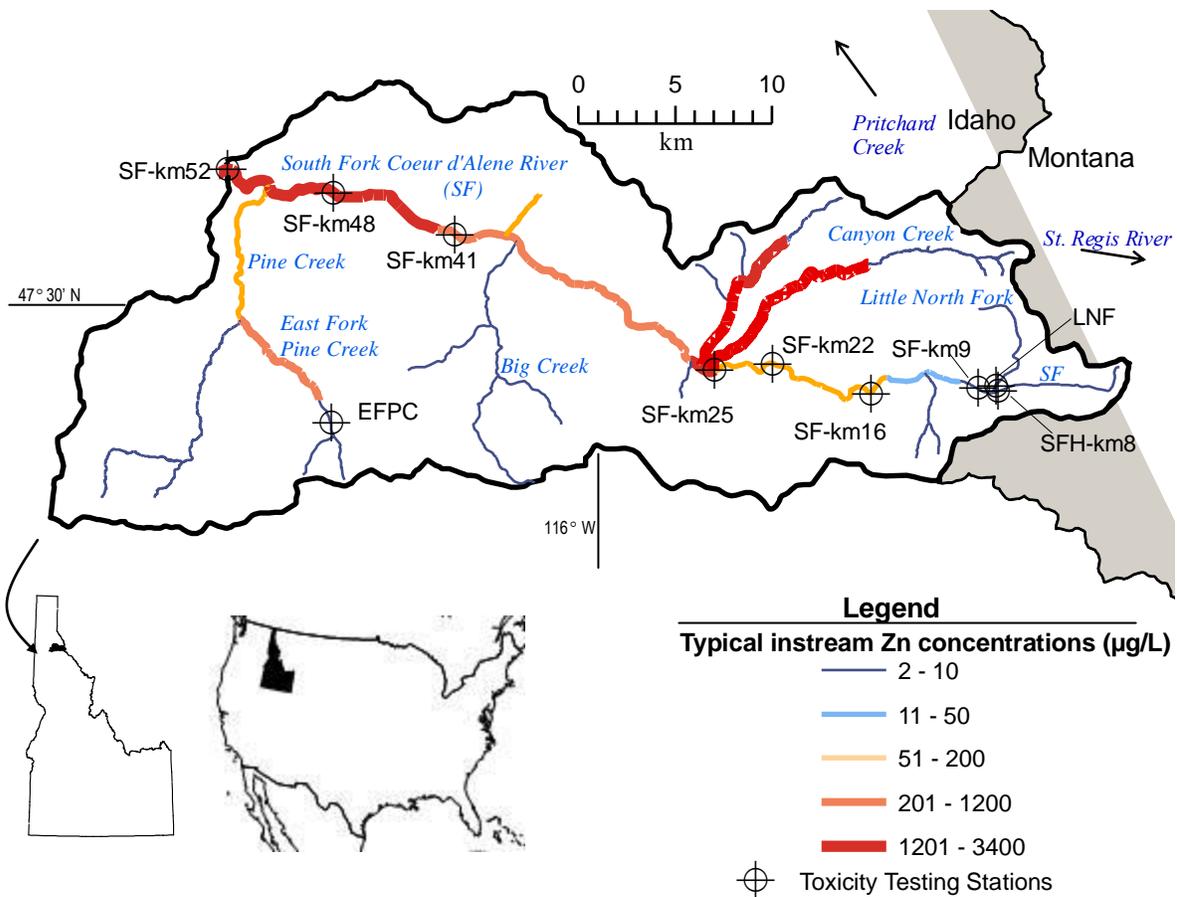
Metal mining operations often have to contend with elevated background metals concentrations in watersheds. By design, mines are located where natural mineralization is anomalously high and natural weathering of mineralized zones could lead to elevated background metals concentrations. When this natural weathering is accelerated by decades or even centuries of mining disturbances, persistently elevated metals concentrations in surface waters often result, sometimes with profound ecological harm [1-3]. This legacy can pose a major practical problem for the management of contemporary mining operations. Wastewaters from mining usually have metals concentrations that are elevated above natural background concentrations as result of runoff from disturbed ground, groundwater pumped from underground workings or pits, mill tailings decanting ponds, and so on [2,3]. Depending on the severity of contamination and the assimilative capacity [4] of streams that receive mining wastewaters, treated or untreated wastewaters may be released into downstream waters. If the background concentrations of metals in these receiving waters are elevated, receiving waters may be judged as having little or no assimilative capacity for new discharges. This can greatly affect the treatment costs of wastewater and create perverse incentives to locate new effluent discharges in more pristine areas rather than expanding or renewing operations in already disturbed watersheds.

In the USA, assimilative capacity of streams is usually operationally defined using broadly applicable, numeric ambient water quality criteria that were derived at a national scale and from which wastewater limits are back calculated. If background concentrations of a chemical exceed these criteria, then the criteria become restoration targets and because by definition there is no assimilative capacity, no dilution to meet criteria is possible. Wastewater discharges may then be required to meet aquatic life criteria at the point of discharge. Because meeting aquatic life criteria at the point of discharge without allowances for dilution may be difficult, it may be important to reduce uncertainties associated with applying broadly applicable national or general criteria to a specific site. Such broad application could lead to great inefficiency through unnecessary compliance or misguided remediation [5]. Alternatively, general criteria could be underprotective if locally important ecosystem components were not captured in broadscale criteria.

Our study area was the South Fork Coeur d'Alene River (SFCdAR) watershed in northern Idaho, USA ([Fig. 1](#)). The watershed has been extensively disturbed by mining and smelting for over 100 years, resulting in elevated cadmium (Cd), lead (Pb), and zinc (Zn) in many streams in the watershed [2,3]. The elevated background metals in streams posed a challenge to contemporary mining operations, for their effluent discharge permits were supposed to meet the same nationally developed water quality criteria that apply in waters without historical mining disturbances. This and the fact that although metals were greatly elevated above natural background concentrations, at least some fish persisted in all but the most severely polluted stream reaches [6], led to the present study to support the development of site-specific criteria (SSC) for the watershed.

We followed a resident species procedure to develop a dataset for SSC. The procedure entails generating a species sensitivity distribution in a similar manner to that done for national criteria in the USA, by generating a separate set of toxicity data for species resident to the site, in site water [5,7]. The proof of concept of the procedure has been demonstrated in previous studies [8-12], although we are not aware of any water quality criteria ever being established as result of these studies. The present article is one of a planned series describing the SFCdAR resident species studies. Here we describe acute toxicity testing of resident fish and invertebrates with Cd, Pb, and Zn individually and in mixtures, and factors that affected toxicity. The main purpose of the present article is to present our original data in sufficient detail to support secondary analyses. We intend to follow with derivative analyses including the derivation of SSC based upon data from the present study along with previously reported chronic testing [13], field validation of the SSC, and development of biotic ligand models for predicting the toxicity of metal mixtures.

The acute toxicity testing had five major elements: 1) pilot testing to develop methods for holding and testing candidate organisms; 2) rangefinding tests to further refine methods and to determine the most sensitive species for more definitive testing; 3) definitive testing of sensitive species using narrower exposure ranges in order to estimate species mean acute values for the most sensitive tested species; 4) testing for spatial and seasonal differences in toxicity within the watershed; and 5) metals-mixture testing to evaluate if potential criteria concentrations that were developed from testing with single metals would likely be protective in mixtures. Because of the scale of the present study, with 150 acute toxicity tests, we present the detailed results in the online supporting information, with the main body of the article focused on our interpretations.



**Figure 1.** Location of toxicity testing stations in the South Fork Coeur d'Alene River watershed, Idaho, USA, in relation to typical ranges of ambient dissolved Zn concentrations under stable flow conditions during 1996-2001. Dissolved Cd and Pb co-occurred with Zn throughout the watershed, averaging near a Zn to Cd mass ratio of 150:1; dissolved Cd and Pb had roughly similar instream concentrations.

## Methods

### Rationale for selecting test species

Candidate resident species for testing were targeted based upon the U.S. Environmental Protection Agency (U.S. EPA) eight family minimum diversity guidelines for criteria development (*Table 1*), resident species testing guidance [14], and consultations with Idaho Department of Fish and Game (IDFG) and Idaho Department of Environmental Quality (IDEQ) field biologists, and U.S. EPA water quality criteria experts. The specified eight-families represent groups that are often sensitive to contaminants, but if the eight family diversity requirement cannot be met because a specified family or group is not represented by resident species, then a substitute from a sensitive family represented by one or more resident species should be tested to meet the eight family minimum data set requirement [7]. Our substitution considerations included whether the species were important ecosystem components, socially valued, likely sensitive to metals, were common enough in unpolluted waters to obtain enough specimens for testing, and were robust to survive for several days of holding and testing.

**Table 1.** Comparison of taxa specified for criteria development in U.S. EPA guidelines [67] versus the resident taxa tested

Taxa Specified by U.S. EPA	Order or Family		Resident Organisms Used to Develop Site-Specific Criteria	Code	Tested with:
The family Salmonidae in the class Osteichthyes	Salmonidae		<i>Oncorhynchus clarki lewisi</i> (Westslope Cutthroat Trout)	WCT	Cd, Pb, Zn
			<i>O. mykiss</i> , (Rainbow Trout (surrogate for native Cutthroat Trout))	RT	Cd, Pb, Zn
A second family in the class Osteichthyes	Cottidae		<i>Cottus confusus</i> (Shorthead Sculpin)	SS	Cd, Pb, Zn
A third family in the phylum Chordata	Too scarce to test <sup>a</sup>				
A planktonic crustacean	Not present <sup>a</sup>				
A benthic crustacean	Not present <sup>a</sup>				
An insect	Ephemeroptera	Baetidae	<i>Baetis tricaudatus</i> (Blue-Winged Olive)	Bt	Cd, Pb, Zn
	(mayflies)	Ephemerillidae	<i>Drunella</i> sp. <sup>b</sup> (Green Drake)	Dru	Pb
		Heptageniidae	<i>Epeorus</i> sp. (Yellow Quill)	Epe	Pb
		Heptageniidae	<i>Rhithrogena</i> sp. (March Brown)	Rh	Cd, Pb, Zn
		Leptophlebidae	<i>Paraleptophlebia</i> sp. (Mahogany Dun)	Para	Pb
A family in any order of insect or any phylum not already represented	Gastropoda (snails)	Planorbidae	<i>Gyraulus</i> sp.	Gyr	Cd, Pb, Zn
		Physidae	<i>Physa</i> sp.	Phy	Pb
	Plecoptera (stoneflies)	Chloroperlodidae	<i>Sweltsa</i> sp. (Little Green Stonefly)	Sw	Cd, Pb, Zn
		Trichoptera (caddisflies)	Hydropsychidae	<i>Arctopsyche</i> sp. <sup>c</sup> (Great Gray Spotted Sedge)	Arc
	Diptera (true flies)	Chironomidae	pr. <i>Tanytarsini</i> (Midge) <sup>d</sup>	Chir	Pb
		Chironomidae	<i>Chironomus dilutus</i> (Midge) (surrogate for chronic effects)	C.d.	Pb
		Simuliidae	<i>Simulium</i> sp. (Black Fly)	Sim	Pb
		Tipulidae	<i>Tipula</i> sp. (Crane Fly)	Tip	Pb
	Coleoptera (beetles)	Dytiscidae	Dytiscidae sp.	Dyt	Pb

Notes: Invertebrate common names are not formally designated but are in wide use in North America (e.g., Hafele and Roederer [28]). <sup>a</sup> Not expected to be present in sufficient numbers to be feasible for use as test organisms. <sup>b</sup> Probably *Drunella doddsii* because this was the only *Drunella* species identified in co-located USGS biosurveys (<http://aquatic.biodata.usgs.gov>, site USGS 12413027 “SF Coeur d’Alene River At Shoshone Park nr Mullan, ID”) <sup>c</sup> Likewise, probably was *Arctopsyche grandis*; <sup>d</sup> Likewise, probably was *Tanytarsini* sp.

Westslope cutthroat trout, *Oncorhynchus clarki lewisi*, were of obvious interest as the only native salmonid present in the low-metals, headwaters areas of the watershed and because they are species of conservation concern [15]. Shorthead sculpin, *Cottus confusus*, was the only other native fish species established in the upper watershed, and biomonitoring had indicated sculpin were apparently sensitive to elevated metals and other disturbances [6,16]. The remaining candidate species were all benthic invertebrates. Mayflies, stoneflies, and

caddisflies were particularly targeted because they are abundant in unpolluted forest streams, hold a fundamental intermediate trophic position in stream food webs, and in biomonitoring are sometimes sensitive to metals [17,18]. Baetid mayflies, other mayflies, chironomid midges, and caddisflies are of particular importance as prey items for cutthroat trout and shorthead sculpin [19,20].

Although the resident-species approach to developing criteria does not distinguish between native species and introduced, naturalized species, we focused our efforts on native species. Brook trout, *Salvelinus fontinalis*, and rainbow trout, *O. mykiss*, have become established in the drainage, but they are considered invasive and a threat to the persistence of native cutthroat trout populations [15,21]. We did not attempt to test brook trout. We tested hatchery rainbow trout extensively, but as a surrogate for cutthroat trout, rather than to protect rainbow trout per se.

## Test interpretations

Test responses were interpreted based on effective mortality, defined as loss of equilibrium, immobilization, or death. Insects and snails were considered effectively dead if no leg or foot movements could be seen following gentle prodding. Concentrations affecting 50% or other  $p$ , percentages of the organisms exposed in a test (EC50 or EC $p$ , respectively) were preferentially estimated using a tolerance distribution analysis using a Gaussian distribution, which is equivalent to a 3-factor probit model [22]. When comparing two or more EC50s, values were considered statistically different if the EC50 estimate for one test was outside the estimated 95% confidence limits of the EC50 of the second test. The geometric means of measured concentrations for a treatment were used as exposure concentrations for modeling EC $p$  values. When evaluating time courses of effects, we estimated ET $p$ 's, effect times in hours for  $p$  percent responses, for the first treatment that was higher than the 96-h EC50s, or if <50% effects occurred, the first treatment higher than the EC20. Modeling of ET $p$ 's was similar to that for 96-h EC $p$ 's except that hours were used as the exposure variable, and corresponding responses from a single treatment were the response variable.

The EC50 is nearly universally used as a summary statistic for reporting acute toxicity testing. However by itself, the EC50 statistic is wasteful of experimental information, as information is discarded on the thresholds for effects, time course of toxicity, or steepness of concentration-responses. Further, EC50s are not observable responses but are model fits of data which can be influenced by different statistical choices such as which model to apply, data transformations, corrections for control mortality, censoring, or the use of treatment mean or replicate responses. These influences are seldom transparent to readers. Thus for the tests which had responses, we also provide as supplemental data, full toxicity response matrices of organisms to measured exposure concentrations by replicate and time, including plots of the time course of mortalities, modeling judgments, EC10 and EC20 estimates, and EC $p$  curve fits.

## Range-finding testing of field-collected and hatchery organisms

We conducted pilot testing to work out techniques for holding and testing field collected invertebrates. Pilot testing with the snail *Gyraulus* sp., caddisfly *Arctopsyche* sp., the stonefly *Sweltsa*, and the mayflies, *Baetis* sp. and *Rhithrogena* sp. was conducted by collecting invertebrates from the upper SFCdAR (station SF-km9, Fig. 1) and transporting them and about 4500 L of site water in a refrigerated truck about 600 km to the University of Washington, School of Fisheries, Seattle, WA USA for testing. Most results of the pilot testing are not shown here except for a few test results that showed that some taxa may be highly resistant and which were therefore eliminated from further testing (e.g., with Cd and Zn, *Rhithrogena* and *Sweltsa*).

Following the pilot testing, all further testing was carried out at the Hale Fish Hatchery near Mullan, Idaho. The Hale Hatchery ("SFH-km8") is located at the confluence of the SFCdAR and the Little North Fork of the South Fork Coeur d'Alene River (LNF), about 8 km downstream of the headwaters of the SFCdAR (Fig. 1). The hatchery is located upstream of most mining disturbances. The Hale Hatchery raceways could take water from either the LNF or SFCdAR in a once-through flow design.

We conducted multiple nested rangefinding tests of field collected organisms, and hatchery rainbow and Westslope cutthroat trout in order to find the most sensitive species to focus more definitive testing on.

Invertebrates were collected from the SFCdAR near the Hale Hatchery using kick nets and Surber samplers. Invertebrates were held in 20-L buckets during collection, replacing the water in the buckets every 15-min with new stream water. Invertebrates were taken to the hatchery within an hour of collection for sorting and identification using the taxonomic keys of Merritt and Cummins [23] for insects and Pennak [24] for snails. Rocks from the collection areas and a small amount of woody debris were placed in the holding aquaria as substrate and for food supply. Water movement, water quality, and temperature were maintained with a flow-through system that provided approximately 100 ml of hatchery water per minute. Prior to test seeding, rocks were removed and the invertebrates were fine-sorted by size to sort out the small instars for distribution to the test chambers. Tests with invertebrates were initiated within 48-h of collections.

Resident westslope cutthroat trout and shorthead sculpin were captured for rangefinding tests by electrofishing. Young-of-year (YOY) cutthroat trout fry ranging in length from 20 to 50 mm (~0.1 - 1.1 g) were retained for testing. No YOY sculpin were captured; sculpin retained for testing were 30 to 60mm in length. Field collected fish were held in raceways with water from the LNF at least 72-h and up to 14-d before testing. We also obtained westslope cutthroat trout fry and Kootenai strain rainbow trout fry from the IDFG Sandpoint Hatchery. The cutthroat trout fry were 15 to 35 mm in total length (~0.08 -0.4 g/fish) and rainbow trout from were 20 to 40 mm (~0.1 - 0.5 g) when received. Because the hatchery trout fry had not been reared in site-water, they were acclimated longer than were the field collected fish. Hatchery trout were held in LNF water for 7, 35, and 42-d prior to testing with Zn, Cd, and Pb, respectively. The Sandpoint cutthroat trout experienced about 1 to 2% mortalities per day during holding whereas very few mortalities occurred with the hatchery rainbow or field collected fish. Higher mortality rates occurred with early-life stage cutthroat trout than rainbow trout at the Sandpoint Hatchery, although the fish were free from common diseases of aquaculture (John Thorp, IDFG, Sandpoint, ID, personal communication). In captivity, cutthroat trout may be intrinsically less robust than rainbow trout. While rainbow trout commonly do well in hatchery environments, cutthroat trout growth and survival during early life stages in a hatchery environment has sometimes been reported as inconsistent and nonrepeatable from year to year [25].

Invertebrate tests were conducted in 800-ml polypropylene beakers nested in 20-L aquaria with the test solutions. Four beakers per aquaria were used. Two rectangular panels approximately 6 x 3 cm were cut in each beaker and covered with Nytex screen. The Nytex-covered panels allowed for the exchange of test solution between the beakers within the aquaria. The panels were cut with the bottom at the 100 ml mark of the beaker. This allowed approximately 80% of the test solution to drain from the beaker during renewal and provided enough test solution during the renewal to cover the organisms.

A maximum of eight treatments could be administered to four invertebrate species during a given test. Each aquarium held four beakers placed in 6.5 L of treatment solution, the volume required in the aquaria to fill the individual beakers to the 500-ml level. Two water baths, holding eight aquaria each, were used to control the temperature of the test solutions during the invertebrate tests. There were two replicates, one for each water bath, per treatment. Seven to 10 organisms per replicate beaker were seeded in most exposures, depending on our collection success. Examples of this nested test design are shown in Supplemental Data, Fig. S1.

All collection, transport, holding, testing, and euthanizing or release of fish was done under permit from, and in consultation with the IDFG. We also arranged project support from the Hale Hatchery Manager because of her expertise with fish husbandry, proximity, and familiarity with IDFG requirements. No permits were required for collecting and testing invertebrates.

## **Exposure waters**

Stream water was collected into acid-washed plastic barrels through polyvinyl tubing with a peristaltic pump. Master batches of the treatment concentrations were prepared from reagent-grade chloride salts of Cd, Pb, or Zn dissolved in hatchery water. All water samples for dissolved metals analysis were filtered through a 0.45- $\mu$ m cellulose filter. Metals samples were analyzed by the Idaho Department of Health and Welfare laboratory using the total recoverable metals digestion procedure. Cd and Pb were analyzed using U.S. EPA Method 200.9 (graphite furnace atomic absorption spectrometry) and Zn was analyzed using U.S. EPA Method 200.7 (inductively coupled plasma/atomic emission spectrometry). Method reporting limits were usually 0.2, 3, and 10

µg/L for Cd, Pb, and Zn, respectively. Hardness and alkalinity were measured onsite by titration. Data quality control steps included analyses of all control waters, and field blanks of filtered deionized water were collected and analyzed twice during each concurrent test group.

The test temperatures were moderated by placing the aquaria into a hatchery water bath. All replicate beakers were continuously aerated through pipettes. The aeration also maintained some water velocity, which was needed since zero velocities are unnatural and stressful to stream insects, and impair test performance [26]. Temperature and dissolved oxygen (DO) concentrations were recorded daily in each replicate aquarium; conductivity and pH were recorded daily in one replicate from each treatment. Concentrations of DO were always greater than 60% of saturation, as specified by guidelines for renewal tests [27]; minimum and average DO concentrations were >7 and >8 mg/L, respectively in all tests. Except as noted, water samples for metals were taken at least at test initiation and termination for all treatments and were interpreted as geometric means.

We initially assumed that water hardness is an acceptable surrogate measure of the ionic constituents in water that influence metals toxicity, especially calcium. However, the timing of the present study overlapped with that of major advances with biotic ligand models to predict metals toxicity and we became more appreciative of the importance of major ion and organic carbon data for interpreting toxicity test results. In the 2001 tests, major ions and organic carbon were also measured. However major ions, and to a lesser extent dissolved organic carbon (DOC), have been monitored extensively in the SFCdAR watershed by the U.S. Geological Survey (USGS) (<http://waterdata.usgs.gov>, hydrologic unit code 17010302). Using USGS data, we developed regressions to predict major ions as functions of water hardness or conductivity, and used these equations to estimate major ion concentrations in our test waters.

### **Cutthroat trout broodstock establishment**

Because cutthroat trout were among the most sensitive organisms in the rangefinding testing, a broodstock was established so that we could obtain resident cutthroat trout fry of known age, in sufficient numbers for more definitive testing, and to reduce concerns of local depletions of wild fish. Our collection permit from IDFG allowed only the use of trapping or hook and line to capture the broodstock fish. Trapping was inefficient, but by fly fishing with barbless hooks, 83 adults were captured with 6 mortalities. Most fish were caught using imitations of aerial adult Baetid mayflies and the adult aerial stages of *Arctopsyche* rock case building caddisflies, the Parachute Adams and Elk-Hair Caddis artificial flies, respectively [28]. These insects were included in the species tested. The fish were captured in Big Creek, upper Canyon Creek, and the upper SFCdAR near the Hale Hatchery (Fig. 1). The broodstock adults were artificially spawned from April to June of each year, with the goal of having swim-up stage fry available for testing from July to September. The spawning and incubation procedures followed the standard guide for conducting early life-stage toxicity tests with fishes [29]. Initially the broodstock were held and reared in LNF water, but in July 1999 we transitioned them to SFCdAR for the duration of the project. While the LNF was free from known mining disturbances, we were concerned that the low hardness waters of the LNF (<10 mg/L during spring runoff) were stressful to the broodstock.

After testing, all fish except control resident cutthroat trout were euthanized with the anesthetic tricaine methanesulfonate (MS-222). Resident cutthroat trout that were used in control treatments were retained after testing for potential recruitment into the broodstock. At the conclusion of the project, custody of the native broodstock was transferred back to the IDFG, who released the fish back into the wild at sites within the Coeur d'Alene River basin.

### **Definitive Testing Using Broodstock Cutthroat Trout and Rainbow Trout Surrogates**

Cutthroat and rainbow trout exposures were conducted in 20-L aquaria placed on racks in hatchery raceways. A raceway supplied with temperature-adjusted stream water was used as a waterbath to control the temperature of the test solutions. Swim-up stage cutthroat trout fry reared from the broodstock were exposed to five to seven treatments; a control and increasing metals concentrations, each with three replicates. The exposure volume in the aquaria was 12 L with approximately 80% of the test solution replaced at 48 h. Air was bubbled into each aquarium via a pipette and each aquarium was seeded with eight to ten fish. Tests were initiated between two to four weeks after hatching in order to test the swim-up life fry stage, which we assumed would be the most

sensitive life stage. The average weights of fry used in the acute tests ranged from 0.06 to 0.86 g, giving fish loadings of about 0.05 to 0.7 g/L which were less than the recommended maximum loading density of 0.8 g/L for renewal testing [27].

We also obtained rainbow trout eggs from Mt. Lassen Trout Farm, Red Bluff, California, because the numbers of fish needed for tests of spatial and temporal variability in toxicity exceeded the broodstock production. Rainbow trout were incubated in the same site waters as were the cutthroat trout, using separate raceways. Test methods were the same as for the cutthroat trout except that usually only two replicates per treatment were used because of space limitations.

### **Spatial and temporal variability of toxicity**

The U.S. EPA's guidance for deriving water quality criteria using resident-species advises that "the frequency of testing (e.g., the need for seasonal testing) will be related to the variability of the physical and chemical characteristics of site water as it is expected to affect the biological availability and/or toxicity of the material of interest. As the variability increases, the frequency of testing will increase [7]" Spatially, during periods of stable flow when all water is from ground water, the calcium hardness of the SFCdAR increases markedly from upstream to downstream (*Table 2*), and temporally, hardness can be very low during spring snowmelt and runoff which usually occurs in May and June. Accordingly, we conducted concurrent tests using waters collected from an upstream-downstream gradient during both stable flows and during snowmelt runoff. The tests were conducted simultaneously with water collected on the same day from four different sites with fish randomly distributed across all tests and treatments. The downstream testing was constrained by increasing ambient Cd and Zn concentrations; metals concentrations downstream of site SF-km25 were far too high to use ambient SFCdAR as dilution water. We also conducted tests using water from a tributary with very low hardnesses (EF Pine Creek, *Fig. 1*).

With Cd and Zn, rainbow trout were used to test the spatial and temporal variability of toxicity. With Pb, we also tested cutthroat trout, *Baetis tricaudatus* mayflies, *Simulium* sp. black flies, and *Gyraulus* sp. snails. In total, we conducted 12 sets of concurrent tests with four tests per set using different dilution waters within each set. We interpret spatial and temporal variability of toxicity in the context of hardness-toxicity relations.

### **Metal mixture testing**

In order to define causality, criteria are derived by testing metals individually, but in ambient waters, metals or other contaminants occur as mixtures. When general criteria are derived, the nearly infinite combinations of substances and ratios defy mixture testing. However, for SSC, the relevant combinations of contaminants of concern may be more tractable and the U.S. EPA SSC guidelines call for mixture testing of prospective SSC [7]. We tested the three metals in combinations close to their prospective acute criteria values. Mixture tests were conducted both as metals additions to low-metals waters collected upstream of mining disturbances, and as dilutions of high-metals waters with reference water collected from a similar-sized reference river, the St. Regis River, MT [6].

## **Results**

### **Water chemistry**

Measured exposure concentrations with Cd and Zn were stable between test initiation and termination. In contrast, measured Pb exposures usually declined after test initiation and after renewal. Percent declines during tests ranged from approximately 0 to 50%, with greater declines with increasing alkalinity and treatment concentration. (Supplemental Data, Fig. S2). This is reflected in the poorer solubility of Pb in downstream waters, where the theoretical solubility limits generally declined upstream to downstream as ionic strength of the water increased (*Table 2*). In some of the downstream waters, not enough Pb stayed in solution to produce toxicity. Measured Pb concentrations in 0.45  $\mu\text{m}$  filtered samples and EC50 estimates often greatly exceeded the calculated equilibrium solubility limits (Supplemental Data, Fig S2). This suggests that operational solubility

limits are higher than theoretical solubility limits estimated from the PHREEQ aqueous model, and or most of the Pb present in the 0.45  $\mu\text{m}$  filtered samples was not truly dissolved but is actually in a suspended colloidal form [30,31]. If so, these colloidal form are not completely non-toxic based on measured concentration-dependent responses.

**Table 2.** Aquatic chemistry collected at stable flows from test sites.

Parameter	Sample Type	Units	LNF	SF-km9	SF-km16	SF-km25	SF-km41	SF-km52
Ionic strength	Calculated	mmol/L	0.46	0.76	1.4	2.0	2.8	5.5
pH	field	pH	6.8	7.2	7.2	7.6	7.4	7.5
Organic carbon	unfiltered	mg/L	<1	<1	<1	<1	2.1	2.6
Hardness	calculated	mg/L	14	24	43	50	70	135
Calcium	filtered	mg/L	3.36	6.28	11.7	13.2	18.8	34.0
Magnesium	filtered	mg/L	1.40	1.93	3.37	4.03	5.68	12.1
Sodium	filtered	mg/L	0.55	0.70	1.51	1.32	6	5.6
Potassium	filtered	mg/L	0.33	0.44	0.70	0.66	1.4	1.9
Silica	filtered	mg/L	9.11	8.71	8.10	8.11	4.24	4.87
Bromide	filtered	mg/L	nm	0.08	0.05	0.06	<0.2	<0.2
Chloride	filtered	mg/L	0.3	1.2	3.1	2.3	5.3	4.6
Fluoride	filtered	mg/L	<0.1	0.05	0.05	0.04	<0.1	0.4
Sulfate	filtered	mg/L	2.3	2.47	9.38	33.8	33.5	118
Alkalinity	unfiltered	mg/L	12	20	25	33	54	54
Aluminum	filtered	$\mu\text{g/L}$	6	9	12	10	<20	20
Barium	filtered	$\mu\text{g/L}$	17	27	55	55	66	50
Copper	filtered	$\mu\text{g/L}$	2	1	<1	<1	<3	<3
Iron	filtered	$\mu\text{g/L}$	5	23	21	8	<20	40
Manganese	filtered	$\mu\text{g/L}$	1	6	51	29	34	755
Nickel	filtered	$\mu\text{g/L}$	3	2	8	<1	<10	<10
Cadmium	filtered	$\mu\text{g/L}$	<0.2	<0.2	0.9	1.8	7.8	8.1
Lead	filtered	$\mu\text{g/L}$	<0.2	1.1	3	2.7	2	1
Zinc	filtered	$\mu\text{g/L}$	<2	8	82	126	1230	1850
Solubility limits								
Pb	modeled	$\mu\text{g/L}$	460	260	250	300	210	210

LNF = Little North Fork of the South Fork Coeur d'Alene River, SF-km9 = South Fork Coeur d'Alene River, about 9km downstream of the headwaters, and so on; nm = not measured; filtered = sample filtered through 0.45  $\mu\text{m}$  capsule filters. Samples were collected April 10, 1995, prior to snowmelt runoff. For LNF, USGS sampling with lower detection limits reported 0.13, 0.06 and 1.3  $\mu\text{g/L}$  for Cd, Pb and Zn respectively (sampled once, 22 May 1999). Solubility limits were estimated using the PHREEQ geochemical model [31] and represent saturation for the most abundant complexed species (for Pb, cerrusite,  $\text{PbCO}_3$ ); estimated limits for saturation of Cd and Zn species were >10 mg/L for all samples.

Regression-based estimates of major ions in the SFCdAR and tributaries were very good with  $r^2$  coefficients of determination ranging from 0.68 for chloride predictions to >0.99 for calcium, magnesium, and sulfate predictions (Supplemental Data, Table S1). These regressions produced results that agreed well with validation data sets. With Balistriieri and Blank's [32] independent data from their most lowest ionic strength water (Pine Creek, hardness of 12 mg/L) their measured values, followed in italics by our regression estimates for the same

samples were: Ca 3.3, 3.4; Mg 1.1, 1.0; Na 1.2, 1.0; K 0.5, 0.3; chloride 1.0, 0.4, and sulfate 5.4, 4.2, all in mg/L. At a higher ionic strength site at the bottom of the watershed, SF-km52 with a hardness of 104 mg/L, their measured and our predicted values were respectively: Ca 27.8, 27.4; Mg 8.4, 8.7; Na 3.5, 5.2; K 1.2, 1.5; chloride 2.9, 3.1, and sulfate 80, 79 mg/L. Because aqueous solutions have to be electrically neutral, cation and anion charges have to be balanced, and charge balance differences indicate errors or the presence of unaccounted for substances. For the samples with estimated ionic compositions, 75% of the charge balance differences were <5%, and >99% of the differences were <10% (Supplemental Data, Table S3).

Our organic carbon data were sparse (Table 2). We also measured total and dissolved organic carbon during our pilot testing but dropped it because most values were nondetects and the data were of little interpretive value. However the USGS has collected DOC time series data from the SFCdAR at site SF-km52. Over seasonally varying streamflows, DOC concentrations ranged only from 0.4 to 1.0 mg/L, with a mean of 0.69 mg/L, n=20 (<http://waterdata.usgs.gov>, site 12413470, values from 1999,2000, and 2007). These USGS data were very similar to those of Balistrieri and Blank [32] who independently collected DOC data throughout the SFCdAR. For site SF-km52, their mean DOC value of 0.74 mg/L (n=3) was very similar to that from the USGS time series monitoring. Thus, we used Balistrieri and Blank's [32] average DOC value for the upper watershed (0.6 mg/L) for estimates of DOC in our test waters, except for the EF Pine Creek tests for which we used their Pine Creek mean of 0.2 mg/L.

### Relative sensitivity of fish and invertebrates to Cd, Pb, and Zn

Results of the single-metals toxicity tests are summarized in Table 3. More details including test water characteristics, EC50 confidence limits, and test quality ratings, and are given in Supplemental Data, Tables S2 and Table S3 for single-metals tests and metal mixture tests respectively. Full time and concentration-response matrices are given in Supplemental Data, Table S4. For brevity in the text, tests are simply referred to by test number.

With Cd and Zn, cutthroat and rainbow trout were consistently more sensitive than were the invertebrates. With Cd, the lowest invertebrate EC50 was about 16 µg/L with the mayfly *Baetis tricaudatus* (Table 3). Other invertebrate tests resulted in few deaths at considerably higher exposures, most notably the stonefly *Sweltsa* with 100% survival up to 5239 µg/L Cd. Cutthroat trout, rainbow trout, and shorthead sculpin showed roughly similar sensitivities to Cd with EC50s ranging only from about 0.3 to 1.5 µg/L (Table 3). In matched tests, Cd EC50s were between 100X and 166X lower than Zn EC50s (Table 4).

With Zn, the lowest invertebrate EC50s were about 1400 µg/L or greater for the mayfly *Rhithrogena* sp. and the snail *Gyraulus* sp. (tests 95 and 118). In contrast, EC50s with either cutthroat or rainbow trout were a factor of 10 or more lower than the most sensitive invertebrate results. In two pairs of side-by-side tests with rainbow and cutthroat trout, rainbow trout were more sensitive to Zn. In the first pair, tests 114 and 129, the Zn EC50s for rainbow and cutthroat trout were 69 and 120 µg/L respectively. The second side-by-side, cutthroat and rainbow trout test pair was a dilution test with ambient Canyon Creek water (tests 147 and 148). With the lowest treatment tested, 10% Canyon Creek water, 17% of the cutthroat trout survived to 96 h with progressively declining survival with stronger blends of Canyon Creek water. In contrast, all rainbow trout were dead in all Canyon Creek treatments by 72 h. Median times to death (ET50s) were about 67 h (51-84 h) for cutthroat trout in 10% Canyon Creek water, compared to about 35 h (32-38 h) for rainbow trout (tests 147 and 148).

With Pb, there was considerable overlap between the EC50s for the invertebrates and trout, and greater variability in the results with all species with Pb than with Cd and Zn. This led to more extensive testing of invertebrates with Pb than with Cd and Zn. For instance, during the early rangefinding tests in LNF water, more sensitive results were obtained with *Baetis* mayflies and *Gyraulus* snails than with field collected cutthroat trout fry (tests 29, 30, 63, and 92). In early side-by-side tests, the field collected cutthroat trout fry were much more resistant to Pb than were cutthroat trout fry obtained from the Sandpoint hatchery (tests 91 and 92). However, the lowest Pb EC50s with resident organisms were ultimately obtained with broodstock cutthroat trout fry (tests 80 and 89).

**Table 3.** Summary of single-metal tests with Cd, Pb and Zn. See Table S-3 for dilution water chemistry, fish sizes, and test quality, and Table S-5 for matrices of concentration-responses over time.

Test	Species	Dilution water		Hard. (mg/L)	EC50 (µg/L)	Test	Species	Dilution water		Hard. (mg/L)	EC50 (µg/L)
		source						source			
Cadmium tests:						Lead tests (continued)					
1	Cd	Arc	SF-km9	28.	>458.	46	Pb	Rh	LNF	15.	>737.
2	Cd	Bt	SF-km9	24.	>444.	47	Pb	Rh	LNF	18.	>985.
3	Cd	Bt	SF-km25	59.	16.	48	Pb	Rh	LNF	19.	>166.
4	Cd	Bt	LNF	21.	74.	49	Pb	Chir	SF-km9	39.	>1035.
5	Cd	Rh	SF-km9	25.	157.	50	Pb	Chir	LNF	22.	>1255.
6	Cd	Rh	SF-km25	57.	85.	51	Pb	C.d.	SFH-km8	32.	1955.
7	Cd	Rh	LNF	21.	>50.	52	Pb	C.d.	SFH-km8	32.	3617.
8	Cd	RT	LNF	21.	0.8	53	Pb	RT	LNF	20.	138.
9	Cd	RT	LNF	7.	0.48	54	Pb	RT	SFH-km8	31.5	127.
10	Cd	RT	SF-km9	13.	0.99	55	Pb	RT	SFH-km8	32.	160.
11	Cd	RT	SF-km16	24.	1.3	56	Pb	RT	SFH-km8	19.	591.
12	Cd	RT	SF-km25	30.	< 2.9	57	Pb	RT	SF-km16	25.	631.
13	Cd	RT	SFH-km8	32.	0.93	58	Pb	RT	SF-km22	32.	916.
14	Cd	RT	SFH-km8	28.5	0.83	59	Pb	RT	SF-km25	33.5	969.
15	Cd	RT, K	LNF	21.	0.34	60	Pb	RT	SFH-km8	28.5	>98.
16	Cd	SS	LNF	21.	0.93	61	Pb	RT, K	LNF	21.	180.
17	Cd	Gyr	SF-km9	24.	>455.	62	Pb	SS	LNF	21.	>855.
18	Cd	Gyr	LNF	21.	>73.	63	Pb	Gyr	LNF	18.	544.
19	Cd	Sw	SF-km9	26.	>5239.	64	Pb	Gyr	LNF	20.	537.
20	Cd	WCT	SFH-km8	32.	1.51	65	Pb	Gyr	LNF	19.	380.
21	Cd	WCT	SFH-km8	30.5	1.2	66	Pb	Gyr	LNF	22.	796.
22	Cd	WCT,S	LNF	21.	0.35	67	Pb	Gyr	SF-km9	39.	981.
23	Cd	WCT,F	LNF	21.	0.94	68	Pb	Gyr	SF-km16	67.	>952.
Lead tests:						69	Pb	Gyr	SF-km25	84.	>683.
24	Pb	Sim	LNF	22.	415.	70	Pb	Gyr	LNF	13.	644.
25	Pb	Sim	SF-km9	39.	961.	71	Pb	Gyr	SF-km9	19.	>1035.
26	Pb	Arc	LNF	22.	>1255.	72	Pb	Gyr	SF-km16	33.	>952.
27	Pb	Tip	SF-km9	39.	>1035.	73	Pb	Gyr	SF-km25	41.	>683.
28	Pb	Dyt	SF-km9	39.	>1035.	74	Pb	Phy	LNF	22.	1159.
29	Pb	Bt	LNF	15.	592.	75	Pb	Sw	LNF	15.	>737.
30	Pb	Bt	LNF	18.	752.	76	Pb	Sw	LNF	19.5	253.
31	Pb	Bt	LNF	20.	664.	77	Pb	Sw	LNF	17.	>494.
32	Pb	Bt	LNF	22.	426.	78	Pb	WCT	SFH-km8	32.	>123.
33	Pb	Bt	SF-km9	39.	1002.	79	Pb	WCT	SFH-km8	31.5	>54.
34	Pb	Bt	SF-km16	67.	>952.	80	Pb	WCT	SFH-km8	32.	215.
35	Pb	Bt	SF-km25	84.	>683.	81	Pb	WCT	SFH-km8	30.5	>72.
36	Pb	Bt	LNF	17.	>494.	82	Pb	WCT	SFH-km8	31.5	362.
37	Pb	Bt	LNF	11.	322.	83	Pb	WCT	SF-km16	56.	487.
38	Pb	Bt	LNF	13.	511.	84	Pb	WCT	SF-km22	68.	>414.
39	Pb	Bt	SF-km9	19.	640.	85	Pb	WCT	SF-km25	72.5	>409.
40	Pb	Bt	SF-km16	33.	>952.	86	Pb	WCT	SF-km16	62.5	>153.
41	Pb	Bt	SF-km25	41.	<1250.	87	Pb	WCT	SF-km16	62.5	>197.
42	Pb	Dru	LNF	19.5	>267.	88	Pb	WCT	EFPC	12.3	67.
43	Pb	Epe	LNF	17.	>494.	89	Pb	WCT	EFPC	11.4	47.
44	Pb	Epe	LNF	11.	>346.	90	Pb	WCT	St.R	40.5	>387.
45	Pb	Para	LNF	11.	>346.	91	Pb	WCT,S	LNF	21.	127.
						92	Pb	WCT,F	LNF	21.	>855.

Table 3. (continued)

Test	Species		Dilution			Test	Species		Dilution		
			water source	Hard. (mg/L)	EC50 ( $\mu\text{g/L}$ )				water source	Hard. (mg/L)	EC50 ( $\mu\text{g/L}$ )
97	Zn	RT	LNF	7.	20.	114	Zn	RT, K	LNF	18.	69.
98	Zn	RT	SF-km9	10.	37.	115	Zn	SS	LNF	18.	>1068.
99	Zn	RT	SF-km16	16.	99.	116	Zn	SS	SFH-km8	38.5	300.
100	Zn	RT	SF-km25	24.	131.	117	Zn	Gyr	SF-km25	62.4	3292.
101	Zn	RT	SFH-km8	22.5	77.	118	Zn	Gyr	LNF	14.	1400.
102	Zn	RT	SF-km16	29.	102.	119	Zn	Gyr	LNF	18.	1451.
103	Zn	RT	SF-km22	40.	174.	120	Zn	Sw	LNF	18.	>1526.
104	Zn	RT	SF-km25	41.	176.	121	Zn	WCT	SFH-km8	32.	>275.
105	Zn	RT	SFH-km8	30.	175.	122	Zn	WCT	SFH-km8	31.5	208.
106	Zn	RT	SF-km16	42.	199.	123	Zn	WCT	SFH-km8	30.5	196.
107	Zn	RT	SF-km22	51.	279.	124	Zn	WCT	SF-km16	62.5	>186.
108	Zn	RT	SF-km25	55.	289.	125	Zn	WCT	SF-km16	62.5	283.
109	Zn	RT	SFH-km8	28.5	83.	126	Zn	WCT	SFH-km8	38.5	280.
110	Zn	RT	SFH-km8	35.	49.	127	Zn	WCT	EFPC	12.3	93.
111	Zn	RT	SF-km16	66.5	111.	128	Zn	WCT	EFPC	11.4	76.
112	Zn	RT	SF-km22	73.	138.	129	Zn	WCT,S	LNF	18.	120.
113	Zn	RT	SF-km25	75.	138.	130	Zn	WCT,F	LNF	18.	309.

Species codes from Table 1 except RT,K - Kootenai strain rainbow trout young-of-year obtained from the Idaho Department of Fish and Game Hatchery Sandpoint, Idaho; WCT,F and WCT,S - young-of-year cutthroat trout that were field collected or obtained from the Sandpoint hatchery respectively. Hard. - water hardness as  $\text{CaCO}_3$

Two pairs of side-by-side cutthroat and rainbow trout tests yielded conflicting results whether which was more sensitive to Pb. In paired tests 61 and 91 with Sandpoint hatchery cutthroat and rainbow trout fry, the cutthroat trout appeared more sensitive than rainbows with EC50s of 180 and 124  $\mu\text{g/L}$  respectively. Yet in paired tests 54 and 80, with Mt. Lassen rainbow trout and SFCdAR broodstock cutthroat trout, the rainbow trout were more sensitive with EC50s of 127 and 215  $\mu\text{g/L}$  respectively. Thus, while in paired tests, rainbow trout were more sensitive to Zn than cutthroat trout, with Pb, results were indeterminate. As follows, the sensitivity of both swim-up stage cutthroat and rainbow trout to Pb and Zn varied with the size of the tested fish, further complicating conclusions of relative sensitivity.

### Hardness-toxicity relations

We generalized the spatial and temporal variability of toxicity with differences in water hardness, which increased from headwaters downstream (Table 2), and which decreased during spring snowmelt. In our tests that were conducted simultaneously with waters from different locations using similar sized fish, there were always strong relations between water hardness and EC50s for Cd, Pb and Zn, with  $r^2$  coefficients of determination of 0.89 to 0.99. However, if the rainbow trout tests were pooled across these groups, with fish sizes ranging from about 0.1 to 0.5g, the relationships were much poorer with  $r^2$  values of 0.04 to 0.38. The slopes of the  $\log(\text{hardness})$  versus  $\log(\text{EC50s})$  were shallower in the pooled regressions (Fig. 2; Supplemental Data Table S5). With cutthroat trout, which ranged in size from about 0.2 to 0.7 g, these differences were less pronounced than for rainbow trout. These differences in response patterns with rainbow trout that were otherwise similarly acclimated and tested in similar waters suggested that differences in sensitivity were attributable to fish size.

**Table 4.** Relative toxicity of Cd and Zn in matched tests. ET<sub>p</sub> – time to cause p-% effects for a given concentration. The concentrations used for ET<sub>p</sub> calculations higher than the EC<sub>p</sub> Chronic test pair is from [13]

Description (fish size tested and dilution water source)	Test no.		EC <sub>p</sub> (µg/L)	95th percentile CIs	Ratio, Zn:Cd EC <sub>p</sub> s	ET <sub>p</sub> (h)	Conc. for ET <sub>p</sub> (µg/L)
<u>Cd and Zn, test pair #1</u>			<u>EC50</u>			<u>ET50</u>	
Rainbow trout	14	Cd	0.83	(0.72-1)		66 (58-74)	1.06
0.36g, SFH-km8, 26Jul2000	109	Zn	83	(71-97)	100	35 (16-53)	133
<u>Cd and Zn test pair #2</u>			<u>EC20</u>			<u>ET20</u>	
Cutthroat trout	20	Cd	0.98	(0.85-1.3)		62 (42-83)	1.47
0.34g, SFH-km8, 26Aug1999	121	Zn	120	(96-149)	122	86 (68-105)	275
<u>Cd and Zn, test pair #3</u>			<u>EC50</u>			<u>ET50</u>	
Cutthroat trout	21	Cd	1.20	(1.05- 1.4)		32 (26-37)	2.1
0.18g, SFH-km8, 09Aug2000	123	Zn	196	(171-225)	166	28 (17-38)	258
<u>Cd and Zn, chronic test pair</u>			<u>EC20</u>				
Rainbow trout [13]		Cd	1.2	(0.9-1.5)	123	not calculated	
Eggs+69d, LNF, 1997		Zn	147	(96-223)			

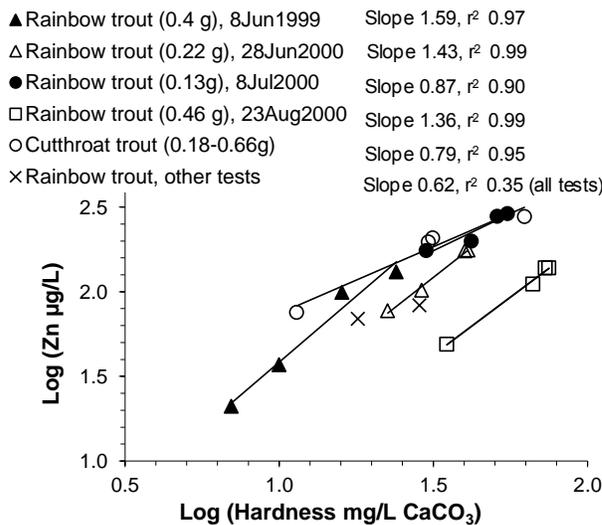
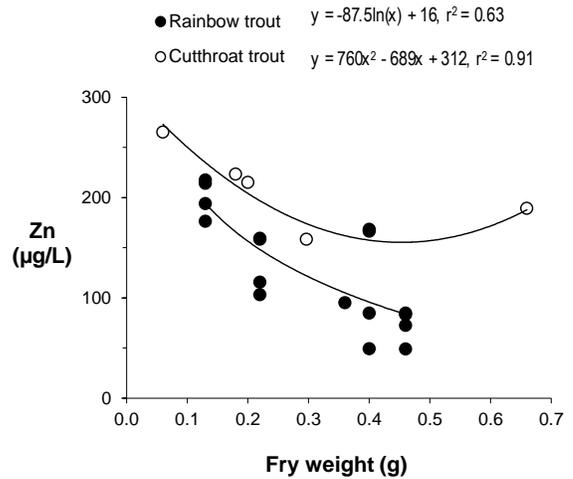
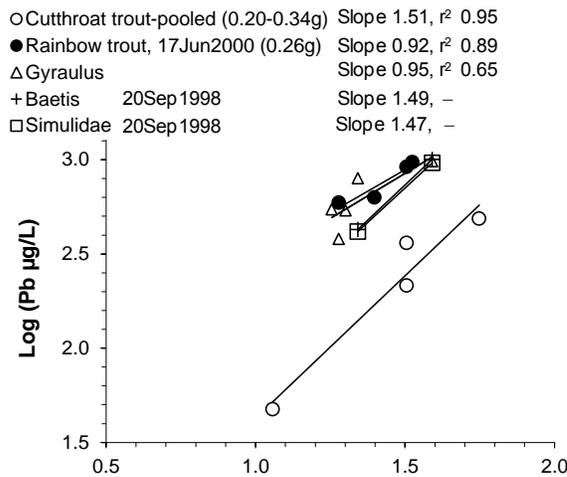
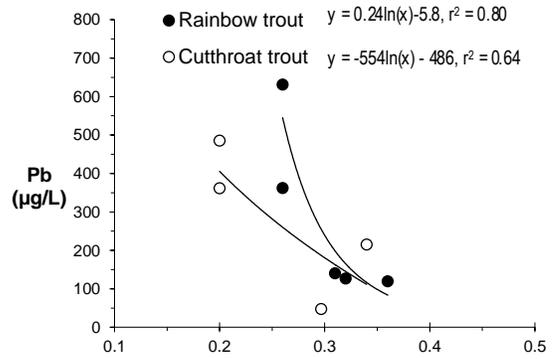
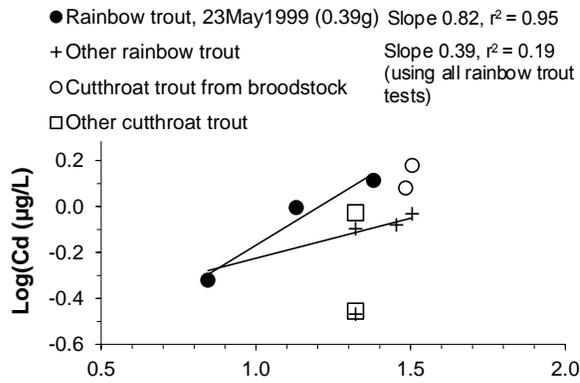
### Size-dependent sensitivity of trout fry to Pb and Zn

The distinct hardness-toxicity patterns within concurrent test groups, particularly with Zn and rainbow trout, show that the trout differed in Zn sensitivity over time (*Fig. 2*). After adjusting the Zn EC50s for differences attributable to hardness using regression no. 20 from Supplemental Data, Table S5, and plotting the adjusted EC50s against the size of the fish, a general pattern of decreasing EC50s (increasing sensitivity) with increasing size appears (*Fig. 3*). The largest rainbow trout tested, about 0.5 g, appear to be the most sensitive size tested. Cutthroat trout and Zn show a similar pattern, with one test suggesting that larger fish near 0.7 g may start becoming more resistant. With Pb, the data hint at a similar pattern of decreasing resistance with increasing size of the fry, although we have fewer data and the results are more scattered than for Zn. With Cd, the differences in sizes were only about 0.1 g per species which was insufficient to make meaningful comparisons.

In addition to these comparisons of EC50s across fish sizes, tests with no- or low-effects also showed that within the swim-up fry life stage, sensitivity to Pb increased with increasing fish size up to at least 0.5 g. Tests 80 and 82 with cutthroat trout differed little except for the size of fish tested. Both used SFH-km8 dilution water with a hardness of 32 mg/L. With the 0.34 g fish in test 80, 100% mortality occurred in the 329 µg/L treatment, whereas with the 0.20 g fish in test 82, only 50% mortality occurred in the 362 µg/L treatment. The largest fish tested with Pb, 0.78 g in test 90, with 100% survival at up to 387 µg/L Pb in hardness 41 mg/L water, were more resistant than the ~0.3 g fish.

### Metal mixture toxicity

The differences in the toxicity of metals in mixtures compared to single metals tests were complex. Zinc was less toxic in tests in which Zn was the primary toxicant and Cd or Pb were also present compared to Zn alone. The strongest evidence of this was from a set of tests conducted over a two-week period with similar sized cutthroat trout, in similar dilution waters with a hardness of 65 mg/L. In this test series, Zn toxicity decreased when tested in the presence of Cd and or Pb, each added at nearly constant concentrations at about half of their expected EC50s (*Table 5*).



**Figure 3** Patterns between mean wet weight of swim-up stage cutthroat and rainbow trout fry and Pb and Zn EC50s, toxicity values normalized to a hardness of 35 mg/L using slopes no. 12 and no. 20 respectively from Supplemental Data, Table S5.

**Figure 2.** Hardness-toxicity relations for Cd (top), Pb (middle) and Zn (bottom), grouping results by concurrent test groups where available. Relations were stronger in concurrent tests.

Decreased Zn toxicity was also suggested from two simultaneous exposures of rainbow trout to Zn alone or to a Zn+Cd mixture in very-soft dilution waters (hardness 10-11 mg/L, tests 98 and 143). In test 98, a Zn exposure of 134 µg/L caused slightly higher mortalities than did a Zn+Cd exposure to 522 µg/L Zn and 4.3 µg/L Cd in test 143. In the Zn-alone test, a 134-mg/L exposure resulted in an ET50 of 70 h (66–74 h) and 95% mortality at 96 h. These responses were similar to the responses from the much higher Zn+Cd exposures of test 143, with an ET50 of 71 h (68-73 h) and 85% mortality at 96 h.

In contrast to the mixture tests in which Zn was the primary toxicant and mortalities decreased with the addition of sublethal Pb (tests 125 and 136), when Pb was the primary toxicant and Zn was added in sublethal concentrations mortalities were marginally more toxic than was Pb alone at similar concentrations (Table 5).

**Table 5.** Toxicity of Zn and Pb singly and in mixtures in closely matched tests. EC20s were estimated for the primary toxicants. The secondary toxicants were added at about 0.5X their expected EC50s.

Series	EC20s (CI)	Test no.	Series	EC20s (CI)	Test no.
Cutthroat trout, 0.65g, hardness 65 mg/L, Sep 12 to 21, 2000	Zn (µg/L)		Cutthroat trout, 0.30g, hardness 11-13 mg/L, Sep 6, 2001	Pb (µg/L)	
Zn	154 (127-186)	125	Pb	38 (34-41)	89
Zn+Pb	251 (210-292)	136	Pb+Zn	23 (15-36)	139
Zn+Cd	368 (304-433)	137			
Zn+Cd+Pb	328 (298-358)	138			

Not all tests resulted in >50% effects so comparisons use EC20s.

## Discussion

### Relative sensitivity of fish and invertebrates to Cd, Pb, and Zn

For Cd and Zn, there were differences in sensitivity among the invertebrates. However, our EC50s with benthic invertebrates were >10X higher than those for trout and we questioned whether the benthic invertebrates were truly that resistant, or whether our 96-h water-only tests with field collected organisms were simply insensitive. Four explanations, which are not mutually exclusive, seem plausible. First, perhaps common benthic stream invertebrates truly are much less sensitive to Cd and Zn than are fish, and perhaps literature reporting that benthic invertebrates are sensitive to “metals” has been overbroad and should have been more specific to copper (Cu). Clements [17] found that effects on heptageniid mayflies from Zn or Cd+Zn mixtures were much less severe than when Cu was included in the mixture. In our study area, diversities and abundances of benthic invertebrates declined with increasing Cd and Zn concentrations that were about a factor of 10 or more lower than invertebrate EC50s or no-effect concentrations in our 96 h exposures. However, taxa that have been considered to be metals sensitive were still reasonably abundant at all but the most severely polluted sites [33]. Second, benthic stream invertebrates have been shown to have strongly size-dependent sensitivity to Cd-dominated metals mixtures, with the smallest individuals being most sensitive. Kiffney and Clements [34] tested early instars too small to be identified by eye by naturally colonizing rock trays and then exposing the colonized trays to metals in artificial streams. Which insects were actually exposed and affected was inferred after the fact based on deviation from reference samples. In contrast, our approach of field collecting, hand-picking and identifying individuals without injury prior to testing likely biased our testing toward larger, older instars that were less sensitive than the earliest instars. In testing of field collected invertebrates in an approach similar to ours, mayflies that have been considered metals-sensitive in field studies were only affected by Cd, Cu, and Zn at environmentally exorbitant concentrations [35,36]. Yet there have been sensitive results reported from short-term tests with field-collected mayflies and Cu [37]. Third, perhaps 96-hour water exposures are simply too short to be strongly correlated with effects from indefinite exposures in the wild. For instance, we observed sublethal responses with the mayfly

*Baetis tricaudatus* to Pb in 10-day exposures that were nearly as sensitive as sublethal responses by rainbow trout in 60-d exposures. In contrast, 96-h EC50s were about six times higher for the mayfly than for rainbow trout [13]. With Cd, Buchwalter et al. [38] found that some aquatic insects take months to reach steady state tissue burdens, while the durations of most toxicity tests with aquatic insects are only a few days to weeks [but see 39]. Fourth, diet may be a more important metal source to grazing benthic invertebrates than water [40-42].

Our conclusion that rainbow trout are more sensitive than westslope cutthroat trout to Zn is consistent with results obtained with other cutthroat trout subspecies. In side-by-side tests with rainbow trout and Colorado River cutthroat trout, *O. clarki pleuriticus*, Brinkman and Hansen [43] also found that rainbow trout were consistently more sensitive to Zn. Two other cutthroat trout subspecies (greenback, *O. clarki stomias*, and Rio Grande, *O. clarki virginalis*) were at least as sensitive as the Colorado River cutthroat trout [36], which together support a generalization that cutthroat trout are more resistant to Zn than rainbow trout.

The variability in results from repeated tests with the same species was striking. For instance, the lowest Pb EC50 for an invertebrate was obtained with the stonefly *Sweltsa* (test 76), and this EC50 (253 µg/L) was in the range of results obtained with cutthroat trout (215 to 362 µg/L). Yet in two other tests (tests 75 and 77), few mortalities to *Sweltsa* resulted at Pb concentrations up to three times greater. With Cd, *Sweltsa* was extremely resistant and we were unable to kill any at Cd concentrations four orders of magnitude higher than EC50s for cutthroat trout (tests 19-23). With fish, we think much of this variability is attributable to differences in the size of the swim-up fry (discussed later). Except for the *Baetis* mayflies, the invertebrate taxa we tested probably have annual or longer life cycles, so tests conducted at different times of the year, likely tested different developmental stages with different sensitivities [23,24]. For instance, Clark and Clements [44] found that in field experiments in a stream with Cd, Cu, and Zn blends, significant mortalities occurred with the mayfly *Rhithrogena hageni* during summer when mayfly populations were dominated by small, early instars. However, no significant mortality was observed during spring when organisms were larger [44]. In the present study, no seasonal patterns in the in EC50 differences were obvious (Supplemental Data, Table S2).

*Relative sensitivity of sculpin and cutthroat trout:* The initial rangefinding tests with field collected fish indicated that with Cd, the sensitivities of resident cutthroat trout and shorthead sculpin were approximately equal (Table 3, tests 16 and 23). However with Zn, cutthroat trout were much more sensitive than were sculpin (tests 115 and 129), and with Pb, neither cutthroats or sculpins suffered any mortalities when exposed to environmentally exorbitant concentrations (tests 62 and 92). However, while the field-collected cutthroat trout were YOY fry, the shorthead sculpin were 30 to 60 mm in length, which were probably yearling to two-year old fish based on length-at-age compilations for the species [16]. Because Woodling et al. [45] found that the closely related mottled sculpin, *Cottus bairdi*, could be much more sensitive to Zn than were the shorthead sculpin in our test no. 115, we conducted a second nested Zn test pair with field collected YOY sculpin that were probably newly hatched (8-12 mm in length, 0.02 g average wt., Supplemental Data, Fig. S1) and broodstock early swim-up cutthroat trout fry (tests 116 and 125). While the results of this second test pair were not definitive because of high control mortalities (30% with both), the test pair did suggest both that the newly hatched shorthead sculpin were much more sensitive to Zn than were the yearling or older sculpin used in test 115, and that the sensitivities to Zn of newly hatched sculpin and cutthroat trout were similar. This conclusion is congruent with results from two other comparative studies. First, in tests with three cutthroat trout subspecies and field collected YOY mottled sculpin, the cutthroat and sculpin Zn EC50s overlapped [36]. Second, with mottled sculpin tested by Besser et al. [46] with Cd, Cu, and Zn, the newly hatched sculpin were consistently more sensitive than older, larger juveniles. Yearling sculpin were very resistant to Cd, Cu, and Zn. In those tests with the newly hatched mottled sculpins, toxicity values were similar to or lower than the most sensitive rainbow trout tested [46]. Our results with Cd differ from those of Besser et al. [46] in that their yearling mottled sculpin were much more resistant to Cd compared to earlier life stages and to rainbow trout fry. In our testing, the yearling or older sculpin tested were as sensitive as were the cutthroat or rainbow trout fry (Table 3).

### Relative toxicity of Zn and Cd in simultaneous tests

Several tests were conducted simultaneously with Cd and Zn, which provides direct comparisons of relative toxicities, avoiding the uncertainties of species mean acute values, which often involve extrapolations across tests

or studies with different test waters. These direct comparisons are useful for screening relative risks from different metals in ambient environments, although mixture interactions can also be important (discussed later). In particular, Zn and Cd are often associated in mining-influenced watershed [2,18,32,47,48]. In the SFCdAR, ambient Zn and Cd concentrations are high enough to be acutely toxic (Supplemental Data, Table S3), and occur in mass ratios averaging about 150:1, ranging from at least 70:1 to about 230:1 (Table 2). While we did not attempt a thorough review, these Zn:Cd mass ratios were somewhat lower than others we noted. In watersheds in the Rocky Mountains in Colorado, USA, Zn:Cd ratios were consistently about 213:1 [48]; streams in southwestern Montana, USA averaged 205:1 (range 147-271) [47]; and in rivers in northern Honshu, Japan, Zn:Cd ratios averaged 167:1 (range 95-600) [18]. Unlike Zn and Cd, Pb occurs primarily in particulate, solid phases rather than the dissolved phase [2] and under stable river flows, dissolved Pb concentrations in the SFCdAR water column are >100X lower than those causing acute toxicity (Tables 2 and 3). Thus, Zn to Cd EC50 ratios in well matched tests could be useful for interpreting which metal is the cause of toxicity in ambient waters.

The ratios of Zn to Cd ECps in three pairs of side-by-side acute tests, and in one pair of previously reported 69-d, side-by-side chronic exposures, ranged from about 100:1 to 166:1 (Table 4). The ratios were greater with cutthroat trout, because they were similarly sensitive to Cd as were rainbow trout, but cutthroat trout were less sensitive to Zn than rainbow trout. These ratios were mostly greater (i.e., Zn was relatively less toxic) than those from other simultaneous Zn and Cd tests that we reviewed. Chapman [49] obtained Zn:Cd LC50 ratios of 72:1 and 54:1 with rainbow trout and Chinook salmon swim-up fry, respectively. With mottled sculpin in multiple tests, Besser et al. [46] obtained matched Zn:Cd EC50 ratios ranging from 14:1 to 249:1, and with rainbow trout from 34:1 to 98:1. Besser et al.'s [46] tests suggested Zn was faster acting than Cd because Cd was more toxic relative to Zn in chronic tests than in comparable 96-h tests, with chronic Zn:Cd EC50 ratios ranging from 40:1 to 249:1.

Other studies [e.g., 50] have found that mortalities caused by Cd have had a slower onset than Zn mortalities. If it were generally the case that Zn effects occurred sooner than Cd effects, that could provide a clue to which metal was the primary cause of toxicity in mixtures. However, the time courses of mortality in our matched Zn and Cd tests were quite variable. There were marked differences in the timing of the onset of effects in matched Cd and Zn exposures, however the direction of the differences was not consistent (Table 4). With rainbow trout in paired tests 14 and 109, effects from Zn occurred sooner than from Cd; with cutthroat trout in paired tests 21 and 123, effects from Zn occurred at similar exposure durations as with Cd, but with cutthroat trout in paired tests 20 and 121, effects from Zn occurred later than did those from Cd. Thus, from these three concurrent Cd and Zn test pairs, there was little support for generally assuming that at concentrations near their 96-h EC50s, deaths from Zn exposures were more likely to occur faster than from Cd.

## Hardness-toxicity relations

For organisms reared in site water, we provided no further acclimation before testing in site waters. However, in the spatial variability testing, each of the 12 sets of tests included one test in which the rearing and test waters were the same (LNF or SFH-km8) and three tests from downstream waters with progressively increasing water hardness. Acclimating organisms to these higher hardness, downstream waters was infeasible because Cd and Zn likewise progressively increased in downstream waters (Table 2). By rearing the organisms in lower hardness water than they were tested in, we believe that the most likely influence was that we low-biased our downstream EC50s slightly. If so, the slopes of the log(hardness) versus log(toxicity) regressions would in turn also be biased low. This thinking is based on two studies of Zn uptake or toxicity in which acclimation in low hardness waters were followed by Zn exposures in higher hardnesses waters (“low-high” scenarios), versus both acclimation and exposures occurring in the higher hardness waters (“high-high” scenarios). First, Barron and Albeke [51] found that rainbow trout accumulated more Zn after a “low-high” hardness exposure scenario than after a “high-high” hardness exposure scenario. Second, we hatched and reared rainbow trout in the very-low and higher hardness waters of the LNF and SFH-km8 respectively, and then tested the responses of both groups to a Cd+Zn mixture in the higher hardness SFH-km8 water. The fish were about twice as resistant in the “high-high” hardness acclimation and toxicity test scenario than in the “low-high” scenario [52]. A “low-high” hardness acclimation pattern is congruent with the natural history of cutthroat trout. Cutthroat trout usually spawn in small,

headwater, low-hardness tributaries of rivers and fry tend to remain in tributaries through their first summer, before moving into larger, higher-hardness, downstream waters with the onset of winter [e.g., 21].

We think the influence of the potential bias introduced by our “low-high” acclimation and testing was probably slight because of comparisons between the slopes of hardness-toxicity regressions we obtained were similar to those obtained from studies which were able to acclimate and test fish in identical hardness water. With Zn, the log(hardness) versus log(toxicity) slopes from our four concurrent series with rainbow trout ranged from 0.87 to 1.59. These are similar to slopes of 0.96 to 1.53 obtained by Brinkman and Hansen [43] in concurrent testing with cutthroat and rainbow trout respectively. With Cd, the slope of 0.82 from our sole concurrent series (Fig. 2) was somewhat lower than slopes from other test series with salmonids (1.0 to 1.5) [53].

While the spatial and temporal variability testing mostly produced expected patterns, the exceptions were curious. The Zn test series with rainbow trout in the softest waters tested (hardness 7-24 mg/L) had unusual concentration-response reversals with one mid or high treatment per test having higher than expected survival, but otherwise having an expected concentration response (Table S4, tests 97, 98, 100). While initially we suspected a mix-up, careful inspection of the raw data convinced us that the responses were real. In each instance, the unexpected reversals were observed across replicates, suggesting that this was more than random variability. No similar reversals were observed in a Cd test series conducted shortly before the Zn series in a similar range of very low hardness water (tests 9-12). While we are unable to explain these unexpected response reversals, the fact that they were only observed during Zn exposures in extremely soft water is intriguing because osmoregulatory stress from low ionic strength water and from Zn covaries, with complex physiological and compensation responses by fish [54].

If criteria are dependent on environmental factors affecting toxicity such as empirical hardness-toxicity relations with metals, the representativeness of the relations to diverse waters and organisms and strength of the relations are fundamental [5]. When developing general criteria, in order to obtain enough data across a wide enough range of water hardnesses to work with, data sometimes must be compiled across studies. In the present study, even though our results for each species were obtained with organisms from the same culture or field site, using the same testing facility, and the tests were conducted by the same people, the variability of EC50s within a species was large, ranging up to a factor of 5 or more (Fig. 2, Table 3). Factors such as differences in sensitivity between species or even between test groups of the same species introduce unwanted variability into and thus dilute the hardness-toxicity regressions. These factors cannot be treated as a continuous variable but can be incorporated into multiple linear regression by using a binary or dummy variable. This essentially blends regression and analysis of variance into an analysis of covariance or ANCOVA [55]. When the hardness-toxicity slopes were pooled through ANCOVA using species as a grouping variable, the overall hardness-acute toxicity slopes for all species tended to be considerably less than 1.0. In contrast, when we used concurrent tests groups as the grouping variables in ANCOVA, hardness-acute toxicity slopes were steeper and strengths of regressions stronger (Fig 2, Table S5). The differences were largest with rainbow trout and Zn. With cutthroat trout and Zn, the differences were less apparent with a hardness-toxicity slope of 0.79 and an  $r^2$  of 0.95 obtained from tests conducted at different times with different sized fish. Whether this reflects real differences between these closely related species or happenstance is uncertain, but the high  $r^2$  value supports pooling the cutthroat trout data. With Pb, for insects and rainbow trout the hardness-toxicity relations were all similar to each other and higher in the concurrent tests than if pooled across tests, but there was little difference for cutthroat trout or snails. With the cutthroat trout, the range in hardness, 11 to 56 mg/L, may have been large enough to overcome some of the variability from using different sized fish. With snails, we are aware of only one study relevant to the variability introduced by testing mixed age, field collected snails. With Cu, juvenile springsnails, *Pyrgulopsis robusta*, 5 to 7 weeks post-hatch, were only slightly more sensitive than mixed age, field collected snails with 28-day survival EC20s varying only by a factor of 1.6 [56]. These results and ours, suggest that size-sensitivity patterns with at least some snails may be less pronounced than with insects or fish.

The strong regressions and reasonably consistent slopes with hardness and toxicity among concurrent tests are consistent with the role of Ca competition reducing of Cd and Zn toxicity to fish and invertebrates [53,57,58]. With Pb, studies of Pb toxicity in relation to specific ions in water have produced conflicting results for different organisms with Ca an important factor mitigating toxicity to fish, yet calcium had little influence on Pb toxicity to an invertebrate compared to alkalinity or ionic strength [59,60]. In our natural waters, Ca, ionic strength,

hardness, and alkalinity were all correlated (Table 2; Table S1), and Pb toxicity was predicted well from hardness in these low DOC waters, even though hardness may not necessarily be directly related to the actual mechanisms of toxicity.

### **Size-dependent sensitivity of trout fry to metals**

Our results show that the general idea that smaller, juvenile organisms are likely to be more vulnerable to chemical stress than larger, older life stages of the same species [e.g., 27] is not always a reliable assumption. Instead, swim-up cutthroat and rainbow trout fry tended to be more vulnerable to Pb and Zn with increasing size up to approximately 0.5 g wet weight. Our results are not unique. All size-sensitivity comparisons located for juvenile *Oncorhynchus* trout and salmon species in the 0.1 to 0.5 g size range showed increasing sensitivity to Cd, Cu, Pb, or Zn with increasing size. The most comprehensive were Hedtke et al.'s [61] weekly tests with coho salmon (*O. kisutch*) across developmental stages from hatching (alevins), through the brief swim-up transition to free swimming and successful foraging, ending with juveniles up to 175 days post-hatch. Resistance to Zn generally increased as fish progressed from the alevin to older juveniles stages, but when same age fish (56 day olds, late alevin to swim-up stage) of different sizes were tested, the larger fish were distinctly less resistant. With Cu and Zn, the least resistant fish were about 0.7-0.8 g (all as wet weight) [61]. Chapman [62] found in weekly tests that coho salmon steadily decreased in resistance to Cd, Cu, Pb, and Zn from 14 to 49 d post hatch (~0.15 to 0.5 g). Paired tests by Hansen et al. [63] with rainbow trout and bull trout, *Salvelinus confluentus*, exposed to Cd and Zn showed that rainbow trout tended to become more sensitive with increasing size from 0.4 to 0.9 g with Zn, and from 0.26 to 0.66 g with Cd. Further growth in juvenile rainbow up to 1.1 and 1.6 g for Cd and Zn had little effect on sensitivity. With bull trout and Cd, size had little effect on sensitivity over a narrow size range of 0.08 to 0.22 g, although with Zn, the smallest bull trout (0.1 g) were also most resistant [63]. Results with older and larger rainbow trout with Zn have been contradictory. Rainbow trout Zn resistance was reported to decrease three-fold over a size range of 1.7 to 29 g [64], yet another study was observed EC50 to increase seven-fold over a size range of 0.17 to 68 g [49].

This pattern of decreasing resistance to metals by salmonids with increasing size during the juvenile stages certainly does not hold with all species. For instance with mottled sculpin, newly hatched fish were distinctly less resistant to Cd, Cu, and Zn than were progressively older and larger fish [46].

### **Metal mixture toxicity**

While a variety of terms have been used to describe the effects of chemical mixtures, the simplest are probably whether the toxicity of the different metals is additive, less than additive, or greater than additive. Additive toxicity may further be defined by concentration addition or response (effects) addition [65]. Concentration addition is based on the concept of toxic units (TUs), where the proportions of chemicals in a mixture relative to an organism's response concentration, e.g., EC50s, are added, and where a sum of 1.0 defines additive toxicity. Then, if at an observed 50% response to a mixture, if the concentrations in the mixture add up to 1.0 TUs, the toxicity of the mixture is additive; if the sum of the TUs is less than 1.0, the mixture toxicity is greater than additive, and if the sum of the TUs is greater than 1.0, the mixture has less than additive toxicity. If instead, the toxic effects are predicted based on response addition, then toxicity of the mixture can be predicted as the product of the survivals observed when each metal is tested individually [65].

In a series of mixtures in moderately-hard water, in which Zn was the principal toxicant and Cd and or Pb were added at concentrations that were expected to kill few fish individually, the EC50s as Zn were higher in all mixture combinations than in the tests with Zn alone test (Table 5). Thus the observed mixture toxicities in that series were clearly less than additive on either a concentration or response additivity basis.

In contrast, for test 139 with Pb+Zn added to very-soft water, whether the toxicity of the Pb+Zn concentrations in the mixture would be considered greater or less than additive would depend on whether toxicity was considered on a concentration or response basis. For a given concentration of Pb or Zn in the mixture, toxicity was greater than that of similar concentrations of Pb or Zn individually (*Table 5* and Zn test 128, *Table S4*). On a concentration additivity basis, the mixture toxicity would be considered less than additive as follows: From the concurrent single metals tests, 1 TU Pb = 48 µg/L and 1 TU Zn = 74 µg/L (tests 89 and 128); the EC50s of the Pb+Zn mixture were estimated as 39 µg/L Pb and 34 µg/L Zn, which could be expressed as 0.83 Pb TUs + 0.46 Zn TUs = 1.29 TUs, indicating that the observed toxicity was less than predicted by concentration addition. Yet on a response addition basis, the observed toxicity in test 139 was at least marginally greater than that expected by taking the product of individual survivals from the two treatments with partial effects. Treatment 2 resulted in 42% survival with 47 µg/L Pb and 35 µg/L Zn present, while in the concurrent single metals exposures, 47 µg/L Pb yielded 50% survival and 36 µg/L Zn yielded 100% survival (tests 89 and 128). Thus 50% effects were predicted were 50% (50% x 100%), versus 58% observed effects. Similarly, in treatment 1 of test 139, predicted effects were 3% assuming response additivity, versus observed effects of 21%.

The mixtures tested as dilutions of the lower SFCdAR which had elevated ambient Zn+Cd (*Table 2*) had no corresponding single-metals tests in the same dilution waters. The main utility of the ambient dilution tests will be to evaluate the protectiveness potential criteria formulations in joint exposures.

The decreased toxicity of Zn in the presence of Cd or Pb suggests competitive metal interactions for gill binding sites. If two or more metals compete for binding to the same site of toxic action on the organism, it would be possible to adapt the biotic ligand model approach and model the total metal bound to that site, and predict metal toxicity [65,66]. With this type of loading or accumulation additivity approach, metals mixtures in water could be linked to inferred critical tissue residues, which would be a more advanced type of concentration addition mixture toxicity model.

## Conclusions

Our testing of resident fish with Cd and Zn produced environmentally relevant data, meaning that response concentrations overlapped ambient concentrations. With Pb, the lowest Pb EC50s were on the order of 100X higher than ambient dissolved Pb concentrations. With Cd and Zn, the lowest EC50s of stream-resident invertebrates were at least an order of magnitude greater than the lowest fish EC50s. In contrast, the Pb EC50s of cutthroat trout and invertebrates overlapped, although the lowest Pb EC50s were obtained with cutthroat trout. Whether the invertebrates are in fact much more resistant to Cd and Zn than are salmonids and sculpin or whether these are artifactual differences that reflect limitations of acute tests with field-collected invertebrates could be informed by careful comparisons with field studies. Future toxicity testing with invertebrates should consider alternative techniques such as microcosms.

Even though trout tests were limited to the swim-up fry stage, resistance to at least Pb and Zn declined with increasing fish size. These differences of up to two-fold with Zn and up to six-fold with Pb could confound judgments of relative species sensitivity, and likewise hardness-toxicity relations. When hardness-toxicity relations were constrained to concurrent toxicity tests of waters with differing hardness, regressions were usually strong with  $r^2$  values > 0.9. This indicates that water hardness can be a good surrogate for calcium and pH influences on Cd, Pb, and Zn toxicity in these waters with low DOC concentrations of approximately 1 mg/L or less.

Metal mixture responses were complex and warrant more attention. Zn was less toxic to cutthroat trout when exposed in moderately-hard water as a mixture with Cd or Pb present but where Zn dominated toxicity. In contrast, Pb was marginally more toxic to cutthroat trout when exposed in very-soft water where Pb dominated toxicity. We plan to report further analyses using biotic ligand models to predict toxicity of metals mixtures, which could inform to what extent each metal contributes to observed effects, and whether the toxic effects of metals mixtures are enough different than those of individual metals to warrant adjusting criteria derived from single metals.

## Supporting Information

Fig. S1. Photographs of test layouts, and of selected SFCdAR test sites

Fig S2. Stability and solubility of exposure solutions

Table S1. Regression equations used to estimate ionic content of exposure waters.

Table S2. Single metals testing: dilution water chemistry, fish sizes, and 96-hr EC50 estimates for exposures to Cd, Pb, and Zn.

Table S3. Metal mixtures testing: dilution water chemistry, fish sizes, and 96-hr EC50 estimates for exposures to Cd, Pb, and Zn mixtures.

Table S4. Full time and concentration-response matrices

Table S5. Toxicity versus hardness: slopes of log(hardness) and log(EC50) relations from regressions of concurrent test data vs. regressions pooled across tests conducted at different times

Fig S1, Fig S2, and Table S5 are in a single PDF file (0.9 MB); Table S1, Table S2, and Table S3 are in a single Excel file (0.7 MB); and Table S4 is in a separate Excel file (1.3MB).

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