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Developing Acute-to-chronic Toxicity Ratios for Lead, Cadmium, and Zinc using Rainbow Trout, a Mayfly, and a Midge

Christopher A. Mebane · Daniel P. Hennessy · Frank S. Dillon

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Abstract In order to estimate acute-to-chronic toxicity ratios (ACRs) relevant to a coldwater stream community, we exposed rainbow trout (*Oncorhynchus mykiss)* to cadmium (Cd), lead (Pb), and zinc (Zn) in 96-h acute and 60+ day early-life stage (ELS) exposures. We also tested the acute and sublethal responses of a mayfly (*Baetis tricaudatus*) and a midge (*Chironomus dilutus, formerly C. tentans*) with Pb. We examine the statistical interpretation of test endpoints and the acute-to-chronic ratio concept. Increasing the number of control replicates by 2 to 3× decreased the minimum detectable differences by almost half. Pb ACR estimates mostly increased with increasing acute resistance of the organisms (rainbow trout ACRs <≈ mayfly < *Chironomus*). The choice of test endpoint and statistical analysis influenced ACR estimates by up to a factor of four. When calculated using the geometric means of the no- and lowest-observed effect concentrations, ACRs with rainbow trout and Cd were 0.6 and 0.95; Zn about 1.0; and for Pb 3.3 and 11. The comparable Pb ACRs for the mayfly and *Chironomus* were 5.2 and 51 respectively. Our rainbow trout ACRs with Pb were about 5–20× lower than earlier reports with salmonids. We suggest discounting previous ACR results that used larger and older fish in their acute tests.

Keywords Acute-to-chronic ratio · ACR · *Baetis tricaudatus* · Cadmium · *Chironomus dilutus* · *Chironomus tentans* · ECp · Lead · NOEC · *Oncorhynchus mykiss* · Statistical power · Zinc

C. A. Mebane (✉)
U.S. Geological Survey,
230 Collins Rd.,
Boise, ID 83702, USA
e-mail: cmebane@usgs.gov

D. P. Hennessy
Anchor Environmental, L.L.C.,
Bellingham, WA, USA

F. S. Dillon
CH2M Hill,
Okemos, MI, USA

1 Introduction

The diversity of aquatic life is such that it is infeasible to acquire test data for all species when developing criteria that are intended to protect aquatic communities. In the USA the approach has generally been to develop species-sensitivity distributions (SSDs) of acute toxicity data to estimate thresholds of acute toxicity for sensitive species which lack data. To account for untested and potentially sensitive species, a hypothetical LC50 for the 5th percentile most sensitive species is calculated from the SSD for a substance (that is, 95% of the species are less sensitive). This hypothetical LC50, the "final acute value" (FAV), is often divided by an acute-to-chronic toxicity

ratio (ACR) to estimate a chronic criterion that would not result in unacceptable adverse effects to aquatic communities. Although SSDs and chronic criteria have been derived directly from chronic data (e.g., EPA 1999; Grosell et al. 2006a; Mebane 2006; Van Sprang et al. 2004), because much fewer chronic data are available than acute data, in practice ACRs have been most commonly used to estimate the chronic sensitivity of untested species and derive chronic criteria in the USA (Mount et al. 2003; Raimondo et al. 2007; Stephan et al. 1985).

The purpose of this testing was to obtain well matched acute and chronic data to calculate ACRs, which would in turn be used to estimate chronic thresholds that were protective of a coldwater, lotic community. For species that are impractical to test directly, estimating chronic thresholds is a problem. For many native fish, invertebrates, or amphibians with long life cycles or poorly understood life histories, culturing or collecting suitable organisms for chronic testing could be very difficult. In contrast, acute test data may be reasonably obtained for many native species because of the much simpler methods (ASTM 1997b). Although the ACR approach has weaknesses for criteria development or risk assessment, a major strength of the ACR approach is that it allows making estimates of chronic values for acutely sensitive species for which no chronic data are available. In such cases, direct analysis of available chronic data may underestimate chronic toxicity, whereas the ACR allows some extrapolation of chronic effects for sensitive species, even though no chronic data exists (Mount et al. 2003).

The use of fixed ACRs for a species is logically warranted if the mechanisms of chronic toxicity were the same as for acute toxicity. For Pb, Cd, and Zn, one mechanism, ionoregulatory disruption, is involved in both acute and chronic toxicity although the sites of action differ. In short-term exposures the site of action is the gill surface, whereas in chronic exposures metals burdens shift from the gills to kidney and liver, leading to kidney impairment, and ionoregulatory disruption. When mechanisms immediately affected by metals are disrupted for a long period of time, general stress reactions (loss of condition, growth impairment, and reduced fecundity) result. These complexities weaken the logic for considering ACRs constant for a species (Alves et al. 2006; Wood et al. 1997). However, until the specific mechanisms for chronic toxicity are better understood, some use of ACRs to estimate risks of chronic toxicity will likely be needed.

Our selection of test species was a compromise between our desire to test sensitive organisms that are relevant to coldwater, streams and the desire to use established protocols. We selected rainbow trout, *Oncorhynchus mykiss*, because it is a common surrogate species for coldwater fishes, they are often ranked among the more sensitive fish species in SSDs in the U.S. Environmental Protection Agency's (EPA's) water quality criteria documents, their eggs are readily available, and procedures for rearing and testing are well developed. The choice of invertebrates was less straightforward. Commonly tested invertebrates such as daphnids or amphipods are atypical in fast water streams. We selected the eurythermal midge *Chironomus dilutus* (formerly *Chironomus tentans* (Shobanov et al. 1999)), because it was an invertebrate that occurs in fast water streams and for which a life cycle method was available (Benoit et al. 1997). Reports on the sensitivity of Chironomids to metals are mixed. In laboratory testing, early instar *Chironomus* were fairly sensitive to copper (Cu) in acute exposures (Gauss et al. 1985), and a life cycle test with Cd placed *Chironomus* in the middle of a chronic SSD for Cd (11th most sensitive of 21 genera (Mebane 2006)). Yet, in field surveys, *Chironomus* are sometimes abundant in metals polluted sites, and apparently may acquire resistance to some metals (Kiffney and Clements 2002; Krantzberg and Stokes 1989; Mebane 2002). We selected a mayfly because mayflies are often sensitive to metals and are an ecologically important in streams (Kiffney and Clements 2002; Mebane 2002). Mayflies in the genus *Baetis* are disproportionately important in coldwater stream food webs (Rader 1997). Reports of the sensitivity of *Baetis* to metals are mixed, with the genus being quite sensitive in stream microcosms (Kiffney and Clements 1994); yet in field surveys *Baetis* were common in streams with elevated concentrations of Pb, Cd, and Zn (Maret et al. 2003). *Baetis* have features that are desirable for toxicity testing. In cool temperate streams, they have a rapid life cycle larval stages lasting around two weeks and with several hatches per summer. Thus early instars can be collected during much of the summer. *Baetis* are widely distributed and often abundant in temperate streams, and others had successfully used *Baetis*

Table 1 Water quality parameters and background metals in the two dilution waters sampled at stable, base flows (filtered – 0.45 μm filtered sample; nm – not measured)

Parameter	Sample Type	Units	LNF	SFH
TOC	Whole	mg/L	<1.5	<1
DOC	Filtered	mg/L	<1.5	2
Alkalinity	Whole	mg/L	12	20
Hardness (mg as $CaCO_3$)	Calculated	mg/L	14	24
Calcium	Filtered	mg/L	3.36	6.28
Magnesium	Filtered	mg/L	1.40	1.93
Ca:Mg mass ratio	Calculated	None	2.4	3.3
Ca:Mg molar ratio	Calculated	None	1.4	2.0
Chloride	Filtered	mg/L	nm	1.17
Potassium	Filtered	mg/L	0.33	0.44
Sodium	Filtered	mg/L	0.55	0.70
Sulfate	Filtered	mg/L	nm	2.47
Aluminum	Filtered	μg/L	6	9
Copper	Filtered	μg/L	2	1
Nickel	Filtered	μg/L	3	2
Cadmium	Filtered	μg/L	<0.2	<0.2
Cadmium	Whole	μg/L	Nm	0.3
Lead	Filtered	μg/L	<0.2	1.1
Lead	Whole	μg/L	Nm	1.7
Zinc	Filtered	μg/L	<2	8
Zinc	Whole	μg/L	Nm	7

LNF – Little North Fork of the South Fork Coeur d'Alene River.

SFH – South Fork Coeur d'Alene River at the Hale Fish Hatchery.

in toxicity testing (Lowell et al. 1995a, b; Wallace and Anderson 1996).

2 Methods

2.1 Test Facility and Water Source Descriptions

All testing was carried out at the Hale Fish Hatchery near Mullan, Idaho, USA (47° 27.8' N, 115° 42.3' W), near the headwaters of the South Fork Coeur d'Alene River (SFCDR). Much of the SFCDR has been polluted by a long history of mining and smelting, but the hatchery is located upstream of most mining disturbances. The Hale Hatchery is located at the confluence of the SFCDR and the Little North Fork of the South Fork Coeur d'Alene River (LNF). This provided us with the choice of two dilution water sources. The first test phase (1997) used the LNF since it was the usual hatchery source and was piped into the hatchery by gravity flow. Use of SFCDR at Hale water source (SFH) was more involved because it required pumping which was subject to interruption due to power failures and beaver (*Castor canadiensis*) activity. However, as testing progressed, we thought that the low hardnesses of the LNF (7–22 mg/L as $CaCO_3$) were stressing some test organisms. The second test phase (1999 and 2000) used water pumped from the SFH, which provided hardnesses in the range of 20–35 mg/L as $CaCO_3$.

Dilution waters were analyzed extensively throughout the testing for standard water quality parameters (hardness, alkalinity, pH, conductivity, dissolved oxygen, and temperature) and background metals concentrations per ASTM (1992) protocols. A detailed chemical screen of non-routine parameters (anions, cations, other metals, organic carbon) was made once during stable base-flow conditions (Table 1). Dilution water was filtered through a paper cartridge filter to remove debris and avoid introducing indigenous organisms into the test chambers. UV sterilization was added to the midge life cycle exposures to reduce the algal growth that occurred in the warm temperatures used in that protocol.

Master batches of the treatment concentrations were prepared from reagent-grade chloride salts of Cd, Pb, or Zn dissolved in hatchery water. Samples were collected for chemical analysis from initial treatment batches and from selected treatment replicates or composites during the course of the testing. All water samples for dissolved metals analysis were filtered through a 0.45-μm cellulose filter. Metals samples were analyzed using the total recoverable metals digestion procedure. Cd and Pb were analyzed using EPA Method 200.9 (graphite furnace atomic absorption spectrometry) and Zn was analyzed using EPA Method 200.7 (inductively coupled plasma / atomic emission spectrometry). Hardness and alkalinity were measured onsite by titration (APHA 1992).

2.2 Mayfly Chronic Methods

Mayfly larvae were exposed for 10 days to evaluate the effect of Pb on survival, growth, and molting using methods adapted from Diamond et al. (1992). *Baetis* tricaudatus mayflies were collected from the SFCDR 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 (1996).

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 quality and temperature was 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 24 h of collection.

Larvae were exposed in 800-mL polypropylene Tri-pour® beakers containing approximately 600 mL of test solution. The experimental design was a randomized block with three replicates of the clean-water control treatment and three replicates each of the six Pb-spiked treatments.

The test temperature was controlled by placing the replicate beakers into a water bath. All replicate beakers were continuously aerated through a clean pipette. The aeration also maintained some water velocity, which was needed since zero velocities are unnatural, stressful to stream mayflies, and affect test performance (Lowell et al. 1995b). A piece of cottonwood leaf approximately 3 cm^2 was added as substrate. Several flakes of TetraMin® fish food were added as a food source and were replaced as they were consumed. The control water and test solutions were replaced every 48 hours during the test. Water samples were collected for dissolved Pb analyses at the beginning of the test and at 4-day intervals, for a total of three time-point measurements for each treatment.

Temperature and dissolved oxygen concentrations were recorded daily in each replicate beaker; conductivity and pH were recorded daily in one replicate from each treatment. Shed exuviae and dead organisms were recorded and removed daily. Head capsule width and body length were measured at the end of the test using a micrometer.

2.3 Midge Life Cycle Methods

The midge life cycle (LC) test was modified from a method developed to assess the sublethal toxicity of contaminated freshwater sediments (Benoit et al. 1997). The experimental design consisted of three replicates each of a control and five Pb-spiked treatments. Each replicate aquarium held four invertebrate cups, one to assess survival and growth at day 21, and the other three for reproductive measurements. All cups contained approximately 15 mL of clean silica sand for substrate. Aquaria were aerated to ensure that the test solutions were mixed within the cups. The day-21 cup was seeded with 12 first-instar larvae. The three reproductive cups were each seeded with eight first-instar larvae. Feeding was 1.5 mL or 1 mL of TetraMin® slurry per day per cup for the growth and reproduction cups respectively.

The *C. dilutus* culture was initially started with egg cases from Aquatic Biosystems in Ft. Collins, Colorado, USA. Two nursery tanks, cultured under static-renewal conditions, were started. The tanks were covered 20-L aquaria with approximately 2 cm of clean silica sand as a substrate. Temperature was maintained at about 23°C using submersible aquarium heaters. Aeration was supplied to the aquaria via a glass pipette inserted through the cover. Cultures were fed a TetraMin® fish food slurry.

Four endpoints were measured: survival, growth, emergence, and fecundity. Percent emergence was

calculated as the sum of viable adults per replicate aquarium divided by the total number of organisms accounted for throughout the duration of the test (emergers, dead pupae, partial emergers, and dead larvae found at test termination). Fecundity was measured as the number of egg cases per treatment; the number of eggs per case was estimated using the ring count method (Benoit et al. 1997).

We initially sought to culture and test *C. dilutus* using water from the LNF, but we believe the low hardnesses of the culture (7–20 mg/L as $CaCO_3$) may have ionically stressed the animals, preventing the cultures from thriving. After changing the culture to the harder SFH water, the cultures appeared to thrive. We tested progeny from a culture that had been reared for a full generation in SFH water.

2.4 Rainbow Trout Early-life-stage Methods

Methods were adapted from the standard guide for conducting early-life-stage toxicity test with fishes (ASTM 1992). The rainbow trout early-life-stage toxicity tests used eggs from the Mt. Lassen Trout Farm, Red Bluff, California. Approximately 5,000 eyed eggs were shipped express freight and allowed to acclimate overnight in hatchery water before seeding the ELS tests.

Temperature-controlled dilution water, targeted at 10°C in the first group of tests and 12°C in the second group, was supplied from a headbox equipped with both a Frigid Units® chiller and a Clepco® heater. Three replicate aquaria, with randomized positions and holding approximately 15 L each, were run for each treatment. There were six or seven treatments: a stream-water control and five or six metal solution additions to stream water in increasing concentrations. The flows of the dilution water and metals stock solutions were measured daily. The target flow rate of 50 mL/min into each replicate aquarium produced about five volume additions per day. Ground trout food was offered to all treatments beginning when about 90% of the control fish were swimming. Fish were fed to satiation. Excess food and feces were siphoned from tanks every other day.

The rainbow trout ELS tests were conducted in two phases: the first from August to October 1997 and a second phase from July to September 1999. In the 1997 tests, following overnight acclimation of the eyed eggs in hatchery water, 45 eggs were randomly distributed into each of the 63 replicate aquaria to begin the early-life-stage tests. The remainder of the eggs was incubated in 20-L aquaria supplied with flowing hatchery water from the LNF. These remaining fish were raised to the fry stage and were used in acute toxicity tests. All fish used in toxicity tests were euthanized with the anesthetic tricaine methanesulfonate (MS-222) after testing. The 1999 tests were conducted similarly except that the hatchery's SFH water source was used and 60 eggs were seeded per aquarium. Through day 7, dead eggs were replaced with spare embryos so that the tests started with the same number of newly hatched alevins (ASTM 1992).

2.5 Acute Methods

Concurrent 96-h static-renewal tests were conducted in order to estimate ACRs. All acute tests used renewal exposures; modifications to the standard renewal protocols (ASTM 1997a) follow: Mayfly acute tests were conducted in 800-mL polypropylene beakers nested in 20-L aquaria containing the test solution. Two rectangular panels approximately 6× 3 cm were cut in each beaker and covered with Nytex screen. The screened panels allowed for the exchange of test solution among the beakers and the aquaria. The panels were cut so that the bottom was 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. Six treatments, with two replicates each, were administered to mayflies. Each aquarium held four beakers, two of which were placeholders, placed in 6.5 L of treatment solution, the volume required in the aquaria to fill the individual beakers to the 500-mL level. Air was bubbled into each beaker via a pipette. A raceway supplied with temperature-adjusted stream water was used as a waterbath to control the temperature of the test solutions during the invertebrate tests. Each replicate beaker was seeded with 10 mayflies.

C. dilutus for the acute tests were cultured from larvae left over after seeding the Pb life cycle tests to start two new culture tanks. These new culture tanks were supplied with the same dilution water supplied to the LC test. The adults from these culture tanks emerged at the same time of the organisms in the LC test and were collected and placed in clean cups to

collect the egg cases. The larvae from these egg cases were used to seed the renewal 96-hour Pb acute test. Modifications to the standard midge *C. dilutus* acute test were made to reduce variability in test concentrations and to avoid cannibalism (ASTM 1997a). At test initiation each jar was filled with 750 mL and 1 mL of TetraMin® slurry was added. At 24 h and 72 h, and additional 250 mL was added to each chamber. At the 48-h refresh, 50% of the test solution was replaced and an additional 1 mL of food slurry was added to each jar. About 15 mL of clean silica sand was used as substrate. The control and seven Pb-spiked treatments were each replicated five times, each seeded with 6 s instar larvae. The jars were randomly placed in rows in a water bath with the temperature set to 22°C. The replicate jars were not aerated because dissolved oxygen concentrations were above 60% saturation. As a precaution against losing organisms, water quality was only measured in a single designated replicate.

Rainbow trout exposures were conducted in 20-L aquaria placed on wood tables 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 rainbow trout fry reared from the same shipment of eggs used in the early-life-stage tests were exposed to seven treatments; a control and six 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. Each aquarium was seeded with 10 fish. Tests were initiated between 2 and 4 weeks after hatching in order to test the swim-up life fry stage, which we assumed would be the most sensitive life stage. Average weights of fry used in the acute tests ranged from about 0.2 to 0.4 g, giving fish loadings of about 0.17 to 0.33 g/L. Temperature and dissolved oxygen concentrations were recorded daily in each replicate aquarium; conductivity and pH were recorded daily in one replicate from each treatment. Water samples for metals were taken at test initiation and at 96-h.

2.6 Data Interpretation

All test results are given for "dissolved" metals (i.e. 0.45 μm filtered). Chronic test concentrations for effects analyses were calculated as time-weighted arithmetic averages (TWA) of measured concentrations. Acute test concentrations were calculated as geometric means of concentrations at test initiation and at 96 h.

Test results were analyzed both by hypothesis testing and regression analyses. Treatment responses were compared to control responses using Dunnett's multiple comparison test using α of 0.05 (one tailed) to define "significant" results (EPA 1993; Zar 1984). If Bartlett's test indicated heterogeneity of variance, we used a nonparametric version of Dunnett's test with rankit-transformed data. Since the rainbow trout ELS tests with the different metals were run side-by-side in the hatchery with the same water source and using eggs from a common cohort, we pooled the control replicates in order to increase the statistical power of Dunnett's test to detect changes from controls (Zar 1984:195). Two values may be derived from Dunnett's test, a no-observed-effect concentration (NOEC) defined as the highest test concentration that resulted in responses that are not statistically different from the controls and a lowest-observed-effect concentration (LOEC) defined as the lowest test concentration that is statistically different from controls. These values are commonly used as "chronic limits;" between them is a hypothetical "maximum acceptable threshold concentration" (MATC) or "chronic value" which is assumed to be a threshold for toxic effects. Its point estimate is the geometric mean of the NOEC and the LOEC. We report NOEC, LOEC, and MATC values for our tests. We also report estimates of the minimum statistically detectable difference (MDD) of the hypothesis testing to detect minimum responses when setting α of 0.05.

The 10th and 20th percentile effects concentrations (e.g. EC10, EC20, ECp) from distribution or regression based dose–response curves were also calculated. Mortality ECp values were calculated using a three-factor probit model (Caux and Moore 1997; Erickson 2002). Growth or reproduction inhibition effects concentrations were estimated by least-squares nonlinear regression analysis (Erickson 2002) or nonparametric linear interpolation (Norberg-King 1993).

Unlike acute tests where the EC50 is the customary ECp value, there is no consensus on the appropriate ECp value for comparing low toxic effects values from chronic testing. We calculated EC10s and EC20s for the following reasons. EC10s are assumed to be near the low limits of an ECp value that could be calculated with confidence using usual test procedures

Table 2 Mayfly *Baetis tricaudatus* mean (S.D.) growth and survival with Pb

Pb (μg/L)	Control (<3)	69	103	160	222	350	546	Minimum detectable difference from control (% of control)
Percent survival	68.7 (20.8)	75.6 (6.1)	68.7 (13.1)	67.3 (10.5)	54.5 (9.1)	37.3[a] (0)	6.1[a] (5.2)	23% (72%)
Number of molts	14.0 (3)	10.7 (2.3)	10.3 (2.3)	8.7[a] (2.5)	9.0[a] (1.7)	7.7[a] (1.2)	5.3[a] (2.3)	4.6 (32%)

[a] Significantly different from control at $P<0.05$ using Dunnett's procedure.

Mean (±SD, range, *n*) hardness 20.7 mg/L as $CaCO_3$ (±0.58, 20–21, *3*); alkalinity 19.8 mg/L as $CaCO_3$ (±1.04, 19–21, *3*); pH 6.64 (±0.18, 6.4–6.9, *16*); conductivity 47.7 μs (±1.72, 45–52, *20*); DO 10.1 mg/L (±0.45, 9.1–10.9, *22*) temperature 9.3°C (±0.67, 8.4–10.6, *22*).

and EC10s have been suggested as a regression-based replacement for the NOEC. (Moore and Caux 1997; van der Hoeven et al. 1997). We also calculated EC20s since they have been used recently in lieu of MATC values to derive criteria or to report sublethal effects (e.g., EPA 1999; Hansen 2002a). Mebane (2006) calculated ECp values for 34 chronic toxicity tests with cadmium. For invertebrates, the average MATCs and NOECs were greater than corresponding EC20 concentrations (28 and 16% greater, respectively). With fish, the average NOEC values were 11%lower than the corresponding EC10 values, and average MATCs were 16% greater than EC10 values. This suggests that at least with Cd, EC10s and EC20s may have roughly similar effects levels as statistical NOECs with common test designs for fish and invertebrate species, respectively.

3 Results and Discussion

3.1 Mayfly Growth Test

Consistent dose-dependent reductions in mayfly growth (as number of molts) and survival were observed with Pb exposures over a range of 69–546 μg/L (Table 2). The reduced molting endpoint was about 3–6× more sensitive than the mortality endpoint with EC20s of about 60 and 213 μg/L respectively (Table 6). The pattern of response in the molting endpoint suggested that the threshold of effects was somewhat lower than the lowest concentration tested (Fig. 1a). The concurrent 96-h EC50 with Pb was calculated at about 664 μg/L.

The sum of molts per test chamber was chosen as the measurement endpoint on which to develop a chronic toxicity value for four reasons: first, molting in mayflies is relevant to whether the organisms are likely to complete their life cycle; second, the endpoint has been found to be a sensitive endpoint for measuring effects of pollutants (Diamond et al. 1992; Lowell et al. 1995a); third, the sum of molts is not confounded by mortality as is the endpoint molts per individual, and fourth, we were less confidant in the accuracy of our measurements of body length and head capsule width. Diamond et al. (1992) also found the number of molts produced was the most sensitive endpoint with the mayfly *Stenonema* and silver. Mayflies in general are often reported to be sensitive to metals, and among the mayflies, baetids are probably intermediate in metals sensitivity. Baetid mayflies were sensitive to Cu and Zn in experimental stream studies (Clements et al. 1992; Kiffney and Clements 1994). However, the surveys of Maret et al. (2003) in the SFCDR vicinity suggested other mayfly taxa such as *Drunella*, *Rhithrogena*, *Paraleptophlebia*, or *Cinygmula* may be more consistently affected by elevated metals than *Baetis*. These or other mayfly taxa should also be amenable to the mayfly growth test, so long as they are abundant enough in unpolluted reference areas to feasibly collect enough early instars.

The mayfly growth test results encourage wider use of this type of testing. The test was less costly to perform than the longer midge life cycle test and the sensitivities of the growth endpoint to Pb overlapped some of the sublethal endpoints of the rainbow trout tests (Table 6). In contrast, the acute mayfly LC50 was about five times greater than the rainbow trout LC50s with Pb. More sublethal testing with mayflies might reduce some of the disparity between field surveys where mayflies have been observed to be sensitive indicators of elevated metals in streams, and acute toxicity values for the same or related taxa that may be well above environmentally relevant concentrations (Buchwalter et al. 2007; Mebane 2002, 2006). Arguably, the mayfly test is more relevant to concerns

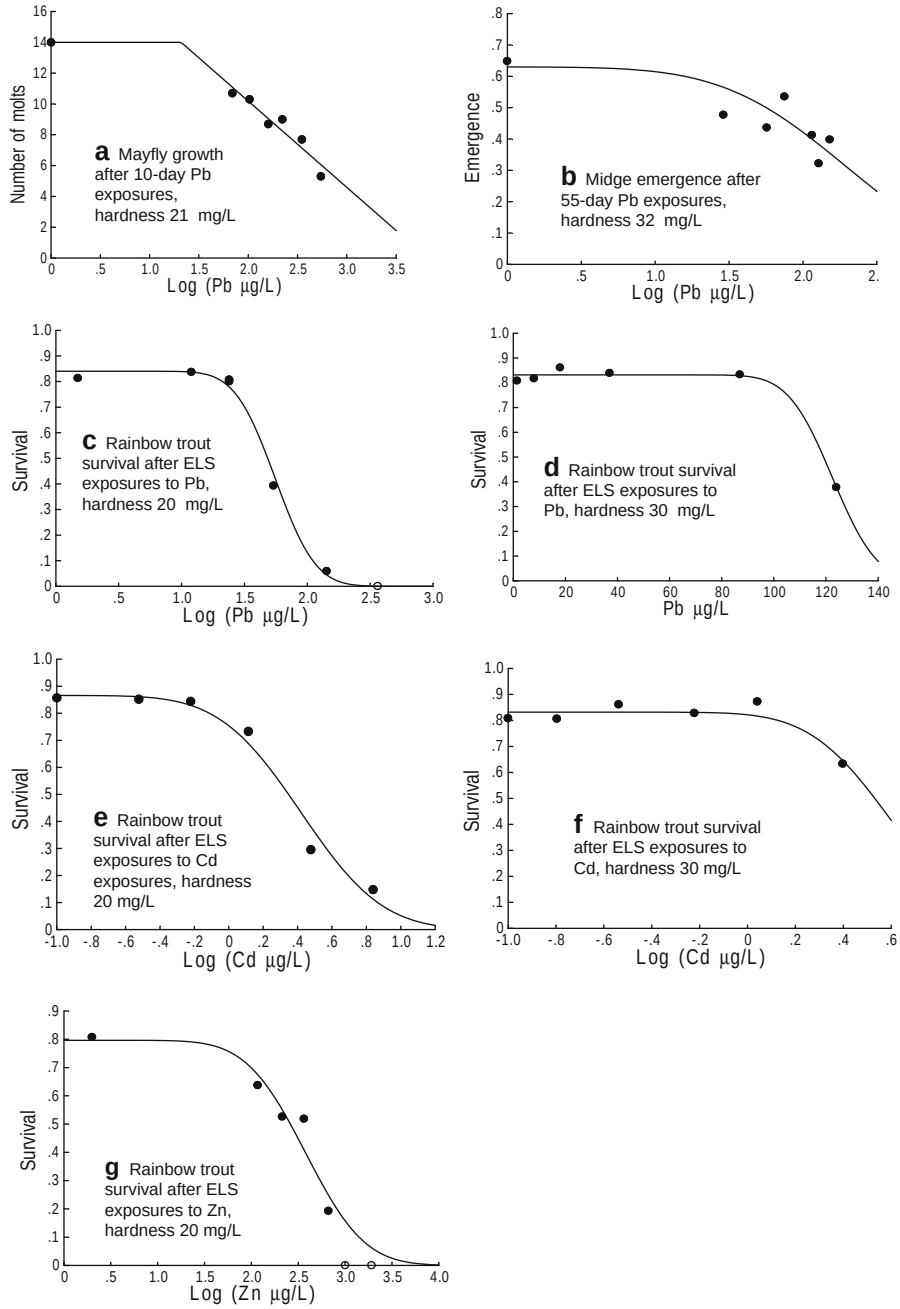


Fig. 1 Chronic toxicity relationships from each test. Response curves for survival and emergence were estimated using 3-parameter probit model, and for growth data, nonlinear regression. Open symbols indicate high effects data points that were censored to improve model fits at the low effects levels

of metals toxicity in stream invertebrate assemblages than are more commonly used invertebrate test methods for midges, amphipods, or cladocerans, since these alternative test organisms may not be important components of lotic assemblages, or may not be sensitive to metals in streams. Advancing techniques such as the mayfly test of Diamond et al. (1992) could provide sublethal effects data for a greater diversity of taxa and lessen the need for the use of acute-to-chronic or taxonomic extrapolations.

3.2 Midge Life Cycle Test

The results of the midge life cycle test showed reduced growth and emergence in Pb exposures over a range of 29–152 µg/L, although the reductions were

Table 3 Midge *C. dilutus* full life cycle mean (S.D.) responses to dissolved Pb concentrations

Pb (SD) μg/L	Control (<3)	29 (9.8)	57 (35)	75 (16)	115 (40)	128 (32)	152 (38)	Minimum detectable difference from control (% of control)
Survival – day 20 (%)	61.1 (18.9)	33.3 (26.2)	58.3 (19.5)	58.3 (22.1)	61.1 (31.2)	38.9 (22.9)	66.7 (25.0)	48% (79%)
Dry weight – day 20 (mg/individual)	2.95 (0.12)	2.24[a] (0.37)	2.4 (0.35)	1.98[a] (0.39)	1.47[a] (0.05)	1.56[a] (0.14)	2.1[a] (0.29)	−0.57 mg (19.6%)
Eggs/case – day 20	690 (144)	746 (181)	792 (242)	762 (210)	646 (169)	751 (190)	798 (202)	−240 (35%)
Percent emergence – day 55	64.8 (10)	47.7 (19)	43.6 (10)	53.5 (19)	41.2 (5.1)	32.2[a] (7.8)	39.8[a] (4.7)	−25% (38%)

[a] Significantly different from control at $P<0.05$ using Dunnett's procedure.

Average (±S.D. *n*) hardness 32 mg/L as $CaCO_3$ (±3.2, 27–35, *8*); alkalinity 31 mg/L as $CaCO_3$ (±3.0, 27–36, *7*); pH 7.26 (±0.21, 6.9–7.7, 18); conductivity 76 (±4.9, 67–83, 18) μs; dissolved oxygen 7.8 (±0.8, 5.9–12.9, *112*) mg/L; temperature 22.2°C (±1.0, 19.7–24.4, *112*). Average (maximum, *n*) dissolved metals in control samples: Cd<0.2 μg/L (<0.2, 9), Pb<3.0 μg/L (5, 9), Zn 12 μg/L (62, 9). nc – not calculable.

not always concentration-dependent. No effects of Pb on mortality or fecundity were observed over the range of concentrations tested (Table 3).

NOEC and LOEC results for growth and emergence were ambiguous because of variable responses. Growth in treatment 1 but not in treatment 2 was significantly lower than controls. For the growth endpoint, treatment 2 (57 μg/L Pb) was considered the NOEC and treatment 3 (75 μg/L Pb) the LOEC since all higher exposures resulted in lower weights than in either the control or treatment 2. We estimated an emergence EC20 of about 55 μg/L, using a 3-parameter probit analysis.

The midge growth and emergence endpoints were fairly sensitive, within the general range of some rainbow trout endpoints from our tests as well as responses reported for other tests conducted in soft water (Tables 6 and 7). This was counterintuitive because chironomids thrive in some metals-contaminated systems (Clements et al. 1992; Kiffney and Clements 1994, 2002; Mebane 2002). However, our results are consistent with reports that chironomids can regulate and thus develop tolerances to elevated exposure to the essential metals, Cu and Zn, but cannot regulate the nonessential metals, Cd and Pb (Krantzberg and Stokes 1989).

Working with this insect in soft water and with Pb presented challenges. While we had previously had good success working with *Chironomus* in sediment toxicity tests, in those tests the characteristics of the overlying water were similar to the culture water which avoided the need for hardness acclimation. In contrast, in our water-only *Chironomus* tests in a natural water that was much softer than the supplier's culture water, acclimation for a full generation in the soft water was necessary. A related challenge is the low solubility of Pb, which decreases with increasing alkalinity. Even in our test water with an alkalinity of 20 mg/L and pH 6.7, of a nominal concentration of 1000 μg/L dissolved Pb, over 50% of the Pb was predicted to form insoluble $PbCO_3$ complexes (using the MINEQL + chemical equilibrium model, http://www.mineql.com). Grosell et al. (2006a) described a compromise between providing suitable chemical conditions for *Chironomus* larval development (supplemented with Ca^{2+}) and being able to maintain high Pb concentrations in solution by maintaining low carbonate levels. In their best of five different test procedures, water of general low-ionic strength was amended with $CaSO_4$ to provide sufficient Ca^{2+} in the water for larval survival and development and yet maintain low carbonate levels to avoid excess Pb precipitation. That procedure yielded 53% survival and emergence in control groups, which was similar to the 61% and 65% respective control survival and emergence in our test (Table 3).

The performance of our *Chironomus* long-term toxicity test in soft water was also within the range

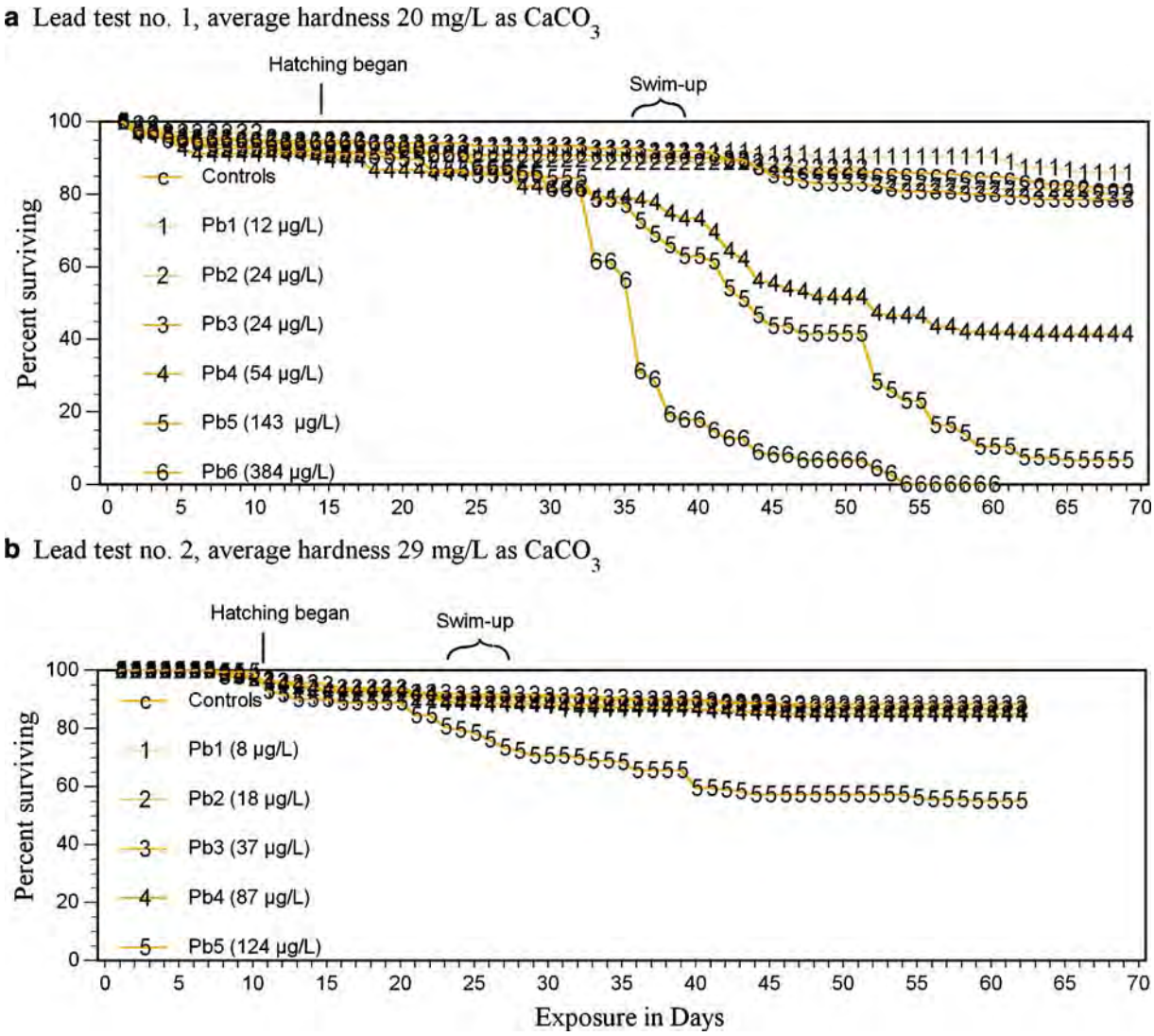


Fig. 2 Cumulative mortality patterns during early-life stage testing of rainbow trout with lead

obtained with *Chironomus* sediment tests in two round robin tests among 8–10 laboratories using overlying water hardnesses of 90–100 mg/L. In two round robin test evaluations, a proposed test acceptability criteria (TAC) of >70% for 20-day control survival was met by 63% to 90% of the laboratories and a TAC of >50% emergence was met by 50% to 70% of the laboratories (Norberg-King et al. 2006). The growth and emergence endpoints were similarly sensitive between our soft water test and the sediment evaluations. Average round robin MDD for growth as dry weight were −0.36 and −0.65 mg/organism (−25 and −64% of control) for two sets of contaminated sediment, compared to our MDD of −0.56 mg (−20% of control). Average emergence MDD for contaminated sediments were −18 and −19% compared to our −25% difference from the controls.

The magnitudes of effects in our test were not extreme in any treatment, with minimum weights and emergence of about 50% of controls, and no reductions in survival or fecundity. The minimum average size of *C. dilutus* larvae at day 20 was about 1.5 mg in dry weight. Sibley et al. (1997) found that when larval dry weights were below about 1.5 mg/individual fecundity increased with increasing larval size, but above this larval size an upper reproductive threshold was evident. This suggests we may not have tested at high enough Pb concentrations to cause profound adverse

Table 4 Early-life stage rainbow trout mean (S.D.) mortality and growth following dissolved Pb, Cd, and Zn exposures, 69 day test (see note), three treatment replicates and nine control replicates (pooled from the three tests)

Pb (±SD)	Control (<3)	12 (2)	24 (3)	24 (9)	54 (25)	143 (20)	Minimum detectable difference from control (% of control)
Survival (%)	81.3 (4.8)	83.7 (6.7)	80.7 (4.6)	80.0 (5.9)	39.3[a] (20)	5.9[a] (4.6)	9.9% (12.2%)
Wet weight (g)	0.369 (0.02)	0.324[a] (0.05)	0.357 (0.07)	0.360 (0.01)	0.282[a] (0.03)	0.108[a] (0.04)	–0.033 g (8.9%)
Length (mm)	31.6 (1.7)	31.36 (1.9)	31.37 (1.3)	32.61 (0.63)	29.07 (1.4)	21.30[a] (2.9)	–2.1 mm (6.7%)
Cd (±SD) μg/L	Control (<0.2)	0.3 (0.05)	0.6 (0.12)	1.3 (0.20)	2.9 (0.47)	6.9 (0.77)	
Survival (%) (53 days, note b)	85.7 (5.9)	85.2 (4.6)	84.4 (6.7)	73.3[a] (11.8)	29.6[a] (6.8)	14.8[a] (8.4)	7.6% (8.9%)
Survival (%) (69 days)	81.3 (4.8)	B	B	67.3[a] (9.7)	14[a] (7.1)	0.74 (0.57)	10.3% (12.7%)
Wet weight (g) (b)	0.218 (0.04)	0.208 (0.01)	0.219 (0.01)	nm	Nm	nm	– 0.048 g (22%)
Wet weight (g)	0.369 (0.02)	B	B	0.342 (0.04)	0.222[a] (0.04)	0.146[a]	– 0.031 g (8.5%)
Length (mm)	31.6 (1.7)	B	B	31.7 (1.9)	28.4 (5.9)	25	–3.0 mm (9.5%)
Zn (±SD)	Control (<10)	117 (16)	214 (28)	365 (27)	665 (204)	1005 (414)	
Survival (%)	81.3 (4.8)	64.7[a] (11.1)	52.6[a] (10.2)	51.9[a] (12.2)	19.3[a] (12.2)	0[a]	10.3% (12.7%)
Wet weight (g)	0.369 (0.02)	0.365 (0.05)	0.317[a] (0.01)	0.307[a] (0.05)	0.350[c] (0.03)		–0.037 g (10.1%)
Length (mm)	31.6 (1.7)	31.38 (1.3)	29.68 (0.40)	27.84[a] (4.73)	29.43 (0.91)		–2.5 mm (7.9%)

[a] Significantly different from control at $P<0.05$ using Dunnett's procedure. [b] Results as of day 53; the two lowest Cd treatments were overdosed on day 54. [c] Increased growth from treatments with low numbers of surviving fish were considered artifacts of the reduced densities and were excluded from statistical comparisons. nm – not measured.

Average (±SD, range, *n)* hardness 19.7 mg/L as $CaCO_3$ (±1.5, 17–21, *17*); alkalinity 19.6 mg/L as $CaCO_3$ (±2.2, 17–25, *17*); pH 6.75 (±0.4, 5.0–7.7, *570*); conductivity 45.8 μs (±2.2, 40.3–57.2, *475*); dissolved oxygen 10.2 (±0.7, 8.3–11.9, *982*) mg/L; temperature 9.8°C (±0.6, 8.4–11.3, *1474*). Maximum metals in control samples (n=12) – Cd<0.2 μg/L, Pb<3.0 μg l^{-}, Zn<10 μg/L.

effects in this species, and thus our effects concentrations from this test may be conservative.

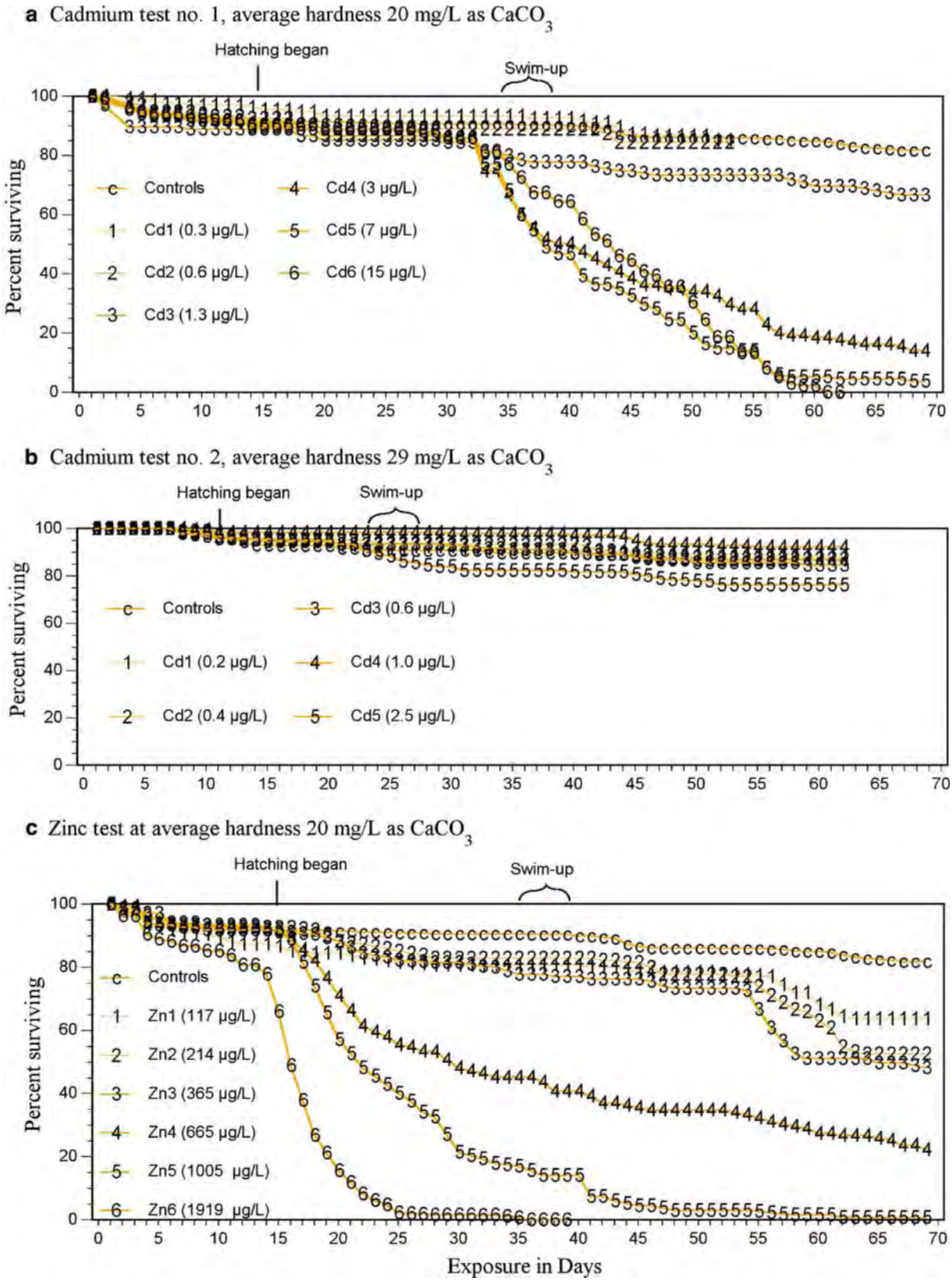
3.3 Rainbow Trout Early-life-stage Tests (ELS)

In the first phase of ELS testing using LNF water with a mean temperature of 9.8°C, the embryos began to hatch at about 14 days, and by 35 days some of the alevins had fully absorbed their egg sacs, were feeding and swimming at the surface of the tanks (i.e. had reached the swim-up stage). By day 39, about 80% of the fish in the controls had reached swim-up stage and the tests were ended 30 days later (69 days total). In the second test phase with a mean test temperature of 12.5°C, hatching began at about 10 days, and about 80% of the fish in the controls had reached the swim-up stage by 27 days The tests were ended at 62 days.

3.3.1 Mortality

Different patterns of responses to metals exposures were apparent with the rainbow trout early-life-stage tests. While evaluating mechanisms of toxicity was not an objective of our study, differences with the timing of the onset and cessation of mortalities suggests differing toxic mechanisms or defenses.

Pb In the first ELS test, concentration-dependent reduced growth and survival was observed with Pb over an exposure range of 12–384 μg/L at an average hardness of 20 mg/L as $CaCO_3$ (Fig. 1c). In the higher treatments, significant mortalities began in the alevin stage, prior to swim-up. In the lowest exposure with reduced survival relative to controls (Pb4, 54 μg/L), mortality increased following swim-up. In this intermediate exposure, mortalities plateaued at day 56,


a Cadmium test no. 1, average hardness 20 mg/L as CaCO3
Hatching began
Swim-up
Percent surviving
c Controls
1 Cd1 (0.3 µg/L)
2 Cd2 (0.6 µg/L)
3 Cd3 (1.3 µg/L)
4 Cd4 (3 µg/L)
5 Cd5 (7 µg/L)
6 Cd6 (15 µg/L)
b Cadmium test no. 2, average hardness 29 mg/L as CaCO3
Hatching began
Swim-up
Percent surviving
c Controls
1 Cd1 (0.2 µg/L)
2 Cd2 (0.4 µg/L)
3 Cd3 (0.6 µg/L)
4 Cd4 (1.0 µg/L)
5 Cd5 (2.5 µg/L)
c Zinc test at average hardness 20 mg/L as CaCO3
Hatching began
Swim-up
Percent surviving
c Controls
1 Zn1 (117 µg/L)
2 Zn2 (214 µg/L)
3 Zn3 (365 µg/L)
4 Zn4 (665 µg/L)
5 Zn5 (1005 µg/L)
6 Zn6 (1919 µg/L)
Exposure in Days

about 12 days after swim-up was complete (Fig. 2a). Treatment Pb6 (384 μg/L) is not included in the Table 4 data summary since complete mortality occurred before the end of the test. In our second test with Pb, over an exposure range of 8–124 μg/L, we saw a similar pattern of mortality with time as in the first Pb test. However, increased mortality was only observed in the highest treatment (124 μg/L). Alevin mortalities began about 10 days after hatching and plateaued about 20 days later. Again, mortalities increased with swim-up (Fig. 2b).

With the Pb exposures, most mortalities occurred around or shortly after the swim-up stage. In the high Pb treatments that ultimately killed most or all fish, there appeared to have been a slight increase in mortalities during the alevin stage (post hatch but prior to swim-up), but by far most deaths occurred within about 10–15 days after swim-up. (Fig. 2a,b). Though not as pronounced, this pattern of early mortalities occurring with swim-up fry is similar to that of fathead minnows exposed as newly hatched fry to Pb for 30 days (Grosell et al. 2006b). In those tests, almost all mortality occurred within day 10 of exposure.

Cd The first Cd ELS test resulted in concentration-dependent survival of rainbow trout with Cd exposures over a range of about 0.3–15 μg/L (Fig. 1e). Little mortality was observed during the embryo phase through hatching until the alevins started to swim-up at about day 32 (Fig. 3a). At an intermediate Cd treatment (treatment 3, 1.3 μg/L), a slight increase (~10%) in mortality was observed over a 4-day period at swim-up. After these initial mortalities, little additional mortality relative to controls occurred over the next month of the test. In the higher three treatments (3–15 μg/L), mortality began at the same time, but in contrast to the intermediate treatment 3, a fairly constant rate of mortality continued until no or only a few survivors remained. No obvious Cd-related mortality occurred in either of the two low treatments (0.3 and 0.6 μg/L). Growth was significantly reduced in the treatments that also experienced high mortality but not at concentrations ≤ 1.3 μg/ at which moderate or no increased mortality was observed (Table 4). In the second Cd ELS test, we used a narrower range of exposure concentrations, with five concentrations ranging from 0.2–2.5 μg/L. Increased mortality was only observed in the highest treatment (2.5 μg/L). Again, almost all mortality in this treatment occurred within about 5-days after swim-up began (Fig. 3b).

Two dosing errors were made during the first Cd test. The first occurred on day 12 when all treatments were overdosed for about 2 days by a factor of about 5×. No increases in embryo mortalities were apparent (Fig. 3a). Metals concentrations resulting from that spike were excluded from the time-weighted average calculations since the spike occurred during the resistant alevin life stage and would have high-biased the average concentrations that the fish experienced during the more sensitive post swim-up stage. The second dosing error occurred on day 54 when treatments Cd1 and Cd2 (0.3 and 0.6 μg/L) were overdosed by a factor of about 10×, killing all fish in both treatments within 48 h. Since this upset occurred 15 days after mortalities ceased in treatment Cd3 (1.3 μg/L), we think that the 53-day exposures were probably long enough to elicit early-life stage responses in treatments 1 and 2. Treatment 6 (15 μg/L) is not included in Table 4, as complete mortality occurred before the end of the test.

In both Cd exposures, mortality patterns in treatments that caused partial mortalities seem consistent with the "damage-repair" model of metals toxicity, recovery, and acclimation of McDonald and Wood (1993). In this model, metals exposure causes a short duration (~2 days) shock phase following exposure of the swim-up stage, followed by a recovery and repair phase, followed by an acclimation phase and increased tolerance to additional exposure. In both of our tests, mortalities began with swim-up and continued for about 5–7 days before ceasing (Fig. 3).

Our inadvertent tests of the responses of rainbow trout embryos and swim-up fry to >5× pulses of elevated Cd resulted in no obvious effects to alevins but killed all swim-up fry. This supported previous reports of the marked differences in sensitivity of the egg and swim-up life stages to Cd toxicity (Chapman 1978b; Van Leeuwen et al. 1985). The interruption of the two low treatments in the first Cd test obviously introduced uncertainty in the results, which is why we repeated the test. However, EC10s and EC20s were

◀ **Fig. 3** Cumulative mortality patterns during early-life stage testing of rainbow trout with cadmium and zinc

similar whether calculated with all seven treatments at 53 days, or the five remaining treatments at 69 days (Table 6). Also, the results of this test were within the range of results from other early life stage or life cycle tests with rainbow trout. When adjusted to a hardness of 50 mg/L $CaCO_3$ hardness, the EC10 or NOEC values from this test at 53-days were intermediate to other values obtained with rainbow trout (Table 7, Mebane 2006).

Zn Concentration-dependent survival of rainbow trout with Zn exposures was observed over a range of 117–1,919 μg/L at average hardness of 20 mg/L as $CaCO_3$ (Fig. 1g). In all Zn treatments except the highest, the onset of mortalities began around day 16 to 22, shortly after hatching began. Mortalities declined about 12-days after their onset and showed no obvious increase for another 30 days, when mortalities increased in treatments 1–3 from around day 56 to 62 (Fig. 3c).

In contrast to the Pb and Cd exposures, mortalities in the Zn exposures did not have any obvious increase with swim-up. Instead, in the intermediate Zn exposures, a few mortalities occurred prior to swim-up, followed by a spike in mortalities about 20 days post swim-up (Fig. 3c). Mortality rates dropped after that time and remained low for the last week of the test in the three mid-range Zn exposures (118–365 μg/L). Because Cd and Zn have similar modes of intoxication, we expected similar patterns in mortality with time. Instead, the pattern observed with the Zn exposures suggests that some Zn acclimation occurred during the embryo stage that delayed, but did not prevent ultimate mortality at these concentrations. Zn acclimation probably did provide some longer lasting protection. The 69-day LC50 was about 3× higher than the 96-hour LC50s, with a 68-day LC50 of 387 μg/L (95th % CI, 309–485) versus 123 μg/L for the comparable 96-hour LC50 (Table 8).

Mortality of fish from Pb, Cd, and Zn in both acute and chronic exposures predominately results from ionoregulatory disturbance following disruption of calcium channels in the gill surface (Grosell et al. 2006b; McGeer et al. 2000; Wood et al. 1997). However, it is possible that exposure to soft water might have resulted in some ionoregulatory disturbance and mortality independent of metals exposure. Taylor et al. (2000) observed that rainbow trout in soft water (hardness 20 mg/L) experienced about 10% mortality after 15 days independent of Cu exposure and that continued at a low rate through their 30-day exposures. Fish exposed to hard water (hardness 120 mg/L) had no mortalities (Taylor et al. 2000). In both of our test groups, control survival rates averaged 81%, suggesting perhaps the hardnesses in our test phases (20–30 mg/L) were similar enough that there was no obvious hardness influence on control mortalities.

3.3.2 Growth

Pb In the first set of tests, decreased growth generally corresponded with increasing concentrations. Fish in the highest Pb treatment were severely stunted (Table 4). Growth as wet weight and survival were equally sensitive endpoints when analyzed by Dunnett's test, growth as length was less sensitive. When analyzed as EC10/EC20 point estimates, survival was the most sensitive endpoint (Table 6). In the second Pb test, slight reductions in length (about 5%) and wet weight (9–14%) were observed in intermediate exposures that had high survival rates (Table 5). However, in the highest treatment in which survival was reduced, growth was increased relative to controls.

This reversal in the expected concentration-growth response was probably due to fish density differences. Similarly, with fathead minnows exposed to Pb, Grosell et al. (2006b) observed increased growth in treatments where final numbers of fish were low due to Pb induced mortality. They concluded that the obvious interpretation was that reduced fish density at higher Pb concentrations allowed remaining individuals to grow faster. Our observations were consistent with Grosell's interpretation. The increased growth occurred in the highest Pb exposure which had less than half the fish density of the controls. Besser (2005b) similarly concluded that reversals of copper concentration–response curves in low-survival treatments suggested a compensatory response of growth to decreased density, decreased competition, or increased food supply. Arthur and Dixon (1994) noted that density can also interact with toxicant-related stress through behavioral size–hierarchy effects. They noted examples where dominant (larger) fish maintained normal growth, where subdominant (smaller) fish grew more slowly, even though there

Table 5 Mean (S.D.) mortality and growth of ELS rainbow trout exposed to Cd and Pb, 62 day test, six pooled control replicates, three treatment replicates

Pb (±SD)	Control (<3)	8.0 (3.3)	18 (7.0)	37 (15)	87 (36)	124 (50)	Minimum detectable difference from control (% of control)
Survival (%)	80.8 (7.8)	81.7 (1.7)	86.1 (4.8)	84.9 (0.9)	82.3 (1.7)	37.8[a] (6.3)	8.8% (9.9%)
Mean wet weight (g)	0.465 (0.02)	0.425 (0.00)	0.400[b] (0.06)	0.422 (0.01)	0.410[b] (0.03)	0.492[c] (0.02)	−0.045 g (9.8%)
Mean length (mm)	36.04 (0.42)	35.63 (0.43)	34.53[b] (1.61)	35.36[b] (0.35)	34.70[b] (0.20)	35.86[c] (0.37)	−1.1 mm (2.9%)
Cd (± SD)	Control (<0.15)	0.16 (0.05)	0.29 (0.17)	0.6 (0.38)	1.1 (0.69)	2.5 (1.6)	
Survival (%)	80.8 (7.8)	80.6 (9.2)	86.1 (5.4)	82.8 (6.9)	87.2 (0.9)	63.3[a] (6.0)	10.3% (12.7%)
Mean wet weight (g)	0.465 (0.02)	0.386[a] (0.03)	0.387[a] (0.03)	0.407[a] (0.05)	0.384[a] (0.01)	0.405[a] (0.03)	−0.043 g (9.3%)
Mean length (mm)	36.04 (0.42)	34.06[a] (0.51)	34.43[a] (0.81)	34.03[a] (1.25)	34.58[a] (0.48)	34.26[a] (0.74)	−1.1 mm (2.9%)

[a] Significantly different from control at $P<0.05$ using Dunnett's procedure. [b] Significantly different from control at $P<0.05$ using the nonparametric rankit data transformation followed by Dunnett's procedure. [c] Increased growth from treatments with low numbers of surviving fish were considered artifacts of the reduced densities and were excluded from statistical comparisons.

Average (±S.D., *n*) hardness 29.4 mg/L as $CaCO_3$ (± 3.6, 13); alkalinity 27 mg/L (±2.1, 9) as $CaCO_3$; pH 7.19 (±0.30, 56), conductivity 69.1 µs (±7.4, 56); dissolved oxygen 9.2 mg/L (±0.9, 248); temperature 12.5°C (±0.9, 248). Average (maximum, *n*) dissolved metals in control samples – Cd<0.2 µg/L (<0.2, *14*), Pb<3.0 µg/L (3, *18*), Zn 13 µg/L (35, *22*).

Underlined values are significantly different than control means at $P<0.05$.

was an excess of food. Instances of increased survival of dominant fish relative to submissive fish were also noted (Arthur and Dixon 1994). Regardless of the exact mechanism, we discounted the increased growth in the less crowded, highest Pb treatment as a test artifact. Thus treatments that had low survival rates and increased growth from treatments with higher survival rates were excluded from statistical analyses.

"Blacktail," darkening of the caudal peduncle, has been reported as a precursor to Pb caused neurotoxicity or skeletal developmental problems in salmonids (Davies et al. 1976; Hodson et al. 1982; Holcombe et al. 1976). While we did not specifically track blacktail occurrence as an endpoint, we did notice that during the first Pb test, some fish in the high treatments which also had growth reductions developed blacktail. Reports of blacktail from other chronic Pb exposures vary. Davies et al. (1976) reported that blacktail began to occur in rainbow trout fry after about 1.5 months of Pb exposures in soft water. Spinal deformities and eroded tails were prevalent about 1 month after the blacktail was observed. Blacktail was the most sensitive effect, occurring more frequently than the deformities (Davies et al. 1976). In contrast, Holcombe et al. (1976), Hodson et al. (1982), and Sauter et al. (1976) observed blacktail only at fairly high Pb concentrations. Holcombe et al. (1976) only observed blacktail and deformities in second and third generation brook trout and Hodson et al. (1982) found that their rainbow trout had to be about 1-year old before blacktail began to appear. Thus, the 60-day early-life-stage test protocol may not be long enough for deformities to develop.

Cd and Zn In the first Cd test, growth as wet weight decreased in a concentration-dependent pattern with the EC10 corresponding to the LOEC. In the second test, with no reduced survival except in the highest Cd treatment, slight (4–16%), but statistically significant reductions in wet weight and length were observed in all treatments (Table 5). In the second Cd test, survival and reduced density in the highest treatment was only reduced by about 20% from controls, compared to the 50% reduction in the highest Pb treatment in the second test. With Zn, growth effects were slight and the growth endpoints were less sensitive than the mortality endpoint (Table 6). Fish weights were progressively lower with increased

Table 6 Comparison of different statistical test endpoints [concentrations in μg/L, except hardness, mg/L]

Metal	Species	Hardness	Days	Endpoint	NOEC	LOEC	MATC	EC10 (95% CI)	EC20 (95% CI)
Pb	Mayfly	19	10	Survival	222	350	279	169 (64–273)	230 (156–304) p
Pb	Mayfly	19	10	Molting	103	160	130	37 (13–104)	66 (28–150) t
Pb	Midge	32	20	Survival	152	>152	>152	nc	nc
Pb	Midge	32	20	Weight	57	75	65	15 (6–35)	28 (11–136) l
Pb	Midge	32	20	Fecundity	152	>152	>152	>152	>152
Pb	Midge	32	55	Emergence	115	128	121	28 (nc)	55 (nc) r
Pb	Rainbow trout	20	69	Survival	24	54	36	26 (20–35)	34 (27–43) p
Pb	Rainbow trout	20	69	Weight	24	54	36	39 (16–92)	55 (29–103) r
Pb	Rainbow trout	20	69	Length	54	143	88	64 (32–126)	98 (68–143) r
Pb	Rainbow trout	29	62	Mortality	87	125	104	108 (0–298)	113 (0–235) p
Pb	Rainbow trout	29	62	Weight	37	87	57	7 (nc)	>87
Pb	Rainbow trout	29	62	Length	8	18	12	>87	>87
Cd	Rainbow trout	20	53	Mortality	0.6	1.3	0.88	0.82 (0.6–1.2)	1.2 (0.9–1.6) p
Cd	Rainbow trout	20	69	Mortality	<1.3	1.3	<1.3	0.89 (0.6–1.2)	1.2 (0.9–1.5) p
Cd	Rainbow trout	20	69	Weight	1.3	2.9	1.9	1.3 (0.07–24)	1.8 (0.15–20) r
Cd	Rainbow trout	20	69	Length	2.9	6.9	4.4	2.6 (nc)	6.8 (nc) l
Cd	Rainbow trout	29	62	Mortality	1.0	2.5	1.6	1.6 (nc)	2.2 (nc) l
Cd	Rainbow trout	29	62	Weight	<0.16	0.16	<0.16	0.15 (0.12–1.6)	>2.5 l
Cd	Rainbow trout	29	62	Length	<0.16	0.16	<0.16	>2.5	>2.5
Zn	Rainbow trout	20	69	Mortality	<117	117	<117	88 (49–159)	147 (96–223) p
Zn	Rainbow trout	20	69	Weight	214	365	279	199 (nc)	387 (nc)
Zn	Rainbow trout	20	69	Length	214	365	279	300 (nc)	>362

nc – no confidence intervals could be calculated. Methods for calculating ECp estimates: p – three factor probit analysis; r – nonlinear regression, using a logistic equation curve (normal distribution); or j – nonlinear regression, using a "jackknife" piecewise linear curve (Erickson 2002); l – non-parametric linear interpolation (Norberg-King 1993).

concentrations except for the highest treatment which only had 19% survival.

A possible explanation for the pattern of slight, non-concentration dependent growth reductions in the second Cd and Pb tests is that they reflect a subtle cost of activating detoxification or repair mechanisms following low-level metals exposures (McDonald and Wood 1993). Once activated, these mechanisms may provide protection at higher exposures, thus no further response would occur until exposures got high enough to overwhelm the detoxification or repair mechanisms. Subsequent experimental evidence seems generally consistent with this explanation. In sublethal exposures of rainbow trout to Zn, Hogstrand et al. (1995) noted increased metabolic cost, increased branchial metallothionein levels, and transient reductions in whole-body growth. Similarly, McGeer et al. (2000) reported that the effects of chronic sublethal exposures to Cu, Cd or Zn followed a pattern of damage, recovery and acclimation. For Cd-exposed fish, the initial damage phase was characterized by a reduced appetite, declines in Na^{+} and Ca^{2+}, and metal accumulation. In Zn exposed trout, there were no changes in feeding or growth although ion loss and gill accumulation occurred. Subsequent to the initial shock phase, ion losses were reversed (McGeer et al. 2000). We have noticed other chronic toxicity tests with metals that similarly reported slight growth reductions in even the lowest treatments so our results may not be that unusual (Chapman 1978a; Hansen et al. 2002a; Hansen et al. 2002b; Hollis et al. 2001; Marr et al.

Table 7 Comparison of the most sensitive endpoints from this study with selected chronic toxicity results (in μg/L) for related species

Species, test type, Endpoint	Metal	Duration	Hardness	NOEC	LOEC	MATC	EC10	EC20	EC10, normalized to hardness 50	Note
Mayfly, *Baetis tricaudatus*, EIL-s	Pb (d)	10 d	20	103	160	130	37	66	88	1
Midge, *Chironomus dilutus*, LC-g	Pb (d)	55 d	32	57	75	65	15	28	87 (n)	1
Midge, EIL-s	Pb (d)	27 d	48	109	497	233	108	149	113 (n)	2
Snail, *Lymnaea stagnalis*, JGS-g	Pb (d)	30 d	102	12	16	14	nc	<4	<2 (EC20)	2
Amphipod, *Hyalella azteca*, LC	Pb (d)	42	138	6.3	16	10	7.2	12	3	3
Daphnid, *Ceriodaphnia dubia*	Pb (t)	7 d	20	51	99	71	nc	nc	121	4
Rainbow trout, ELS-s	Pb (d)	69 d	20	24	54	36	26	34	62	1
Rainbow trout ELS-g	Pb (d)	62 d	29	8	18	12	7	>87	12	1
Rainbow trout ELS-g	Pb (t)	60 d	35	71	146	102	79	99	110	5
Rainbow trout ELS-d	Pb (d)	1.6 yr	28	nc	nc	nc	8.8	10.5	15	6
Rainbow trout JGS-d	Pb (d)	1.5 yr	28	nc	nc	nc	8.2	10.5	14	6
Brook trout, *Salvelinus fontinalis*, LC-d,g	Pb (d)	3 y	44	39	84	57	nc	nc	44 (n)	7
Fathead minnow, *Pimephales promelas*	Pb (d)	30 d	19	17	62	32	nc	21.6	54	8
Rainbow trout, ELS-s	Cd (d)	53 d	20	0.6	1.3	0.88	0.89	1.2	1.6	1
Rainbow trout, ELS-s	Cd (d)	62 d	29	1.0	2.5	1.58	1.61	2.2	2.3	1
Rainbow trout, ELS-s	Cd (t)	100 d	46	1.25	1.74	1.47	nc	nc	1.3 (n)	9
Chinook salmon, *O. tshawytscha*, ELS-s	Cd (t)	120 d	25	1.3	1.9	1.56	1.2	1.8	1.9	10
Bull trout, *Salvelinus confluentus*, JGS-s	Cd (t)	55 d	30	0.38	0.78	0.55	0.49	0.64	0.67	11
Brook trout, LC-gm	Cd (t)	3 y	44	1.7	3.4	2.4	nc	nc	1.8 (n)	12
Brook trout ELS-s	Cd (t)	~60 d	37	1	3	1.7	1.7	2.8	2.1	5
Rainbow trout ELS-s	Zn (d)	69 d	20	<117	117	<117	88	147	191	1
Rainbow trout ELS-s	Zn (t)	62 d	30	112	220	157	140	210	216	13
Chinook salmon ELS-s	Zn (t)	120 d	25	272	511	371	407	623	732	10
Rainbow trout ELS-s	Zn (t)	2 yr	25	nc	nc	nc	318	391	572	14
Rainbow trout JGS-s	Zn (t)	25 d	25	nc	nc	nc	68	87	122	14
Rainbow trout JGS-s	Zn (t)	30-d	30	52	104	74	75	91	116	13
Cutthroat trout JGS -s	Zn (t)	30-d	30	95	190	134	114	125	176	13
Rainbow trout JGS-s	Zn (t)	30-d	29	79	166	114	35	nc	55	15
Brook trout LC-s	Zn (t)	3 y	46	534	1368	854	448	794	481	16
Brook trout JGS-s	Zn (t)	62 d	46	716	1368	989	973	1122	1044	16

Metal: (d) – "dissolved" concentration; (t) – total concentration; nc–not calculable, either because treatments were not replicated precluding statistical NOEC/LOECs (references 5 and 13); inadequate partial responses occurred (ref. 6,14) or because treatment responses were not reported (refs. 2,4,8,9,15); *Test type*: EIL – early instar larval test; ELS – early-life stage test; JGS – juvenile growth and survival test; LC–life cycle test; *Most sensitive endpoint responses*: s – survival, g – growth, d – spinal deformity, b – biomass, u – unknown. Hardness in mg/L as $CaCO_3$. To improve comparability, the 10th percentile effects concentrations (EC10) were normalized to a common hardness value of 50 mg/L using the most comprehensive hardness–toxicity relations available: Mebane (2006) for Cd, Windward (2002) for Pb, and EPA (1987) for Zn. An "n" following the normalized value indicates that the NOEC was used instead of the EC10 because of inability to calculate EC10 values.

Numbered table notes: 1. This study; 2. Grosell et al. (2006a); 3. Besser et al (2005a); 4. Jop et al. (1995); 5. Sauter et al. (1976); 6. Davies et al. (1976); 7. Holcombe et al. (1976); 8. Grosell et al. (2006b); 9. Davies et al. (1993); 10. Chapman (1982); 11. Hansen et al. (2002b); 12. Benoit et al. (1976); 13. Brinkman and Hansen (2004); 14. Sinley et al. (1974); 15. De Schamphelaere and Janssen (2004); and 16. Holcombe et al. (1979).

1996; McGeer et al. 2000; McKim and Benoit 1971; Sinley et al. 1974).

3.4 Statistical and Biological Interpretation of Test Endpoints

In the tests we reviewed for comparison with our results, hypothesis testing was by far the most commonly used statistical analysis approach to establish chronic values and a few studies used no statistical analyses whatsoever (Table 7). However, regression techniques have a number of advantages over hypothesis testing for making point estimates of low toxic effects (Moore and Caux 1997; Stephan and Rogers 1985), and so where possible we calculated comparable ECp values from the previous reports.

In our tests and in most of the tests we reviewed and calculated ECp values from, NOECs were about as high or higher than EC10 values (Tables 6 and 8). In other words, NOECs corresponded with low-effect concentrations, not no-effect concentrations. This blurs the notion of using statistical significance to gauge the biological importance of low toxic effects. Because we were able to pool controls from concurrent tests we lowered the minimum detectable difference from hypothesis testing by about 0.5×. As a result, in our second phase Cd test, small reductions in fish weights from controls were statistically significant where they would not have been significant if evaluated in the usual fashion with the same number of replicates in controls and treatments. The biological importance of slight (3–9%) weight reductions is unclear. For example, EPA guidance for interpreting chronic toxicity data for criteria derivation advised that "the amount of effect that is considered unacceptable is often based on a statistical hypothesis test, but might also be defined in terms of a specified percent reduction from the controls. A small percent reduction (e.g., 3%) might be considered acceptable even if it is statistically significantly different from the control, whereas a large percent reduction (e.g., 30%) might be considered unacceptable even if it is not statistically significant" (Stephan et al. 1985). In other words, they advocate using judgment to select what is an unacceptable amount of adverse effect, rather than rote reliance on a customary but arbitrary statistical level of significance. Likewise, here we sought to decrease the method detection limit of our ELS tests in order to detect threshold effects. Yet rather than simply report statistical detections of differences as therefore adverse, we made informal judgments of the overall test effects concentrations (*sensu* Stewart-Oaten (1995). We describe our rationales and report our data by treatment in addition to just summary statistics in order to provide future compilers the option to accept our data interpretations or to make their own.

For our second Cd test, the statistical LOEC for reduced growth was 0.17 μg/L. However, we think the reduced survival at the highest (2.5 μg/L) Cd exposure is probably the most biologically meaningful overall test effect since it was apparently concen-

Table 8 Acute/chronic ratios (ACR, unitless) resulting from the most sensitive endpoints of each chronic test, calculated by dividing the corresponding 96-h LC50 in μg/L by the chronic MATC, NOEC, EC10, EC20, and LC50 endpoint values in μg/L, (chronic endpoint values in parentheses)

Metal	Species and chronic endpoint	96-h LC50 (95% CI)	ACR calculated using the chronic:				
			NOEC	MATC	EC10	EC20	LC50
Pb	Mayfly – 10 day molts	664 (368–1,315)	6.4 (103)	5.2 (130)	32 (21)	11 (60)	2.0 (333)
Pb	Midge – 20 day weight	3,323 (1,983–8,971)	58 (57)	51 (65)	221 (15)	118 (28)	nc (>152)
Pb	Rainbow trout – 69 day survival	120 (104–138)	5 (24)	3.3 (36)	4.6 (26)	3.5 (34)	2.2 (55)
Pb	Rainbow trout – 62 day length	133 (a)	17 (8)	11 (12)	19 (7)	1.3 (102)	1.1 (120)
Cd	Rainbow trout – 53 day survival	0.84 (0.62–0.95)	1.4 (0.6)	0.95 (0.88)	0.96 (0.89)	0.60 (1.2)	0.3 (2.5)
Cd	Rainbow trout – 62 day survival	0.89 (0.8–0.98)	0.9 (1.0)	0.56 (1.58)	0.56 (1.6)	0.4 (2.2)	nc (>2.5)
Zn	Rainbow trout – 69 day survival	123 (b)	nc (<117)	nc (<117)	1.4 (88)	0.8 (147)	0.3 (387)

(a) Geometric mean of two acute tests conducted concurrently with the chronic test with Pb LC50s of 127 (102–151) and 140 (118–163) μg/L. (b) Concurrent Zn acute test failed test acceptability criteria; value is the geometric mean of two later acute tests which used eggs from the same supplier, eggs and fry were incubated in the same acclimation water source, and were tested in similar hardnesses to the as the Zn ELS. The two Zn LC50s (μg/L) were 117 μg/L (87–161) at hardness 16 mg/L as $CaCO_3$ and 130 (113–149) at hardness 24 mg/L as $CaCO_3$. nc–not calculable.

tration dependent and the growth reductions were not. Further, most other studies have found that unless mortality results in the early stages of exposure, the long-term physiological or metabolic costs of sublethal Cd exposures in juvenile fish may be slight (Hollis et al. 1999; McGeer et al. 2000). Benoit et al. (1976) observed that adult male brook trout that showed no adverse effects of long-term exposure to 3.4 μg/L Cd in soft water became hyperactive and died when attempting to spawn. However, because this concentration was 3–20× higher than our exposures in question, it did not seem reasonable to us to predict similar potential life cycle risks from ELS effects.

In contrast to Cd, where the fry that are not killed within a few days of exposure may suffer few lasting ill effects, some research on the effects of long-term Pb exposure to fish suggests that the usual ~60-day ELS exposures may not be long enough for physiological effects of Pb poisoning to fully materialize (Davies et al. 1976; Hodson et al. 1982). In continuous exposures of 3-generations of brook trout (*Salvelinus fontinalis*), at low-effect treatments, adverse effects (deformities and reduced growth) were not detected until the second and third generations (Holcombe et al. 1976). With rainbow trout, deformities have developed near concentrations that caused subtle growth effects in our tests (Table 7). Thus with Pb exposures, it seemed prudent to consider even subtle effects observed in ELS testing as potentially important in predicting life cycle effects from ELS exposures.

3.5 Comparisons of Chronic Values with Previous Studies

We selected what we considered were the best summary endpoints from our tests (i.e., the most sensitive endpoints that had the best concentration response pattern) and compared them to effects from selected studies that tested related organisms in soft water. To improve the comparability of chronic low-effect thresholds across tests, where EC10 values were reported or could be reliably estimated, they were adjusted to a hardness of 50 mg/L using previous hardness-toxicity regressions. EC20s or NOECs were used if EC10s could not be calculated with confidence (Table 7).

With Pb and invertebrates, low-effect values from our mayfly and midge tests fell within the range of the other chronic studies with invertebrates that we compared. Low-effect Pb values for the mayfly and midge happened to both be around 87–88 μg/L after hardness adjustment. These low-effect values are intermediate to values from sensitive invertebrates ranging from about <2–3 μg/L (*Lymnaea* and *Hyalella*) and the relatively high *Ceriodaphnia* dubia value of 121 μg/L (, Table 7). With Pb and salmonids, our lowest effects concentrations with rainbow trout were nearly as low as the most sensitive values located (i.e., EC10s of about 7 and 8.8 μg/L for our 62-day test and the 570-day test of Davies et al. (1976), respectively). A Pb fathead minnow value that was obtained using very similar dilution water characteristics as our first Pb test with rainbow trout yielded similar low effects values (EC20s of 22 and 34 μg/L for the fathead minnow fry and rainbow trout ELS survival respectively, Table 7, both tested at hardness 19–20 mg/L, pH 6.7, and DOC about 1.3 mg/L). Grosell et al. (2006a) compiled a SSD of mean species chronic NOECs for 19 species without hardness adjustments. Our midge and mayfly NOECs would fall near the mid-range of their SSD, with ranks around the 8th and 13th most sensitive species respectively. Our mean rainbow trout NOECs would fall between the 2nd and 3rd most sensitive species, as opposed to their 5th most sensitive ranking in the SSD of Grosell et al. (2006a).

With Cd and salmonids, values were remarkably similar across species and tests, with hardness-adjusted low effects values differing by less than 3× for all seven test values compared (0.7 to 2.3 μg/L) and less than 2× for six of the seven tests (range 1.3 to 2.3 μg/L, Table 7). With Zn and salmonids, our results using the hatchery's LNF water source were lower than any we had located for salmonids at the time. With Zn, although we used a range of exposure concentrations that encompassed the range of previously reported chronic effects concentrations with salmonids at the time, we observed increased mortality at the lowest concentration tested. Subsequently, we have reviewed several long-term Zn exposures with salmonids where exposures began with fry instead of eggs (i.e., juvenile growth and survival (JGS) instead of ELS tests. Most of the JGS tests were more sensitive (Table 7).

The life stage at which the tests were started and opportunity for subsequent acclimation appears to be a major influence of test sensitivity in the fish tests

compared. The lower low-effect values (more sensitive) results from the fish tests initiated with swim-up fry compared to ELS tests was an obvious pattern from our comparisons of low-effect values for Cd and Zn. (Table 7). The increased sensitivity of swim-up fry to metals is probably related to the increased rates of respiration corresponding with the increased body activity as the fry turn from non-free-living to the free-living stage and have to capture food. Much greater water volumes move across gills to meet gas exchange needs in swim-up fry stage than sac fry stage (Van Leeuwen et al. 1985). However, although ELS tests encompass this vulnerable life stage, if acclimation is induced from exposure to metals at the insensitive egg and alevin stages, then starting a test at the acutely vulnerable swim-up stage may provide more sensitive results than ELS testing. A series of Cd and Zn tests with three species of trout that were initially exposed as either fry or eggs, consistently showed acclimation and resistance developed during the ELS exposures (Brinkman and Hansen 2004, 2007). Similar patterns occur with Cd and other fish species (Mebane 2006). Chapman (1994) tested the combined effects of acclimation and life stage sensitivity to copper during early life stages of rainbow trout. Fish were exposed continuously starting at four distinct stages: fertilization, eye pigmentation, hatch, and swim-up. The most sensitive test was exposure beginning at swim-up with no prior exposure to provide acclimation. The least sensitive test was to begin exposure at either at hatch or at the eyed stage prior to hatch (i.e., as it is done in ELS tests). In all exposures, a pulse of mortality followed the fish reaching swim-up in all exposure scenarios. The egg exposure provides some opportunity for acclimation prior to reaching the sensitive swim-up stage (Chapman 1994). Chapman (1978a) also noted similar patterns with Zn and sockeye salmon, and Guadagnolo et al. (2000; 2001) found that with rainbow trout, the egg was nearly impenetrable to acute or chronic silver exposures, with the chorion preventing 83–99% of the metal from reaching the embryo. An exception to this pattern was the test pair of Besser et al. (2005b) with rainbow trout and copper in a 60-day ELS test and a 30-day fry test which had similar results. For Pb, it seems likely that life stage and acclimation would affect results similarly, and indeed the authors of the sole comparative study with Pb located made similar conclusions (Davies et al. 1976). However we calculated nearly identical ECp values from their data (Table 7), suggesting a lesser effect of acclimation with Pb prior to swim-up. This is consistent with our observations of a slight increase in mortalities after hatching followed by a pulse of mortality around swim-up (Fig. 2).

This issue suggests that the environmental exposure scenarios and life histories should indicate relevant test exposure scenarios rather than simply defaulting to customary methods. For example, if salmonid spawning habitats are expected to be nearly continuously exposed to metals, the ELS exposure regime would be appropriate. But if salmonids spawned in headwater habitats with low metals and initial exposures occur as post-swim-up fry migrate downstream into urbanized or industrialized areas, then initiating tests at the latter stage could be more relevant and sensitive.

Dissolved organic carbon (DOC) and calcium/magnesium ratios are factors that may have affected the toxicity of metals in our test waters. DOC may be an important modifier of Pb bioavailability and toxicity, less so for Zn and Cd (Bringolf et al. 2006; Macdonald et al. 2002; Playle 2004). Prior to conducting the tests described here, pilot tests were conducted over several months at the University of Washington, School of Oceanography and Fisheries Laboratories, Seattle, Washington USA using dilution water collected from the upper SFCDR with resident invertebrates collected from the upper SFCDR or hatchery rainbow trout. In these pilot tests, ambient DOC was not strongly correlated with the acute toxicity of Pb, Zn, or Cd. DOC concentrations measured in SFH dilution waters at test initiation and after renewal were fairly low across different seasons (median DOC concentration <1.5 mg/L, 95th percentile 1.96 mg/L, range <1.5 to 4.0 mg/L, n=25). Because DOC did not obviously help explain the toxicity at the low concentrations encountered, as an economic compromise we did not continue DOC analyses through the tests described here. Monthly DOC sampling by the U.S. Geological Survey from the SFCDR and the adjacent NFCDR watersheds that spanned our second phase of testing ranged only from 0.4 to 1.3 mg/L, median 0.6 mg/L, n=25 (http://waterdata.usgs.gov, sites 12413470 and 12413000). Thus we think that DOC concentrations were close to 1 mg/L during our tests, and were almost certainly less than 2 mg/L. These concentrations were probably similar to those in test results compared in Table 7

that were conducted in laboratories using surface water sources or blends such as Lake Superior water (about 1.3 mg/L, (Benoit et al. 1976; EPA 2007; Holcombe et al. 1979; Holcombe et al. 1976; Maier and Swain 1978)) and EPA's Western Fisheries Toxicology Station in Oregon (about 1.6 mg/L DOC, (Chapman 1982; EPA 2007).

The low Ca/Mg ratio of the LNF water source may have been another factor in the relatively low effects concentrations from the first group of ELS tests. Toxicity of metals generally decreases as water hardness increases and water hardness is a function of calcium and magnesium concentrations. However, calcium hardness probably contributes more protection to fish from Cd and Zn toxicity than does magnesium hardness (Alsop and Wood 1999; Carroll et al. 1979; Davies et al. 1993). The molar ratio of Ca/Mg in the LNF water source was about 1.4 (Table 1), which was lower than those from the other tests summarized in Table 7 except for the juvenile growth and survival test of Hansen et al. (2002b) with bull trout and Cd (Ca/Mg ratio of 1.1).

3.6 Acute-to-chronic Ratios

The practice of using ACRs to estimate chronic thresholds that are protective of aquatic communities has various interpretations. Most interpretations use an acute LC50 as the numerator, and the lowest chronic response (or highest non-response) that can be related to survival, growth, or reproduction is used as the denominator. However, at least five different of statistical endpoints for the chronic denominator have been reported, which results in very different ACR values. As commonly used in the United States for deriving water quality criteria for aquatic life, ACRs are commonly calculated as an acute LC50/MATC (Brix et al. 2001; Mount et al. 2003; Raimondo et al. 2007; Stephan et al. 1985). ACRs have also been reported as LC50/EC20s. EPA (2007) used chronic EC20 values as a replacement of the MATC in ACRs to derive water quality criteria for Cu and Hansen et al. (2002a) selected the EC20 for calculating ACRs for Cu with rainbow trout because a growth EC20 usually occurred at a concentration that caused little mortality to salmonids. In the European Union, Australia and New Zealand, ACRs are usually calculated as LC50/NOEC when used to derive water quality guidelines or to register industrial substances for use. Canada has a national goal for environmental quality of no observable adverse effects on aquatic and terrestrial ecosystems over the long-term, and uses chronic NOECs as the corresponding statistic for that goal (Kent et al. 2003; Länge et al. 1998). If chronic EC10 values are substituted as point estimates to replace NOECs as suggested by Moore and Caux (1997) and van der Hoeven et al. (1997), it follows that ACRs would be calculated as LC50/EC10. Finally, ACRs have been reported as an acute LC50/long-term LC50 (Grosell et al. 2006b). In this context by using the same endpoint over different durations, ACRs reflect the influence of duration or acclimation on lethality, rather than a ratio to estimate protective concentrations for untested species.

With Pb and the mayfly and midge tests, ACRs calculated by EC20s, NOECs or MATCs resulted in about 2× differences. With Pb and the rainbow trout tests, differences between ACRs calculated by dividing the LC50 by the MATCs, NOECs, EC20s, or EC10s ranged from 1 to 4×. In the first Pb test conducted at hardness 20 mg/L, for both the mayfly and rainbow trout the long-term LC50s declined by half, providing LC50/LC50 ACRs of about 2. In contrast, in the second Pb test conducted at a higher hardness, the 4-day and 60+ day LC50s were nearly identical, with an ACR of 1.1. This is consistent with the observation of Taylor et al. (2000) that in soft waters (20 mg/L in their tests), the combined effect of increasing Cu toxicity and the added stress associated with soft-water exposure appear to favor lethality over acclimation.

ACRs were from <1 to 2 for both Cd and Zn regardless of calculation endpoint. With Cd, we believe this is likely because its waterborne toxicity to fish is short acting. With moderate Cd exposures, fish either died within a few days after exposure to the swim-up stage, or else they survived for the duration of the test with slightly (~10%) lower growth than controls. This pattern is similar to other reports of chronic exposures of salmonids to Cd that began after the egg or alevin life stage (Hansen et al. 2002b; Hollis et al. 1999). With both Cd and Zn, LC50s from the long-term ELS exposures were about 3× higher than 4-day LC50s from the static-renewal acute exposures.

ACR studies can be seriously confounded by using resistant fish in the acute tests. Generally for metals, swim-up fry are thought to be the most acutely sensitive life stage (discussed earlier). However, some

older ACR studies used larger fish in acute tests which were resistant to metals, resulting in high LC50s and unrealistically high ACRs. For example, Davies et al. (1976) obtained similar lowest chronic effect concentration with rainbow as did we in waters of similar hardness (lowest EC10s were 8.8 and 7 μg/L respectively, Table 7), but their acute LC50 was about 10× higher than ours. Davies et al. (1976) obtained a 96-h dissolved Pb LC50 with rainbow trout of 1,170 μg/L at a hardness of 32 mg/L as $CaCO_3$. Using their own judgments of what constituted an adverse effect (which differed from the authors' published judgments) EPA (1984) calculated an ACR of 62 from the Davies et al. (1976) tests. The Davies et al. (1976) rainbow trout averaged 165 mm in length, and were probably about 1-year old fish. In contrast, using smaller and younger rainbow trout that were about 30 mm in length, 0.2 to 0.4 g, and 2-to 4 weeks post-swim-up, we obtained a 96-h dissolved Pb LC50 of 120 μg/L at a hardness of 29 mg/L as$CaCO_3$ and our ACRs with comparable chronic endpoints ranged from 3 to 17 (Table 8). Similarly, Holcombe et al. (1976) obtained a dissolved Pb MATC of 57 μg/L in a 3-year, 3-generation study with brook trout, a value only about 2–4× higher than our MATC estimates of 12–36 μg/L with rainbow trout. Yet using 72-week old, 102 g brook trout in an acute test, they obtained a 96-h dissolved Pb LC50 of 3,362 μg/L which yields an ACR estimate of 59. The Davies et al. (1976) and Holcombe et al. (1976) partial and full life cycle tests with 2–3 year exposures were important studies with durations that are unlikely to often be repeated. However, we would not use their insensitive acute results for estimating ACRs or for inclusion in SSD datasets.

(Raimondo et al. 2007) analyzed EPA databases and found that ACRs derived from ELS tests had significantly lower median values than those derived from life cycle tests, which implies that ACRs derived from ELS tests are underprotective. However for Pb, their data source was a dataset that included the insensitive acute values for fish discussed here. Since chronic thresholds from life cycle and ELS tests overlap (Table 7), this pattern of higher ACRs from life cycle tests may not hold for Pb.

A practical constraint when developing water quality criteria using EPA guidelines is that "final" (overall) ACRs for a substance cannot be <2.0. This is because acute criteria are defined using the LC50 of the 5th percentile most sensitive species (FAV), which is then divided by 2 to extrapolate from a lethal concentration to a low-effects concentration for sensitive organisms (Stephan et al. 1985). Thus if an ACR <2 were used for a chemical criterion, the illogical result would be a higher criterion for long-term exposures than for short-term exposures. Only for Pb were our ACR estimates consistently >2 (Table 8).

For Pb, our overall MATC-based estimate of a Pb ACR relevant to a coldwater, stream community was about 12: the geometric mean of the species mean ACRs for the mayfly, midge, and rainbow trout. If in lieu of MATCs, Pb ACRs were calculated using NOECs, EC10s, or EC20s, our overall estimates were about 15, 40, and 14 respectively. These estimates are considerably lower than the MATC-based estimate of EPA (1984) of 51 for aquatic communities, which was influenced by the ACR test pairs previously discussed that used insensitive fish in their acute tests. For Cd, Mebane (2006) estimated an overall ACR of 3.4 for aquatic communities based on data from 17 species (38 test pairs, ACR ranged from 0.35 to 435). Similarly to our results, among tests with salmonids, ACRs in 11 test pairs ranged only from 0.35 to 3.4 with the species mean ACRs all <2. For Zn, the overall ACR estimate of EPA (1987) for aquatic communities was <2, which was supported by our results with rainbow trout.

4 Conclusions

We were pleased with the results from the mayfly growth and survival test, and recommend expanding the technique to other species and contaminants. The test provided reasonably sensitive results that were much lower than acute values and were intermediate to results obtained with other species (Tables 7 and 8). By testing field collected mayflies in their native water, the problem of hardness acclimation was avoided. Because of the short life span of baetid mayflies, the test was much shorter and less costly than the *C. dilutus* life cycle test.

The ability to substantially increase the statistical power of detecting changes from controls by increasing control replicates may have practical benefit to testing programs. In our tests, this increased statistical power was accomplished at no additional cost because two or three ELS tests were being conducted in parallel. Because conducting concurrent tests with common dilution water and test organisms is doubt-

fully unusual in testing laboratories, because Dunnett's test is often used to analyze differences from controls, and because of the long standing advice of Zar (1984), we are surprised how seldom studies report pooling controls to improve the statistical power of Dunnett's test. However, by reducing minimum detectable effects, investigators may have to more often judge which detectable effects are important adverse effects rather than simply relying on rote statistical significance. This is analogous to improvements in method detection limits for banned or hazardous chemicals. Where previously, investigators could simply report "not detected," as detections at lower concentrations are made investigators have to make judgments of their importance.

Our Pb ACR estimates of 51, 5, and 6 for the midge, mayfly, and rainbow trout, respectively (calculated with MATCs and a geometric mean of the two trout tests) were roughly consistent with previous observations that ACRs follow patterns with the highest ACRs for the acutely resistant species, decreasing with decreasing acutely resistant species (Brix et al. 2001; de Zwart 2002; Duboudin et al. 2004; Stephan et al. 1985). However, our results with rainbow trout were not consistent with previously derived Pb ACR values with salmonids. When calculated with MATCs, our Pb ACRs with rainbow trout ranged from 3.3 to 11; similarly calculated ACRs from previous studies with brook trout and rainbow trout ranged from 58 to 123, respectively (Davies et al. 1976; Holcombe et al. 1976). These previous Pb ACR estimates were much higher than ours because our acute tests produced LC50s that were 10 to 25× lower than the earlier studies. The differences in acute sensitivity were probably related to the size of fish used in the acute tests. Our tests were conducted using two to four week old fry, where the previous tests used fish that were considerably larger and were probably subadult, yearling fish. Our Cd and Zn ACRs of about 0.5 to 2 with rainbow trout suggest little practical difference in the sensitivity of rainbow trout to Cd and Zn in short- or long-term exposures, at least for growth and survival.

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