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Responses of Juvenile Mussels to Metals in Sediment and Water of the Tri-State Mining District

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U.S. Department of the Interior
U.S. Geological Survey

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Conversion Factors

U.S. customary units to International System of Units

Multiply	By	To obtain
	Flow rate	
foot per second (ft/s)	0.3048	meter per second (m/s)

International System of Units to U.S. customary units

Multiply	By	To obtain
	Length	
micrometer (µm)	3.93701×10^{-5}	inch (in.)
centimeter (cm)	0.3937	inch (in.)
millimeter (mm)	0.03937	inch (in.)
meter (m)	3.281	foot (ft)
meter (m)	1.094	yard (yd)
	Area	
square meter (m^2)	0.0002471	acre
	Volume	
nanoliter (nL)	3.3814×10^{-8}	ounce, fluid (fl. oz)
milliliter (mL)	0.033814	ounce, fluid (fl. oz)
liter (L)	33.81402	ounce, fluid (fl. oz)
liter (L)	2.113	pint (pt)
liter (L)	1.057	quart (qt)
liter (L)	0.2642	gallon (gal)
liter (L)	61.02	cubic inch (in^3)
	Mass	
milligram (mg)	3.5274×10^{-5}	ounce, avoirdupois (oz)
gram (g)	0.03527	ounce, avoirdupois (oz)

Temperature in degrees Celsius (°C) may be converted to degrees Fahrenheit (°F) as follows:

$$°F = (1.8 \times °C) + 32.$$

Concentrations of chemical constituents in water are given in either milligrams per liter (mg/L), micrograms per liter (µg/L), or grams per liter (g/L).

Molar concentrations are given in micromoles per gram (µmol/g).

Simultaneously extracted metals concentrations are given in micrograms per gram (µg/g).

Abbreviations

ANOVA	analysis of variance
AVS	acid-volatile sulfide
CCVS	continuing calibration standards
CERC	Columbia Environmental Research Center
CPUE	catch per unit effort
CRM	concentration-response model
DOC	dissolved organic carbon
EC20	20-percent effect concentration
EPA	U.S. Environmental Protection Agency
ESA	U.S. Endangered Species Act
GM–EC20	geometric mean of 20-percent effect concentrations
ICP–MS	inductively coupled plasma-mass spectrometer
ICVS	initial calibration standards
LCS	laboratory control standard
MT	metallothionein
NRDAR	Natural Resource Damage Assessment and Restoration
p	probability
PEQ	probable effect quotient
PES	polyethersulfone
PWP-zinc	zinc in pore-water peepers
QA/QC	quality assurance/quality control
SE	standard error
SEM	simultaneously extracted metals
[SEM–AVS]	simultaneously extracted metals normalized to acid-volatile sulfide
[ΣSEM–AVS]	summed molar concentrations of simultaneously extracted metals relative to acid-volatile sulfide
sum-PEQ	summed probable effect quotient values

T20	20-percent effect concentration for survival
TAC	test acceptability criterion
TOC	total organic carbon
TR	total recoverable
USGS	U.S. Geological Survey
XRF	X-ray fluorescence
>	greater than
≥	greater than or equal to
<	less than

Responses of Juvenile Mussels to Metals in Sediment and Water of the Tri-State Mining District

By John M. Besser,[1] Chris D. Ivey,[1] James L. Kunz,[1] Nile E. Kemble,[1] Danielle M. Cleveland,[1] Jeffery A. Steevens,[1] Heidi Dunn,[2] and Ryan Foley[2]

Abstract

The U.S. Geological Survey and collaborators from EcoAnalysts, Inc., completed field and laboratory studies during 2016–19 to evaluate the toxicity of metals to freshwater mussels in streams draining the Tri-State Mining District. This project consisted of (1) sampling and analysis of metals in water and sediment, (2) surveys of mussel assemblages at sites with suitable mussel habitat, (3) toxicity tests with juvenile mussels exposed to zinc or to a mixture of metals (zinc, lead, and cadmium) in water, and (4) toxicity tests to evaluate the contributions of metals in sediment and metals in overlying water to toxic effects on mussels. Field sampling at sites in the Spring River and Neosho River and their tributaries demonstrated wide ranges of metal contamination in water and sediment. Zinc was the predominant toxic metal in water, and concentrations of lead and cadmium were much lower. Mussel areal density and species richness were greater at reference sites with low sediment metal concentrations (for example, zinc, 29–141 micrograms per gram) than at test sites that had higher concentrations of sediment zinc (416–3,420 micrograms per gram) as a result of effects of upstream mining activity. Juvenile mussels were highly sensitive to zinc in water in 12-week toxicity tests compared to previous water-only tests, and adding low levels of waterborne lead and cadmium typical of their occurrence in Tri-State Mining District streams produced greater toxicity. Thresholds for mussel toxicity were at or less than waterborne metal concentrations detected in Tri-State Mining District streams, and sites with waterborne metal concentrations exceeding thresholds had decreased mussel density and decreased mussel species richness. The 12-week toxicity tests with juvenile mussels in Tri-State Mining District sediments also demonstrated negative mussel responses with metal exposure. Thresholds for reductions in survival, growth, or biomass were at sediment metal concentrations less than thresholds reported for previous 4-week tests. We documented strong associations between reduced survival in laboratory tests and reduced species richness in community surveys. Attempts to estimate combined toxicity thresholds for metals in sediment and overlying water were not successful. These inconclusive results may be attributable to several factors, including (1) unexpected losses of waterborne metals from solution, (2) differences in sensitivity of different age/size classes of juvenile mussels, (3) disruption of sediment-water equilibria and changes in metal bioavailability, and (4) behavioral or physiological responses allowing juvenile mussels to temporarily reduce or avoid metal exposure. We also observed differences in metal toxicity thresholds between sediment toxicity tests started with different ages/sizes of test organisms. A followup study that combined exposure to Tri-State Mining District sediments with exposures to multiple levels of waterborne metals demonstrated toxic effects of sediments with low metal concentrations; however, some treatments also indicated unexpected reversals of concentration-response trends and reduced toxicity in treatments that had high metal concentrations in overlying water. These unusual responses may reflect development of physiological tolerance to metal toxicity by induction of metal-binding proteins (for example, metallothionein) in response to high metal levels in water.

Results of laboratory and field studies indicated strong associations between metal exposure in Tri-State Mining District streams and toxic effects on juvenile freshwater mussels. Mussel community characteristics corresponded to differences in metal concentrations in sediment and water among Tri-State Mining District sampling sites. Responses of juvenile mussels in 12-week water and sediment exposures were strongly correlated with the status of mussel assemblages in Tri-State Mining District streams. The combined results support the hypothesis that exposure to metals from historical mining activities adversely affects freshwater mussel communities in the Spring River/Neosho River drainage.

1. Introduction and Scope

Freshwater mussels (Unionidae) are sensitive to toxic effects of many aquatic contaminants, including metals (Wang and others, 2010, 2013, 2020; Besser and others, 2015). Mussels also are frequently listed as endangered or threatened under the U.S. Endangered Species Act (ESA; Williams and

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others, 1993; Haag and Williams, 2014). Effects of pollution on ESA-listed mussels can be a legal basis for recovery of natural resource damages by the U.S. Department of the Interior and State and Tribal natural resource trustees through the Natural Resource Damage Assessment and Restoration (NRDAR) program. Two historical mining areas in Missouri, the Southeast Missouri Lead District (Roberts and others, 2016) and the Tri-State Mining District are currently subjects of NRDAR investigations of potential injury to ESA-listed freshwater mussels. Studies of mussel communities in streams draining these areas have documented declines in resident mussel communities, which correspond to metal pollution from historical mining (Angelo and others, 2007; Ingersoll and others, 2008; Besser and others, 2015). In the Tri-State Mining District, Angelo and others (2007) reported reduced mussel taxa richness at sites downstream from mining areas, compared with upstream sites, and determined statistically significant negative correlations between mussel taxa richness and metal concentrations in sediments. Laboratory sediment toxicity tests completed by the U.S. Geological Survey (USGS) in 2007 with metal-contaminated sediments from streams documented adverse effects on several species of benthic invertebrates (Ingersoll and others, 2008), but toxic effects on juvenile mussels (*Lampsilis siliquoidea* [Barnes, 1823]; fatmucket) were less severe than effects on other test organisms, notably the freshwater amphipod (*Hyalella azteca* [Saussure, 1858]; Besser and others, 2015). *Lampsilis siliquoidea* has been widely used for laboratory toxicity testing and is a reliable surrogate for responses of other unionid mussel species, including ESA-listed species (Wang and others, 2007).

The purpose of this report is to document metal toxicity thresholds associated with injury to mussels based on long-term exposure to metals in water and in sediment. To accomplish this, we designed and completed studies that included four components:

1. Sampling and analysis of metals in water and sediment at selected sites in the Tri-State Mining District,
2. Surveys of mussel assemblages at sites sampled for water and sediment,
3. 12-week toxicity tests to evaluate thresholds for toxic effects of metals in Tri-State Mining District stream water to juvenile mussels and amphipods, and
4. 12-week toxicity tests to evaluate thresholds for toxic effects of metal-contaminated sediment to juvenile mussels, with and without added metals in overlying waters.

Sites for collection of water and sediment for chemical analysis and toxicity testing were colocated with sites of mussel community surveys whenever possible to enable more direct comparisons of mussel responses in the laboratory and in the field. Exposure of mussels to metals in laboratory studies and at field survey sites was characterized by chemical analyses of metals in sediment, pore water, and overlying water. Low-metals reference sites and test sites potentially affected by metals from mining were characterized to ensure they represented suitable physical habitat for freshwater mussels (EcoAnalysts, Inc., 2018). Toxicity tests with juvenile mussels (*L. siliquoidea*) included exposure to metals in field-collected sediments and laboratory-prepared waterborne mixtures typical of streams in the study area, separately and in combination. Mussel toxicity tests were started with smaller juvenile mussels and had a longer (12-week) exposure period, compared to previous tests with sediments (Ingersoll and others, 2008). For comparison with results of the previous 4-week amphipod tests, 6-week sediment toxicity tests also were completed with amphipods (*H. azteca*; MacDonald and others, 2009; Besser and others, 2015). Data generated during this study are available as a USGS data release (Ivey and Besser, 2022).

2. Metal Concentrations in Water and Sediment and Status of Mussel Community

Mining in the Tri-State Mining District took place over a large geographic area (fig. 1) in adjacent parts of Missouri, Kansas, and Oklahoma (Johnson and others, 2016). In southwestern Missouri, large-scale mining took place primarily in lands drained by west-flowing tributaries of the Spring River, including Center Creek, Turkey Creek, and Shoal Creek. Mining-affected areas of southeastern Kansas included small tributaries and the main stem of the Spring River, including an impoundment, Empire Lake. In northeastern Oklahoma, mining-affected streams included highly contaminated Tar Creek and downstream reaches of the Neosho River, as well as the lower Spring River downstream to its confluence with the Neosho River and its terminus in Grand Lake O' the Cherokees.

Subsurface mining throughout the Tri-State Mining District until the 1970s left many large deposits of fine-grained tailings near ore-processing locations. Tailings were distributed across the landscape by wind and water erosion and by human activities, resulting in metal contamination of stream sediments and leaching of metals into groundwater and surface water. Unlike some other mining areas where Mississippi Valley type ores were mined primarily for lead, ores and mine wastes in the Tri-State Mining District were predominantly enriched with zinc rather than lead, and the greater solubility of zinc led to potentially greater toxicity hazards via exposure to waterborne metals (Besser and others, 2015; Gutiérrez and others, 2020). To date, remediation of contaminated areas in the Tri-State Mining District has primarily been completed under the auspices of the Comprehensive Environmental Response, Compensation, and Liability Act.

Remediation efforts have focused on removal of metal-contaminated residential soils, consolidation of mine wastes and capping with uncontaminated soil in upland habitats,

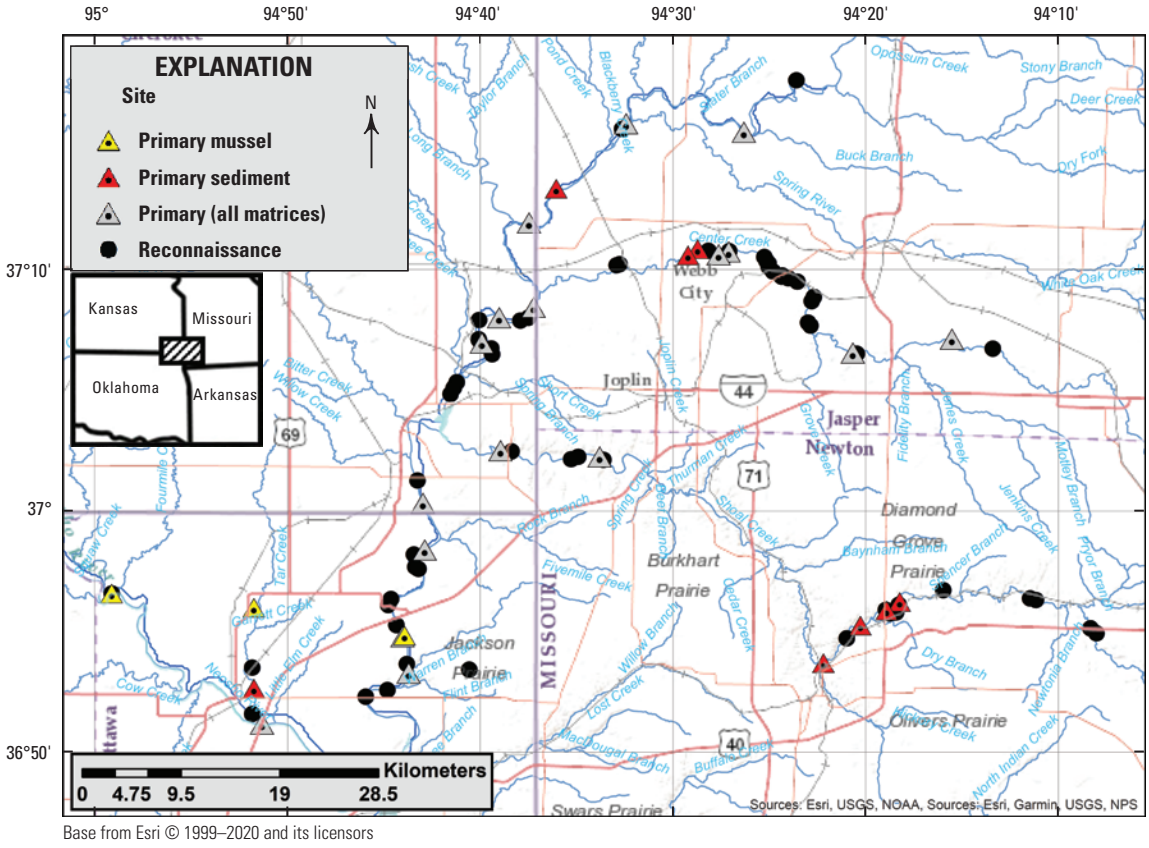

Figure 1. Locations of sampling sites. Map modified from EcoAnalysts, Inc. (2018). Inset shows the approximate location of the Tri- State Mining District (hatched area). Inset data from U.S. Environmental Protection Agency (2021a).

reburial and capping of mine wastes in subsurface mine workings, and pH neutralization and addition of organic amendments to contaminated subsoils to encourage revegetation (Juracek and Drake, 2016). Additional remedial efforts involving removal of mine waste from ephemeral streams were completed in Missouri and Kansas; however, remedial efforts involving removal or isolation of solid mine wastes from stream channels, floodplains, and impoundments are in the planning stages. Despite these ongoing efforts to control sources of metal contamination, many miles of streams remain contaminated by metal-contaminated sediments and or by dissolved metals leached from mine wastes and contaminated soils.

Data from the 2007 USGS investigation of sediment toxicity in the Tri-State Mining District (Ingersoll and others, 2008) were analyzed by MacDonald and others (2009) with the goal of establishing objectives for cleanup based on assessment of residential human health risk and risk of toxicity to aquatic biota. Targets for remediation of stream and reservoir sediments were based mainly on toxicity tests with *H. azteca*, which was the most sensitive species tested. Despite the evidence of effects of metals on mussel communities of the Spring River drainage reported by Angelo and others (2007), the previous sediment toxicity studies determined that juvenile *L. siliquoidea* were less sensitive to toxicity of metals than were *H. azteca* (MacDonald and others, 2009).

This study was part of the ongoing NRDAR cases by Trustees Councils in Kansas, Missouri, and Oklahoma and was focused on characterizing toxic effects of metals on freshwater mussels rather than effects of other pollutants or habitat degradation. Therefore, sampling sites for mussel community surveys were limited to sites that currently supported a mussel assemblage or sites judged to be suitable mussel habitat based on site characteristics (EcoAnalysts, Inc., 2018). This restriction meant that sampling would not include some highly contaminated stream reaches, because the habitat of those areas was highly degraded, making it difficult to distinguish between effects of habitat degradation and toxic effects of metals.

This study used the "bulk" fraction of sediment particles less than 2 millimeters (<2 mm) in diameter for sediment toxicity testing. This strategy differed from the 2007 USGS study, which sieved sediments so that only the "fine" fraction

(<0.25-mm diameter) would be used for toxicity testing, to ensure that live small juvenile mussels could be recovered quantitatively from fine sediments after 28 days. Sediment metal concentrations differed between the <0.25-mm fine fraction and the <2.0-mm fraction, however, making it difficult to compare results of mussel sediment tests to results of tests with other test organisms. The decision to test mussels with <2-mm sediments in the current study also had the advantage of making it easier to collect the large volumes of sediment needed for toxicity testing from sites chosen for mussel surveys.

Objectives of Reconnaissance Sampling

The objectives of the reconnaissance sampling were as follows:

1. To identify about 25 primary sampling sites widely distributed in the study area with habitat suitable for freshwater mussels (Unionidae);
2. To collect samples of surface water, pore water, sediments, and tissues of the nonunionid freshwater clam, *Corbicula fluminea* (O.F. Müller, 1774; Corbicula), at primary sampling sites to characterize metal concentrations and bioavailability and to provide samples for sediment toxicity testing; and
3. To complete qualitative and quantitative surveys of abundance and species richness of unionid mussels at primary sampling sites.

Methods

Selection of Reconnaissance Sites

The selection of primary sampling sites for sediment toxicity testing and mussel community surveys started with a list of about 49 candidate sites, based on published sediment chemistry and toxicity data (for example, Ingersoll and others, 2008; MacDonald and others, 2009; Besser and others, 2015) and mussel community data (Angelo and others, 2007). Sites were also selected using a geographic information system project that delineated historically stable stream reaches that were most likely to support populations of long-lived freshwater mussels (EcoAnalysts, Inc., 2018; table 1). These original reconnaissance sites were identified by four-digit IDs, consisting of two letters to identify stream plus two numbers to indicate upstream/downstream sequence. During reconnaissance sampling, additional alternate sites were selected near the original sites in order to find suitable habitat; these supplemental sites are indicated by site IDs with letters added to the end of the original site numbers (table 1).

Sites for reconnaissance sampling were characterized for stream order, gradient, and channel stability using geographic information system data, and the suitability of each candidate site as mussel habitat was assessed using a mussel habitat checklist (appendix 1). This checklist included factors such as distance to bluffs or bedrock exposures; geomorphic stream features (for example, riffles, pools, bars, terraces, and [or] islands); and features that indicate stream stability (for example, evidence of channel migration compared to historical maps, incised channels, and cut banks), distance from the mouth of the stream, degree of sediment embeddedness, general observations of sediment grain size, presence of woody debris, condition of riparian corridor, presence or absence of unionid mussels and Corbicula, and distance to sediment sampling locations.

The availability of sediments in the sand-sized and smaller (<2 mm) size fraction in wadeable habitats (<1-meter depth) was assessed at each reconnaissance site. At each of these sites, about 0.5 liter (L) of sediment was collected with a polyvinyl chloride scoop sampler from several potential sampling areas, composited, homogenized, and stored in a precleaned high-density polyethylene container. Sediment samples were dried in an oven at about 60 degrees Celsius (°C). After the total dry weight of the composite sample was determined, the sample was crushed and passed through a 2-mm stainless-steel sieve, and the fraction of the sample in the <2-mm fraction (on a dry mass basis) was determined. A part of each sediment sample was placed in a plastic bag for screening analysis of zinc and lead by X-ray fluorescence (XRF; U.S. Environmental Protection Agency [EPA], 2007). Cadmium concentrations in sediments were typically less than XRF detection limits. Samples were analyzed using a Thermo Niton XL2 GOLDD XRF analyzer (Thermo Scientific, Waltham, Massachusetts) for 1 minute by placing the instrument window directly against a part of the bag that was in full contact with the sediment. Three readings were taken for each sample to ensure a representative sample. Concentrations of metals were expressed as probable effect quotients (PEQs) by dividing the mean concentration of each metal by its probable effect concentration (MacDonald and others, 2000). PEQs for the three metals were assumed to be additive, such that a sediment with a single metal producing a PEQ of 1.0 (that is, one metal at a concentration equal to its probable effect concentration) poses a toxicity hazard equal to a sediment with summed PEQ values (sum-PEQ) of 1.0 for multiple metals. These preliminary hazard quotients were used to help select primary sampling sites for sediment toxicity testing. Samples of stream water and pore water also were collected from reconnaissance sites for analysis of water quality properties and metals concentrations.

Selection of Primary Sampling Sites

The 25 primary sampling sites each included suitable mussel habitat and had enough fine sediments to allow assessment of sediment toxicity. Within these constraints, site selections sought to include broad geographic coverage of the Spring River and Neosho River drainages basin and broad ranges of metal contamination. Five sites were selected to

Table 1. Sites inspected and sampled during reconnaissance, 2016–17.

[Dates are given in month/day/year format; NF, North Fork; MO, Missouri; X, site sampled for water, sediment, and mussel surveys; --, no data or not applicable; KS, Kansas; OK, Oklahoma]

Stream	Site	State	Water	Sediment	Mussel	Date	Latitude (in degrees north)	Longitude (in degrees west)
N. Fork Spring	NF01	MO	X	--	--	7/19/2016	37.2969	94.3930
N. Fork Spring	NF02	MO	X	X	X	7/19/2016	37.2612	94.4385
Spring	SP01A	MO	X	X	X	7/19/2016	37.2670	94.5403
Spring	SP01B	MO	--	--	--	7/19/2016	37.2633	94.5438
Spring	SP02	MO	X	X	--	7/20/2016	37.2222	94.6010
Spring	SP03	KS	X	X	X	10/19/2016	37.1988	94.6242
Spring	SP05	KS	X	X	X	10/18/2016	37.1406	94.6203
Spring	SP06	KS	X	--	--	10/18/2016	37.1333	94.6249
Spring	SP07	KS	X	--	--	10/17/2016	37.1313	94.6318
Spring	SP08	KS	X	X	X	10/19/2016	37.1334	94.6501
Spring	SP09	KS	X	--	--	10/5/2016	37.1317	94.6675
Spring	SP10A	KS	--	--	--	10/14/2016	37.1184	94.6678
Spring	SP10B	KS	--	--	--	10/14/2016	37.1181	94.6676
Spring	SP10C	KS	X	X	X	10/14/2016	37.1160	94.6651
Spring	SP10D	KS	--	--	--	10/14/2016	37.1122	94.6569
Spring	SP10E	KS	--	--	--	10/14/2016	37.1081	94.6562
Spring	SP11A	KS	--	--	--	10/3/2016	37.0889	94.6868
Spring	SP11B	KS	--	--	--	10/3/2016	37.0849	94.6100
Spring	SP11C	KS	--	--	--	10/3/2016	37.0808	94.6922
Spring	SP12A	KS	X	--	--	10/5/2016	37.0210	94.7207
Spring	SP12B	KS	X	X	X	10/5/2016	37.0054	94.7161
Spring	SP13	OK	X	X	X	8/23/2016	36.9732	94.7144
Spring	SP13A	OK	--	--	--	8/23/2016	36.9699	94.7239
Spring	SP14	OK	--	--	--	8/23/2016	36.9614	94.7226
Spring	SP15	OK	X	--	--	8/23/2016	36.9599	94.7198
Spring	SP16	OK	--	--	--	8/24/2016	36.9391	94.7437
Spring	SP17	OK	--	--	--	8/24/2016	36.9350	94.7460
Spring	SP18	OK	X	--	--	8/24/2016	36.9211	94.7390
Spring	SP19	OK	--	--	X	8/24/2016	36.9140	94.7321
Spring	SP20	OK	X	X	X	8/24/2016	36.9132	94.7336
Spring	SP21	OK	X	--	--	8/25/2016	36.8942	94.7299

Table 1. Sites inspected and sampled during reconnaissance, 2016–17.—Continued

[Dates are given in month/day/year format; NF, North Fork; MO, Missouri; X, site sampled for water, sediment, and mussel surveys; --, no data or not applicable; KS, Kansas; OK, Oklahoma]

Stream	Site	State	Water	Sediment	Mussel	Date	Latitude (in degrees north)	Longitude (in degrees west)
Spring	SP22	OK	X	X	X	8/25/2016	36.8879	94.7284
Spring	SP23	OK	--	--	--	8/25/2016	36.8762	94.7468
Spring	SP24	OK	--	--	--	8/25/2016	36.8715	94.7655
Center	CC00	MO	X	X	X	10/6/2016	37.1187	94.2586
Center	CC01	MO	--	--	--	7/20/2016	37.1119	94.2233
Center	CC01G1	MO	--	--	--	7/26/2016	37.1086	94.3409
Center	CC01G2	MO	X	X	X	7/26/2016	37.1091	94.3443
Center	CC01A2	MO	--	--	--	7/24/2016	37.1278	94.3809
Center	CC01A1	MO	--	--	--	7/24/2016	37.1299	94.3830
Center	CC01B1	MO	--	--	--	7/24/2016	37.1448	94.3798
Center	CC01B2	MO	--	--	--	7/24/2016	37.1477	94.3780
Center	CC01C	MO	--	--	--	7/24/2016	37.1581	94.3921
Center	CC01D	MO	--	--	--	7/24/2016	37.1605	94.4000
Center	CC01E	MO	--	--	--	7/24/2016	37.1624	94.4052
Center	CC01F	MO	--	--	--	7/24/2016	37.1617	94.4060
Center	CC03	MO	X	--	--	7/21/2016	37.1652	94.4133
Center	CC04	MO	--	--	--	7/20/2016	37.1684	94.4170
Center	CC05D	MO	--	--	--	7/20/2016	37.1708	94.4171
Center	CC05DA	MO	--	--	--	7/20/2016	37.1724	94.4188
Center	CC05DB	MO	--	--	--	7/20/2016	37.1751	94.4208
Center	CC05DC	MO	--	--	--	7/21/2016	37.1791	94.4511
Center	CC05D	MO	X	X	X	7/21/2016	37.1787	94.4517
Center	CC06	MO	--	--	--	7/21/2016	37.1753	94.4552
Center	CC07	MO	X	X	X	7/21/2016	37.1768	94.4604
Center	CCO8	MO	X	--	--	7/21/2016	37.1795	94.4692
Center	CC09	MO	--	X	--	7/21/2016	37.1807	94.4786
Center	CC10	MO	--	X	--	7/21/2016	37.1764	94.4869
Center	CC15A	MO	--	--	--	7/26/2016	37.1702	94.5461
Center	CC16	MO	X	--	--	7/26/2016	37.1696	94.5485
Shoal	SH01	MO	--	--	--	7/20/2016	36.9395	94.1909
Shoal	SH01A	MO	X	--	--	7/22/2016	36.9452	94.2662
Shoal	SH02	MO	--	X	--	7/23/2016	36.9371	94.3039

Table 1. Sites inspected and sampled during reconnaissance, 2016–17.—Continued

[Dates are given in month/day/year format; NF, North Fork; MO, Missouri; X, site sampled for water, sediment, and mussel surveys; --, no data or not applicable; KS, Kansas; OK, Oklahoma]

Stream	Site	State	Water	Sediment	Mussel	Date	Latitude (in degrees north)	Longitude (in degrees west)
Shoal	SH02A	MO	--	--	--	7/23/2016	36.9352	94.3045
Shoal	SH03A	MO	--	--	--	7/23/2016	36.9336	94.3054
Shoal	SH04A	MO	--	--	--	7/23/2016	36.9298	94.3069
Shoal	SH04B	MO	--	--	--	7/23/2016	36.9297	94.3090
Shoal	SC04C	MO	--	--	--	7/23/2016	36.9291	94.3125
Shoal	SH05	MO	--	X	X	7/23/2016	36.9313	94.3152
Shoal	SH05A	MO	X	--	--	7/23/2016	36.9317	94.3158
Shoal	SH06A	MO	X	X	--	7/22/2016	36.9208	94.3378
Shoal	SH06	MO	--	--	--	7/22/2016	36.9119	94.3496
Shoal	SH07	MO	X	X	--	7/22/2016	36.8950	94.3697
Shoal	SH09A	MO	--	--	--	7/25/2016	37.0353	94.5599
Shoal	SH10	MO	X	X	X	7/25/2016	37.0373	94.5633
Shoal	SH11	MO	--	--	--	7/25/2016	37.0387	94.5656
Shoal	SH12	MO	--	--	--	7/25/2016	37.0394	94.5774
Shoal	SH13	MO	--	--	--	7/25/2016	37.0384	94.5805
Shoal	SH14	MO	X	--	--	7/25/2016	37.0373	94.5818
Shoal	SH15	MO	--	--	--	7/25/2016	37.0361	94.5832
Shoal	SH15A	MO	X	--	--	7/25/2016	37.0357	94.5878
Shoal	SH16	KS	X	--	--	10/6/2016	37.0412	94.6000
Shoal	SH16A	KS	--	X	X	10/6/2016	37.0416	94.6490
Shoal	SH17	KS	--	--	--	10/6/2016	34.0436	94.6575
Shoal	SH18	KS	X	--	--	10/6/2016	36.9430	94.9852
Neosho	NRSF	OK	--	--	X	[missing data]	[missing data]	[missing data]
Neosho	NR02	OK	X	--	X	8/26/2016	36.9430	94.9852
Neosho	NR03	OK	X	--	X	8/26/2016	36.9331	94.8623
Neosho	NR04	OK	X	--	--	8/26/2016	36.8917	94.8637
Neosho	NR05	OK	X	--	--	8/26/2016	36.8903	94.6760
Neosho	NR06	OK	X	X	X	8/27/2016	36.8527	94.8552
Tar	TC01	OK	X	X	--	8/25/2016	36.8773	94.8623
Tar	TC02	OK	X	--	--	8/27/2016	36.8597	94.8637
Lost	LC01	OK	--	--	--	8/25/2016	36.8087	94.6760

represent reference conditions, which were defined as sediments with sum-PEQs of <1.0 for zinc, lead, and cadmium and with habitat characteristics comparable to sites that were potentially affected by metals from mining (test sites). One additional low-metal sample was collected from the Spring River at Waco, Missouri (site SR), to serve as a control sediment to document performance of the sediment bioassay. Good performance of juvenile mussels in the site SR sediment has been documented in previous studies (Ingersoll and others, 2008; Besser and others, 2015).

Mussel Surveys

Mussel surveys were completed at reconnaissance sites (qualitative surveys) and at primary sampling sites (quantitative surveys). During the reconnaissance sampling, the status of the mussel community at sites with suitable habitat was assessed by timed searches. The area of the site was delineated with a survey-grade Trimble Global Positioning System, and the site was qualitatively sampled with timed searches to determine unionid distribution, community composition, and catch per unit effort (CPUE). Search effort per site ranged from 30 to 360 person-minutes and averaged 76 minutes.

Primary sampling sites were sampled quantitatively for mussels in 2017. At each site, as many as 100 randomly distributed 0.25-square meter quantitative quadrats were sampled within the delineated unionid habitat area to characterize species richness, density, and age distribution. The number of quadrat samples per site ranged from 15 to 100, depending on the size of the delineated area. Substrate in each quadrat was excavated to a depth of about 15 centimeters (cm), and material was placed into an attached mesh bag. Each sample was washed through a 6-mm sieve and searched for unionids and *C. fluminea*. All live unionids were identified to species, measured (length in millimeters), and aged (external annulus count). Fresh dead shells were identified to species and counted. Weathered dead and subfossil shells were identified to species and noted as present. Additional details of methods for the mussel community and habitat survey are presented in appendix 1 and in EcoAnalysts, Inc. (2018).

Water and Sediment Sampling

Metal concentrations and water quality properties affecting metal bioavailability were determined in samples of stream water and in situ pore water collected at reconnaissance sites and primary sampling sites. Samples of stream water were collected by subsurface grabs with precleaned polypropylene sample vials. The vials were opened, ultrapure water in the vials was discarded, and the vials were rinsed with and then filled with stream water and sealed below the water surface. Samples of surficial pore water were collected by push-point sampling (Zimmerman and others, 2005) in or near mussel beds. The fritted tip of the sampler was inserted into the surface sediment to a depth of 10 cm, pore water was extracted from the sampler with a syringe, and the first aliquot from each location was used to rinse the sampler tubing and the sample vial and then discarded. The first set of reconnaissance water samples was collected in early summer 2016 (about 40 sites), and a second set of water samples was collected during low flow conditions in late summer and early fall 2016 (25 sites). Metal concentrations from these samples were used to establish metal concentration ranges and ratios of metal mixtures for water-only toxicity tests completed in winter 2017.

Sediments for toxicity testing were collected at 25 primary sampling sites in summer 2017 following methods used for the previous sediment study (Ingersoll and others, 2008; Besser and others, 2015). Sediments were extracted from depositional areas with a 7.5- or 10-cm diameter polyvinyl chloride scoop and were wet sieved through a wash bucket (Wildco Wash Bucket with US no. 10 stainless-steel mesh) to separate sand-sized and smaller particles (<2 mm) for toxicity testing. The use of the <2-mm sediment fraction differed from the <0.25-mm fraction used for testing and analysis in 2007 (Ingersoll and others, 2008). Sediments were sealed in precleaned high-density polyethylene buckets with site water and substantial head space, transported to the USGS laboratory in a refrigerated trailer, and stored in a walk-in cooler at 4 °C. Sediment was stored 2 months or less before the first sediment test in 2017, then an additional 5 months before the second sediment test in spring 2018.

About 1 week before the start of sediment toxicity tests, the sieved (<2 mm) sediments from 24 sites plus the control sediment were homogenized with an electric drill equipped with a stainless-steel auger, and subsamples were collected for analysis of total recoverable (TR) metals, simultaneously extracted metals (SEM), acid-volatile sulfide (AVS), total organic carbon (TOC), and particle-size distribution. Porewater samples were separated from sediments by centrifugation and by passive diffusion samplers ("peepers"; Brumbaugh and others, 2007; Cleveland and others, 2017). Sediments were added to 250-milliliter (mL) polyethylene conical centrifuge bottles and centrifuged for 30 minutes at 5,000 revolutions per minute or 7,000 times standard gravity (Sorvall LYNX 400 with Bio-flex HC rotor) to generate pore water for analyses of cations, anions, dissolved organic carbon (DOC), and conventional water quality properties (pH, conductivity, alkalinity, hardness, total ammonia). Pore water also was sampled before toxicity tests using large-volume peepers (30 mL; Cleveland and others, 2017) deployed in 1,000-mL beakers containing 100 mL of homogenized sediment and equilibrated for 10 days at 4 °C. Pore water from the large peepers was analyzed for metals and DOC for comparison to pore water obtained from centrifuged samples.

Water Analyses

Concentrations of filterable metals (zinc, lead, cadmium) and major cations (calcium, potassium, magnesium, sodium) were determined in filtered (0.45-micrometer [µm]

pore diameter) water from grab samples, peeper samples, and push-point samples. Samples were analyzed for metals using an inductively coupled plasma-mass spectrometer (ICP–MS) (ELAN DRC–e; Perkin-Elmer, Waltham, Mass.). Stream water and overlying waters from toxicity tests were filtered with a 0.45-µm polyethersulfone (PES) filter disc and preserved by acidification with subboiling distilled nitric acid (1 percent nitric acid by volume).

Concentrations of anions in stream water, in overlying waters of toxicity tests, and in sediment pore waters were filtered (0.45-µm polyproplylene/polyethersulfone) and analyzed with an ion chromatograph (ICS–1100, Ionpac AS22 anion exchange column, and suppressed conductivity detector; Dionex, Arcade, New York). Anion samples were stored in a laboratory refrigerator at 4 °C for no more than 30 days before analysis.

Concentrations of DOC in stream water, overlying waters of sediment toxicity tests, and sediment pore waters (grab, peeper, and [or] push-point samples) were filtered (0.45-µm glass/PES), dispensed into precleaned low-TOC amber glass vials, and acidified with 9 N high-purity sulfuric acid (BDH Aristar Ultra; VWR International, Radnor, Pennsylvania) to a pH of 2 or less within 48 hours of receipt. Acidified samples were refrigerated and held no longer than 28 days before DOC analysis. DOC was measured as nonpurgeable organic carbon by high temperature combustion catalytic oxidation-nondispersive infrared spectroscopy using a TOC analyzer (model TOC–L CSH; Shimadzu Scientific Instruments, Inc., Columbia, Maryland). Samples were air sparged to remove inorganic carbon species before the nonpurgeable organic carbon measurements.

Sediment and Tissue Analyses

Metal analyses were completed on samples of sediment (<2 mm) and Corbicula tissue. Sediment was analyzed for TR metals, SEM and AVS, TOC, and particle-size distribution. Sediments were microwave digested in a mixture of nitric and hydrochloric acids to extract the TR metal fraction, and tissues were digested with nitric acid and hydrogen peroxide. Metal concentrations in these extracts were determined by ICP–MS. Sediment samples for measurement of SEM and AVS were refrigerated and analyzed within 28 days of receipt using deoxygenated reagents in a two-part process. First, the sediment was mixed with 1 N hydrochloric acid (20:1 mixture of sediment to acid on a wet weight basis) in a nitrogen-purged oxygen-free atmosphere to release sulfide as hydrogen sulfide gas. The hydrogen sulfide was bubbled through an antioxidant trapping solution with a pH of 12 to form free sulfide, which was measured using an ion-selective electrode (Brumbaugh and others, 2011). After sulfide trapping, overlying liquid and the upper 1 cm of sediment were removed and discarded, and the remaining sediment was gently stirred for as much as 1 minute to produce a uniform consistency before sampling the SEM extract. The sediment-hydrochloric acid mixture was settled for a maximum of 15 minutes, and then 40 mL of the extract were filtered through a 0.45-µm PES membrane, diluted in nitric acid, and (or) further digested and (or) volume reduced as needed for ICP–MS analysis. The SEM fraction was analyzed for cadmium, copper, lead, nickel, and zinc.

Metal concentrations in Corbicula tissues were used as a surrogate for metal bioaccumulation by unionid mussels. Corbicula collected during quantitative mussel surveys were held in site water at ambient temperatures for 24 hours to allow them to depurate gut contents before they were frozen for storage (appendix 2). In the laboratory, soft tissues were separated, lyophilized, homogenized, and microwave digested in nitric acid and hydrogen peroxide before ICP–MS analyses.

TOC content and particle-size distribution of sediments were analyzed at the University of Missouri Soils Laboratory using methods 4H2 and 3A1a from the U.S. Department of Agriculture Kellogg Soil Survey Laboratory (Natural Resources Conservation Service, 2014). The sieved sediment fraction (<2 mm) was characterized for particle-size distribution using a dispersion and suspension method. The sample was pretreated with peroxide and other reagents to remove organic matter and soluble salts, then ovendried to obtain an initial weight. The particles were then dispersed with a sodium hexametaphosphate solution and mechanically shaken to create a suspension. The sand fraction was removed from the suspension by wet sieving and then fractionated by dry sieving. The clay and fine silt fractions were determined using the suspension remaining from the wet sieving process. This suspension was diluted to 1 L in a sedimentation cylinder and stirred, and then 25-mL aliquots were removed with a pipette at predetermined intervals. The aliquots were dried at 110 °C and weighed. For total carbon analysis, the sieved sediment was packed in foil, weighed, and analyzed for total carbon by an elemental analyzer. The elemental analyzer used catalytic combustion in an oxygenated atmosphere and high temperature to liberate carbon as carbon dioxide, which was detected by a thermal conductivity detector. Inorganic carbon was determined by treatment of the soil with hydrochloric acid and subsequent manometric measurement of the evolved carbon dioxide, and TOC was estimated by difference of the total and inorganic carbon fractions.

Quality Assurance

A minimum of three external calibration standards plus a calibration blank were used to calibrate instrument response, and established laboratory quality assurance/quality control (QA/QC) procedures (for example, laboratory spikes, duplicates, control samples) were used to verify instrument performance throughout the analyses. A summary of QA/QC methods and results is included in appendix 2.

Results and Discussion

Selection of Primary Sampling Sites

The USGS and EcoAnalysts, Inc., visited 97 locations in the Spring River-Neosho River drainages between August and October 2016 and completed reconnaissance sampling of water and sediment plus qualitative surveys for freshwater mussels at 42 sites (table 1). Screening analyses of metals in sediment samples by XRF were used to select 25 primary sampling sites for sediment toxicity testing (table 2). These primary sampling sites included one site for collection of control sediment from the Spring River at Waco, Mo. (Ingersoll and others, 2008; Besser and others, 2015); 5 reference sites (3 in upper Spring River, 2 in upper Center Creek); and 19 test sites (8 in Spring River, 4 in Center Creek, 6 in Shoal Creek, and 1 each in Tar Creek and Neosho River; table 2). Most primary sampling sites were included in both sediment toxicity testing and quantitative mussel community surveys, but several sites in Center Creek (CC09) and Shoal Creek (SH02, SH06, and SH07) were sampled only for sediments for sediment toxicity tests. Physical habitat at these sediment-only sites was judged to be inadequate to support mussel assemblages, and these sites were therefore excluded from quantitative mussel surveys. These sites were included to avoid having long stream reaches without sediment toxicity data and to ensure that the selection of primary sampling sites included the full range of metal concentrations detected in the study area. Only two samples from the Neosho drainage were included as test sites in the sediment study: one from Tar Creek and one from the Neosho River downstream from Tar Creek. Quantitative mussel surveys also included two samples that were not included in the sediment toxicity tests: a reference site in the Neosho drainage (NRSF) and a test site in the lower Spring River (SP19) that was excluded from toxicity testing because it was too close to mussel site SP20.

Water Chemistry and Metal Concentrations

About one-half of water samples from the reconnaissance sites sampled in 2017 (26 pore-water samples, 23 surface water samples) had measurable zinc concentrations (table 3). Filterable zinc concentrations in surface water and pore water were greater in samples from mining-affected tributaries than in samples from main stem sites in the Spring and Neosho Rivers (fig. 2). Median zinc concentrations in surface water and pore-water samples were 5.6 micrograms per liter (µg/L) as zinc and 8.1 µg/L as zinc, respectively (table 3), and several sites produced samples of surface water or pore water with filterable zinc concentrations greater than (>) 100 µg/L, including Center Creek sites CC05D and CC16, Spring River sites SP06 and SP07, Shoal Creek site SH06, and Tar Creek site TC01. Lead (limit of detection, 0.1 µg/L) and cadmium (limit of detection, 0.05 µg/L) were detectable in fewer than one-half of all surface-water and pore-water samples (44 percent of samples for lead; 28 percent for cadmium). We estimated ratios of zinc:lead (158–310) and zinc:cadmium (451–489) based on water samples in which all three metals were detected (fig. 3).

Water chemistry did not differ widely among sites; median DOC concentrations were 3.6 and 6.0 milligrams per liter (mg/L) for surface water and pore water, respectively, and median hardness was 165 mg/L for both sample types. Samples from Tar Creek (TC01) had the greatest concentrations of major ions in surface water and pore water, including more than 30 mg/L for sodium, more than 900 mg/L for sulfate, and about 1,000 mg/L for total hardness (table 3).

Sediment Metal Concentrations and Toxicity Hazards

The 25 sediments selected for toxicity testing included 1 control sediment to characterize the performance of the test organisms and the exposure regime, 5 reference sediments to represent responses of mussels at low-metal sites, and 19 test sediments. Test sediments were further classified as tributary sites, which received direct input of metals from mining areas, and main stem sites on the Spring River and Neosho River, which were downstream from metal-contaminated tributaries (fig. 4). The control sediment and all five reference sediments had sum-PEQ hazard indices (for zinc, lead, and cadmium) of less than 1.0 (table 4), indicating that sediment toxicity was not likely. Sediments from the mining-affected reach of Center Creek had sum-PEQ indices as high as 7.3 (CC07), and the highest sum-PEQ index for Shoal Creek was 4.7 (SH05). Samples from the middle reach of the Spring River, which received inputs from Center Creek (and Turkey Creek—not sampled) had sum-PEQ indices ranging from 1.3 to 1.7 (SP10), and sediments from lower Spring River between Shoal Creek and the confluence of the Neosho River had sum-PEQ indices ranging from 1.7 to 3.4 (SP20). The Tar Creek site (TC01) had the highest sum-PEQ index measured in this study (8.8), but the main stem of the Neosho River below Tar Creek (NR06) had a sum-PEQ index within the range of the reference sites. Across all sites, zinc was the greatest contributor to the sum-PEQ index (median contribution, 72 percent of total); lead made up almost all of the remainder (median, 28 percent), and cadmium made a minimal contribution (<1 percent).

Other measures of metal bioavailability and toxicity hazards indicated similar patterns across the sampling sites. Normalizing summed molar concentrations of five divalent metals (cadmium, copper, nickel, lead, and zinc), expressed as SEM, relative to AVS ([ΣSEM–AVS], expressed as micromoles per gram dry weight) did not greatly affect predictions of metal bioavailability, relative to predictions of the simple PEQ index, across the 25 sites (fig. 4*B*). Although copper and nickel were detected at relatively low concentrations and were not the focus of this study, analysis of concentrations of these metals in SEM extracts was necessary to evaluate the

Table 2. Primary sampling sites for mussel community surveys and sediment toxicity testing.

[ID, identifier; sum-PEQ, summed probable effect quotient values (see footnote); Hwy, Highway; Rd, Road; Dr, Drive; SP, State park; --, no data or not applicable; Lk, Lake; R, River]

Laboratory ID	Site	Stream or reach	Description	Site type	Mussel survey?	Sediment toxicity?	[1]Sum-PEQ
1	NF02	Upper Spring	North Fork	Reference	Yes	Yes	0.35
2	SP01A	Upper Spring	Hwy 270	Reference	Yes	Yes	0.19
3	SP03	Upper Spring	Lawton	Reference	Yes	Yes	0.15
4	CC00	Center	Hwy 110	Reference	Yes	Yes	0.49
5	CC01G2	Center	Chapel	Reference	Yes	Yes	0.26
6	CC05D	Center	Above Ben's Branch	Test	Yes	Yes	2.3
7	CC07	Center	Below Ben's Branch	Test	Yes	Yes	7.3
8	CC09	Center	Center Creek park	Test	No	Yes	4.6
9	CC10	Center	Below Hwy JJ	Test	Yes	Yes	5.5
10	SP05	Middle Spring	Below Center	Test	Yes	Yes	1.3
11	SP08	Middle Spring	Below Turkey	Test	Yes	Yes	1.3
12	SP10	Middle Spring	Above Empire	Test	Yes	Yes	1.7
13	SH02	Shoal	Indian Springs	Test	No	Yes	4.6
14	SH05	Shoal	Cherry Corner	Test	Yes	Yes	4.7
15	SH06	Shoal	Nighthawk Rd	Test	No	Yes	3.3
16	SH07	Shoal	Lime Kiln Dr	Test	No	Yes	2.4
17	SH10	Shoal	Tipton Ford	Test	Yes	Yes	1.5
18	SH16A	Shoal	Below Schermerhorn Park	Test	Yes	Yes	1.3
19	SP12B	Lower Spring	Baxter Springs	Test	Yes	Yes	1.7
20	SP13	Lower Spring	Blue Springs SP	Test	Yes	Yes	2.1
--	SP19	Lower Spring	Hatchery main channel	Test	Yes	No	[2]2.7
21	SP20	Lower Spring	Hatchery side channel	Test	Yes	Yes	3.4
22	SP22	Lower Spring	Above Grand Lk	Test	Yes	Yes	2.1
23	TC01	Neosho	Tar Creek	Test	Yes	Yes	8.8
--	NRSF	Neosho	Stepps Ford	Reference	Yes	No	[2]0.20
24	NR06	Neosho	Below Tar	Test	Yes	Yes	0.29
25	SP02	Spring	Spring R at Waco	Control	Yes	Yes	0.20

[1]Probable effect quotient=sediment metal concentration/probable effect concentration (MacDonald and others, 2000); summed for zinc, lead, and cadmium.

[2]Sum-PEQ for mussel-only sites (SP19, NRSF) are X-ray fluorescence estimates from reconnaissance surveys (EcoAnalysts, Inc., 2018).

Table 3. Concentrations of metals, major ions, and dissolved organic carbon in samples of surface water and pore water from reconnaissance sites.

[ID, identifier; µg/L, microgram per liter; Zn, zinc; Cd, cadmium; Pb, lead; mg/L, milligram per liter; Na, sodium; Mg, magnesium; K, potassium; Ca, calcium; Fe, iron; DOC, dissolved organic carbon; Fl, fluoride; Cl, chlorine; SO_4, sulfate; <, less than; --, no data; %, percent; nd, not determined]

Site ID	[1]Metals (µg/L)			[1]Cations (mg/L)					[1]Anions and DOC (mg/L)				[2]Hardness (mg/L)
	Zn	Cd	Pb	Na	Mg	K	Ca	Fe	Fl	Cl	SO_4	DOC	
						Surface water							
CC00	<5	<0.05	<0.1	6.1	3.5	2.1	63	<0.5	--	--	--	2.5	174
CC01G2	<20	<0.05	<0.1	6.7	3.5	1.9	61	<0.5	<0.2	10	5.9	2.0	168
CC03	<20	<0.05	<0.1	6.8	3.4	1.9	59	<0.5	<0.2	10	8.3	3.4	162
CC05D	218	0.76	33.60	6.5	3.3	1.9	62	2.5	<0.2	9	13	2.2	169
CC07	64	0.29	0.24	6.3	3.3	1.8	66	<0.5	<0.2	9	24	1.8	179
CC08	73	0.24	0.39	6.7	3.3	1.8	66	<0.5	<0.2	10	23	2.1	179
CC16	148	0.21	0.72	7.8	3.6	2.1	72	<0.5	0.2	11	36	2.2	196
NF01	<20	<0.05	0.19	10.0	3.5	6.6	40	<0.5	<0.2	15	18	8.9	115
NF02	<20	<0.05	0.17	9.3	3.7	6.2	47	<0.5	<0.2	13	18	7.9	134
NR02	<5	<0.05	<0.1	7.7	8.3	5	42	<0.5	0.2	9	32	8.3	141
NR03	<5	<0.05	<0.1	7.5	8.2	4.9	41	<0.5	0.2	9	32	7.1	138
NR04	<5	<0.05	<0.1	7.5	8.2	5	41	<0.5	0.2	9	32	8.2	139
NR05	<5	<0.05	<0.1	7.5	8	5	41	<0.5	0.2	9	32	8.0	138
NR06	<5	<0.05	<0.1	7.7	8.1	4.9	41	<0.5	<0.2	9	32	8.0	138
SH01	<20	<0.05	<0.1	13.0	3.9	3	56	<0.5	<0.2	15	12	2.0	157
SH05	26	<0.05	0.11	9.4	4	2.5	53	<0.5	<0.2	12	9.4	1.9	150
SH06	21	<0.05	0.12	9.2	3.9	2.4	54	<0.5	<0.2	12	9.7	1.7	152
SH07	<20	<0.05	<0.1	8.9	3.9	2.4	54	<0.5	<0.2	12	9.5	1.9	152
SH10	<20	<0.05	<0.1	8.5	3.6	2.2	55	<0.5	<0.2	12	9	2.2	153
SH14	21	<0.05	0.16	8.4	3.4	2.4	51	<0.5	<0.2	12	9	2.4	142
SH15	62	<0.05	0.20	8.0	3.8	2.9	64	1.5	0.2	12	6	5.0	177
SH16A	32	0.06	<0.1	17.0	3.8	3.1	53	<0.5	--	--	--	2.3	148
SH18	93	0.27	0.15	16.6	3.8	3	52	<0.5	--	--	--	2.0	148
SP01A	<20	<0.05	<0.1	11.0	3.9	2.8	61	<0.5	<0.2	15	14	3.9	170
SP02	<20	<0.05	<0.1	11.0	4	3.8	55	<0.5	<0.2	14	17	4.5	155
SP03	<5	<0.05	<0.1	11.4	5.1	6.9	51	<0.5	<0.2	16	28	6.9	149
SP05	35	0.07	<0.1	12.1	7	6.6	51	<0.5	<0.2	15	52	6.5	157
SP06	187	1.02	0.23	17.3	5.9	4.9	70	0.5	<0.2	23	58	6.1	200
SP07	302	1.94	0.79	22.2	5	4	86	0.6	<0.2	27	63	7.0	237

Table 3. Concentrations of metals, major ions, and dissolved organic carbon in samples of surface water and pore water from reconnaissance sites.—Continued

[ID, identifier; µg/L, microgram per liter; Zn, zinc; Cd, cadmium; Pb, lead; mg/L, milligram per liter; Na, sodium; Mg, magnesium; K, potassium; Ca, calcium; Fe, iron; DOC, dissolved organic carbon; Fl, fluoride; Cl, chlorine; SO_4, sulfate; <, less than; --, no data; %, percent; nd, not determined]

Site ID	[1]Metals (µg/L)			[1]Cations (mg/L)					[1]Anions and DOC (mg/L)				[2]Hardness (mg/L)
	Zn	Cd	Pb	Na	Mg	K	Ca	Fe	Fl	Cl	SO_4	DOC	
Surface water—Continued													
SP08	50	0.13	0.12	13.2	7	5.8	57	<0.5	<0.2	15	54	7.9	172
SP09	25	<0.05	<0.1	14.2	6.2	3.8	64	<0.5	--	--	--	3.0	186
SP10	9	0.05	<0.1	13.4	6	4	62	<0.5	--	--	--	3.5	181
SP11	6	<0.05	0.11	12.8	5.8	4.8	57	<0.5	--	--	--	4.3	168
SP12A	10	<0.05	<0.1	13.2	5.3	4.4	57	<0.5	--	--	--	4.7	166
SP12B	7	<0.05	<0.1	14.3	5.5	4.5	58	<0.5	--	--	--	3.9	170
SP13	<5	<0.05	<0.1	14.2	5.3	3.6	57	<0.5	<0.2	17	38	3.7	167
SP15	<5	<0.05	<0.1	13.9	5	3.5	57	<0.5	<0.2	17	38	3.5	165
SP18	<5	<0.05	<0.1	13.6	5	3.4	56	<0.5	<0.2	17	37	3.4	163
SP20	5	<0.05	<0.1	13.7	5.2	3.5	55	<0.5	<0.2	17	38	3.3	161
SP21	8	<0.05	<0.1	13.6	5.2	3.5	59	<0.5	<0.2	17	39	3.6	169
SP22	6	<0.05	<0.1	13.7	5.2	3.5	57	<0.5	<0.2	17	39	3.2	166
TC01	62	<0.05	<0.1	31.9	38.3	5.4	344	2.1	1.4	19	929	5.9	1,028
TC02	<5	<0.05	<0.1	13.8	13.5	4.7	99	0.6	0.5	14	208	7.1	306
% detected	**53%**	**26%**	**35%**	**100%**	**100%**	**100%**	**100%**	**14%**	**19%**	**81%**	**81%**	**100%**	**100%**
Median	**5.6**	**nd**	**nd**	11	5.0	3.5	57	nd	nd	nd	nd	3.6	165
Minimum	5	0	0	6	3	2	40	1	0	9	6	2	115
Maximum	302	2	34	32	38	7	344	3	1	27	929	9	1,028
Pore water													
CC00	<5	<0.05	<0.1	5.7	3.3	2	61	<0.5	--	--	--	3.4	166
CC01G2	<20	<0.05	<0.1	6.4	4.4	2.2	68	<0.5	<0.2	9	4.4	3.3	189
CC03	<20	<0.05	<0.1	6.8	3.5	1.9	61	<0.5	<0.2	11	6	3.3	168
CC05D	83	0.39	0.22	5.2	2.4	1.2	63	<0.5	<0.2	6	17	1.9	168
CC07	55	0.37	0.11	6.4	3.3	1.9	64	<0.5	0.2	9	25	2.2	174
CC08	51	<0.05	0.69	6.9	3.3	1.9	65	<0.5	0.2	10	23	4.5	177
CC16	167	0.34	5.75	7.0	3.7	2.4	67	0.9	0.2	11	34	6.7	184
NF01	<20	<0.05	<0.1	10.0	3.7	7.2	42	<0.5	<0.2	15	17	9.9	121
NF02	<20	<0.05	0.13	9.3	3.7	6.1	46	<0.5	<0.2	14	18	8.1	131

Table 3. Concentrations of metals, major ions, and dissolved organic carbon in samples of surface water and pore water from reconnaissance sites.—Continued

[ID, identifier; µg/L, microgram per liter; Zn, zinc; Cd, cadmium; Pb, lead; mg/L, milligram per liter; Na, sodium; Mg, magnesium; K, potassium; Ca, calcium; Fe, iron; DOC, dissolved organic carbon; Fl, fluoride; Cl, chlorine; SO_4, sulfate; <, less than; --, no data; %, percent; nd, not determined]

Site ID	[1]Metals (µg/L)			[1]Cations (mg/L)					[1]Anions and DOC (mg/L)				[2]Hardness (mg/L)
	Zn	Cd	Pb	Na	Mg	K	Ca	Fe	Fl	Cl	SO_4	DOC	
NR02	<5	<0.05	<0.1	7.5	7.9	4.6	40	<0.5	0.2	9	33	8.5	136
Pore water—Continued													
NR03	<5	<0.05	<0.1	7.6	8	4.8	41	<0.5	0.2	9	32	8.7	136
NR04	<5	0.06	<0.1	9.9	17.7	5.9	91	1.2	<0.2	9	9	9.9	305
NR05	107	<0.05	<0.1	10.8	37	8.1	203	29.3	0.4	8	16	26	670
NR06	<5	<0.05	<0.1	8.7	8.3	4.8	43	<0.5	<0.2	10	33	9.6	143
SH01	<20	<0.05	<0.1	12.0	3.9	2.9	55	<0.5	<0.2	14	12	2.4	155
SH05	<20	<0.05	0.20	9.5	4	2.5	54	<0.5	<0.2	12	9	6.2	153
SH06	218	1.26	16.10	9.1	4	2.6	53	0.9	<0.2	12	9.3	2.0	150
SH07	<20	0.07	0.15	8.7	3.9	2.3	53	<0.5	<0.2	12	9.2	2.1	150
SH10	27	<0.05	0.34	7.8	5.3	3.3	86	4.8	<0.2	11	1.6	6.5	238
SH14	<20	<0.05	0.18	8.5	3.6	2.6	54	0.5	<0.2	13	5.7	3.3	151
SH15	47	<0.05	0.51	8.2	3.7	2.7	61	1.9	<0.2	12	2.6	4.7	169
SH16A	12	<0.05	0.22	17.2	4	3.2	57	<0.5	--	--	--	3.7	159
SH18	74	0.13	0.10	15.7	3.7	3	52	<0.5	--	--	--	3.6	--
SP01A	<20	<0.05	<0.1	11.0	4.1	2.9	58	<0.5	<0.2	15	13	3.6	163
SP02	<20	<0.05	<0.1	10.0	4.1	3.9	54	<0.5	<0.2	15	18	4.7	153
SP03	<5	<0.05	<0.1	10.8	4.9	6.8	48	<0.5	<0.2	16	29	11.9	142
SP05	22	0.06	0.12	11.2	6.5	5.9	48	0.7	<0.2	15	50	7.6	149
SP06	59	0.26	0.12	15.4	5.1	4.5	61	<0.5	0.2	16	59	6.0	174
SP07	169	0.58	0.33	16.8	5.6	5	71	0.6	<0.2	21	48	6.4	202
SP08	11	<0.05	0.19	12.3	7	5.6	57	1.1	<0.2	15	41	13	172
SP09	10	<0.05	<0.1	15.4	7.1	4.5	70	0.5	--	--	--	21	205
SP10	5	<0.05	0.30	13.2	6.7	5.2	60	0.6	--	--	--	6.1	179
SP11	4	<0.05	<0.1	13.6	6.1	5.9	56	<0.5	--	--	--	7.5	166
SP12A	11	<0.05	0.31	12.0	9.1	5.2	91	11.4	--	--	--	13	266
SP12B	19	<0.05	<0.1	12.8	5.3	4.6	54	<0.5	--	--	--	4.5	157
SP13	44	0.30	2.21	13.8	5.1	3.4	57	0.5	<0.2	17	40	5.2	165
SP15	28	0.17	1.47	14.2	5.1	3.5	56	<0.5	<0.2	18	39	4.4	162
SP18	6	<0.05	<0.1	13.5	5.1	3.4	57	<0.5	<0.2	17	39	4.5	164

Table 3. Concentrations of metals, major ions, and dissolved organic carbon in samples of surface water and pore water from reconnaissance sites.—Continued

[ID, identifier; µg/L, microgram per liter; Zn, zinc; Cd, cadmium; Pb, lead; mg/L, milligram per liter; Na, sodium; Mg, magnesium; K, potassium; Ca, calcium; Fe, iron; DOC, dissolved organic carbon; Fl, fluoride; Cl, chlorine; SO_4, sulfate; <, less than; --, no data; %, percent; nd, not determined]

Site ID	[1]Metals (µg/L)			[1]Cations (mg/L)					[1]Anions and DOC (mg/L)				[2]Hardness (mg/L)
	Zn	Cd	Pb	Na	Mg	K	Ca	Fe	Fl	Cl	SO_4	DOC	
SP20	12	0.11	0.15	13.8	5.2	3.4	54	<0.5	<0.2	17	39	4.3	159
Pore water—Continued													
SP21	23	<0.05	0.11	12.5	6	4.5	58	<0.5	<0.2	16	28	4.5	171
SP22	8	<0.05	<0.1	13.5	5.1	3.5	57	<0.5	<0.2	17	40	8.2	165
TC01	167	<0.05	<0.1	30.8	35.8	6	333	6.6	1.6	19	904	12	990
TC02	<5	<0.05	<0.1	11.7	11.3	4.8	77	<0.5	0.4	13	146	8.8	241
% detected	60%	30%	53%	100%	98%	100%	100%	35%	21%	81%	81%	100%	98%
Median	8.1	nd	nd	11	5.1	3.5	57	nd	nd	13	25	6.0	165
Minimum	4	0	0	5	2	1	40	1	0	6	2	2	121
Maximum	218	1	16	31	37	8	333	29	2	21	904	26	990

[1]Samples filtered through 0.45-micrometer filter before analysis.

[2]Hardness expressed as milligrams per liter equivalent of calcium carbonate.

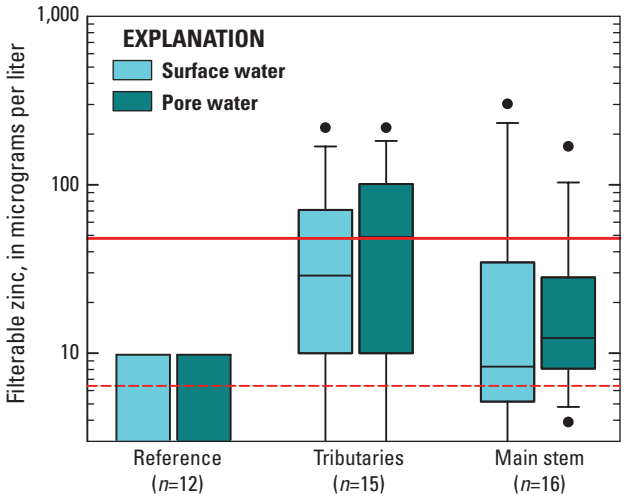

Figure 2. Zinc concentrations in reconnaissance samples of surface water and pore water, 2016–17. Boxes show median and upper and lower quartiles; error bars indicate 5th and 95th percentiles. Red solid and dashed lines are 12-week 20-percent effect concentrations for survival and growth, respectively, from water-only tests with juvenile mussels (*Lampsilis siliquoidea*). [*n*, number of samples]

balance between concentrations of AVS and concentrations of divalent metals potentially controlled by AVS (Ankley and others, 1996).

Concentrations of filtered zinc in pore-water peepers (PWP-zinc; fig. 4*C*) varied widely among sampling sites and indicated that the greatest zinc bioavailability was in Center Creek, upper Shoal Creek, and lower Spring River.

Mussel Community Surveys

Results of the mussel surveys reported by EcoAnalysts, Inc. (2018), are summarized in table 5 and figure 5. Species richness and measures of abundance and areal density of live mussels varied widely among sites but were generally higher at reference sites. The greatest density and species richness of mussels were detected at reference sites in the upper Spring River and North Fork Spring River. The remaining reference sites in Center Creek and Neosho River had mussel densities and species richness that overlapped with test sites, but all reference sites had some live mussels and at least three live species. Mussel assemblages at sites in Center Creek, middle Spring River, and Shoal Creek followed similar patterns, with lower mussel density and species richness in metal-contaminated stream reaches downstream from mining activity. The percentage of juvenile unionids differed widely among sites, from less than 1 percent at several sites in Center Creek and Shoal Creek to more than 50 percent at several sites in the middle reach of Spring River. The spatial pattern of Corbicula density was nearly opposite that of juvenile unionids, and

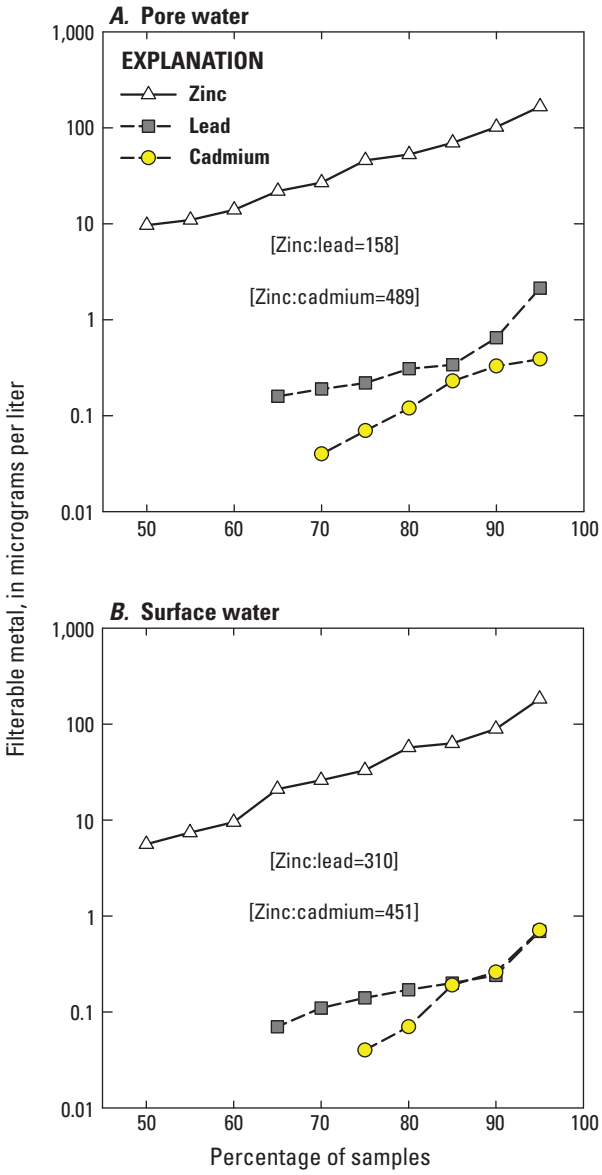

Figure 3. Concentrations and ratios of zinc, lead, and cadmium in pore water and surface water from Tri-State Mining District streams, 2016–17. *A*, pore water; *B*, surface water. Symbols indicate percentage of samples with each metal at or less than specified concentrations, calculated at 5-percent intervals. Ratios of zinc:lead and zinc:cadmium in brackets are means for intervals where both metals of a pair were detectable.

Corbicula density exceeded 40 per square meter at several tributary sites. The predominance of juvenile mussels in the middle reach of the Spring River (SP08/SP10/SP12B) indicates that recruitment at these sites benefitted from relatively favorable conditions, such as close connectivity with upstream reference sites, whereas repopulation of mussel assemblages in tributary sites may be limited by lack of unionid parental stock upstream and (or) lack of host fishes.

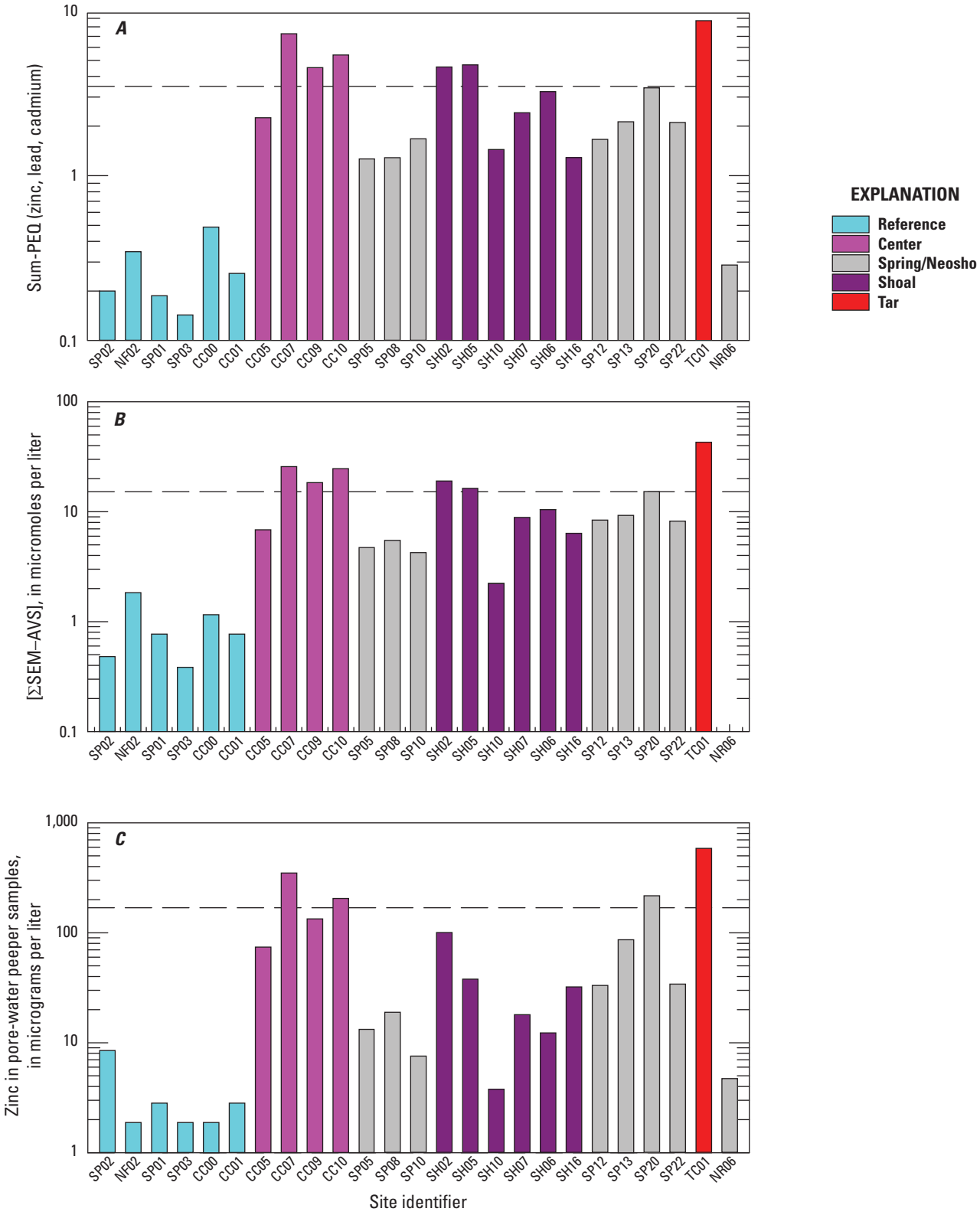

Figure 4. Metal concentrations in sediment and pore water, fall 2017. *A*, sediment hazard quotients (summed probable effect quotient values [sum-PEQ]) for zinc, lead, and cadmium; *B*, summed molar concentrations of simultaneously extracted metals relative to acid-volatile sulfide ([ΣSEM–AVS]); *C*, zinc in pore-water peeper samples. Colors of bars indicate different stream segments. Dashed lines are geometric mean of 20-percent effect concentration estimates for juvenile mussels (table 10).

Table 4. Physical and chemical characteristics of sediments from primary sampling sites, fall 2017.

[ID, identifier; TOC, total organic carbon; %, percent; SEM, simultaneously extracted metals; µg/g, microgram per gram; Ni, nickel; Cu, copper; Zn, zinc; Cd, cadmium; Pb, lead; SEM–PEQ, simultaneously extracted metals probable effect quotient; PEQ, probable effect quotient; sum-PEQ, summed probable effect quotient values (sediment metal concentration/probable effect concentration); ΣSEM, sum of simultaneously extracted metals; µmol/g, micromole per gram; AVS, acid-volatile sulfide; [ΣSEM–AVS], summed molar concentrations of simultaneously extracted metals relative to acid-volatile sulfide; µg/L, microgram per liter]

ID	Site	TOC (%)	Clay (%)	Silt (%)	Total sand (%)	Coarse sand (%)	SEM (µg/g)					SEM–PEQ			Sum-PEQ	ΣSEM (µmol/g)	AVS (µmol/g)	[ΣSEM–AVS] (µmol/g)	Peeper Zn (µg/L)
							Ni	Cu	Zn	Cd	Pb	Zn-PEQ	Cd-PEQ	Pb-PEQ	(Zn+Cd+Pb)				
1	NF02	0.36	3.2	4.0	93	27	1.5	1.08	141	0.35	5.56	0.31	<0.01	0.04	0.35	2.2	0.3	1.9	2.3
2	SP01A	1.11	10.6	17.6	72	20	3.18	3.45	49.2	0.49	10.5	0.11	<0.01	0.08	0.19	0.9	0.2	0.8	2.6
3	SP03	0.33	3.9	4.1	92	20	3.02	1.85	29.2	0.27	10.4	0.06	<0.01	0.08	0.15	0.6	0.2	0.4	1.5
4	CC00	2.28	14.3	60.6	25	2	4.83	4.20	171	1.40	15.2	0.37	<0.01	0.12	0.49	2.9	1.7	1.2	2.2
5	CC01G2	1.05	11.3	21.7	67	41	3.28	2.99	68.3	0.69	14.0	0.15	<0.01	0.11	0.26	1.2	0.4	0.8	3.3
6	CC05D	0.48	3.2	5.2	92	55	4.56	2.12	477	3.57	156	1.04	0.01	1.22	2.3	8.2	1.1	7.1	78
7	CC07	0.54	4.7	8.8	87	43	3.24	5.31	1,780	14.3	435	3.88	0.04	3.4	7.3	29.6	3	27	367
8	CC09	0.28	2.8	3.7	94	47	4.28	2.24	1,310	5.45	218	2.85	0.01	1.7	4.6	21.3	2.3	19	141
9	CC10	0.42	7.0	7.6	85	50	5.81	3.33	1,670	8.28	231	3.64	0.02	1.8	5.5	26.9	1.4	26	216
10	SP05	0.56	3.5	6.1	90	67	5.10	2.54	416	2.05	46.9	0.91	0.01	0.37	1.3	6.7	1.8	4.9	14
11	SP08	0.22	1.6	1.9	97	74	3.28	1.38	432	3.08	44.3	0.94	0.01	0.35	1.3	6.9	1.2	5.7	20
12	SP10	0.90	6.3	10.4	83	2	4.61	3.34	557	3.85	59.3	1.21	0.01	0.46	1.7	9	4.6	4.4	8.2
13	SH02	1.37	8.2	30.3	62	42	3.63	5.16	1,570	4.68	151	3.42	0.01	1.18	4.6	25	5.3	20	106
14	SH05	2.22	12.6	41.6	46	8	4.27	6.05	1,680	5.83	137	3.66	0.01	1.07	4.7	26.6	9.7	17	40
15	SH06	1.28	8.2	31.0	61	19	4.78	4.83	1,080	4.30	116	2.35	0.01	0.91	3.3	17.3	6.5	11	13
16	SH07	1.37	10.6	32.1	57	29	4.84	5.24	803	3.87	86.4	1.75	0.01	0.68	2.4	12.9	3.7	9.2	19
17	SH10	1.52	12.4	37.2	50	14	4.16	4.11	459	2.76	57.6	1	0.01	0.45	1.5	7.5	5.2	2.3	3.5
18	SH16A	0.28	2.8	2.3	95	59	4.49	1.18	417	2.35	49.5	0.91	0.01	0.39	1.3	6.7	0.2	6.6	34
19	SP12B	0.44	3.2	4.2	93	67	5.35	2.83	599	2.36	47.3	1.31	0.01	0.37	1.7	9.6	0.9	8.7	35
20	SP13	0.72	6.6	10.7	83	14	5.75	4.36	737	4.19	68.2	1.61	0.01	0.53	2.1	11.8	2.2	9.6	91
21	SP20	0.94	10.1	16.5	73	11	6.41	5.25	1,170	6.46	113	2.55	0.02	0.88	3.4	18.7	2.9	16	228
22	SP22	0.91	7.8	12.3	80	8	6.66	4.63	737	4.40	65.1	1.61	0.01	0.51	2.1	11.8	3.3	8.5	36
23	TC01	1.02	6.3	7.8	86	54	30.5	7.45	3,420	9.84	168	7.45	0.03	1.31	8.8	53.9	9.6	44	615
24	NR06	0.52	12.8	20.3	67	0	4.98	3.52	93.7	0.40	11.1	0.2	<0.01	0.09	0.29	1.6	1.7	−0.1	4.6
25	SP02	0.77	5.1	10.4	85	15	3.17	2.62	51.2	0.37	11.6	0.11	<0.01	0.09	0.2	0.9	0.4	0.5	8.9

Table 5. Summary of qualitative and quantitative mussel surveys, 2016 and 2017.

[CPUE, catch per unit effort; min, minute; SE, standard error of preceding mean; %, percent; --, no data]

Site[1]	Qualitative survey				Quantitative survey											
								Mean density per square meter, with standard error								
	Live mussels	Live species	Total species	CPUE (per 10 min)	Live mussels	Live species	Total species	Total	SE	Adult	SE	Juvenile	SE	Corbicula	SE	Juvenile (%)	Mortalities (%)
NF02	122	11	12	24.4	3	11	13	6.7	1.2	6.4	1.2	0.2	0.2	0.4	0.2	3.6	0.6
SP01A	199	12	15	39.8	217	16	21	8.7	1.3	8	0.3	0.6	--	2.5	--	7.4	3.6
SP01B	135	15	15	45	--	--	--	--	--	--	--	--	--	--	--	--	--
SP02	216	14	14	43.2	48	10	10	3.2	1.3	3.1	1.3	0.1	0.2	2.1	0.8	4.2	0
SP03	59	9	11	9.8	--	--	--	--	--	--	--	--	--	--	--	--	--
SP05	43	8	8	7.2	72	11	13	3.8	0.1	3.1	1.1	0.7	0.4	2.9	1.3	19.4	2.7
SP06	44	9	9	7.3	--	--	--	--	--	--	--	--	--	--	--	--	--
SP08A	3	3	6	0.5	--	--	--	--	--	--	--	--	--	--	--	--	--
SP08B	6	2	3	1	1	1	1	0.1	0.1	0	0	0.1	0.1	0.4	0.3	100	0
SP10C	9	5	8	1.1	2	2	4	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.2	50	0
SP12A	9	3	8	0.3	--	--	--	--	--	--	--	--	--	--	--	--	--
SP12B	7	4	5	1.2	18	6	6	0.7	0.3	0.5	0.3	0.2	0.2	1.2	0.8	33.3	0
SP13	10	4	4	1.7	8	5	7	0.3	0.2	0.3	0.2	0	0	1.3	0.5	12.5	11.1
SP19	36	6	6	9	25	5	5	1.3	0.5	1	0.5	0.4	0.3	1.1	0.6	28	7.4
SP20	--	--	--	--	19	5	9	0.8	0.4	0.6	0.3	0.2	0.2	1.4	0.5	21.1	13.6
SP22	2	3	3	0.3	7	3	5	0.3	0.2	0.3	0.2	0	0	0.6	0.3	0	0
CC00	12	5	7	2	6	3	3	1.6	1.7	0.3	0.5	1.3	1.3	58.7	54	0	0
CC01F	1	1	1	0.2	--	--	--	--	--	--	--	--	--	--	--	--	--
CC01G2	6	3	8	1	3	2	6	0.2	0.2	0.2	0.2	0	0	2.3	1.4	--	0
CC03	2	2	3	0.4	--	--	--	--	--	--	--	--	--	--	--	--	--
CC05D	12	4	5	2.4	0	0	0	0	0	--	--	--	--	0.3	0.4	--	--
CC07	0	0	12	0	0	0	5	0	0	--	--	--	--	21.3	10.7	--	--
CC10B	0	0	0	0	0	0	0	0	0	--	--	--	--	0	0	--	--
SH02A	1	1	6	0.3	--	--	--	--	--	--	--	--	--	--	--	--	--
SH05A	121	7	8	6.7	54	6	6	2.2	0.9	2.2	0.9	0	--	26.4	6.1	0	0
SH06A	8	3	3	0.4	--	--	--	--	--	--	--	--	--	--	--	--	--
SH07	7	4	4	1.4	--	--	--	--	--	--	--	--	--	--	--	--	--
SH08	35	4	6	4.4	19	6	7	1.5	0.8	1.4	0.8	0	--	123.1	18.2	0	5.3
SH10B	1	1	4	0.2	0	0	2	0	0	--	--	0.1	--	40.8	17.7	--	--

Table 5. Summary of qualitative and quantitative mussel surveys, 2016 and 2017.—Continued

[CPUE, catch per unit effort; min, minute; SE, standard error of preceding mean; %, percent; --, no data]

Site[1]	Qualitative survey				Quantitative survey												
								Mean density per square meter, with standard error								Juvenile (%)	Mortalities (%)
	Live mussels	Live species	Total species	CPUE (per 10 min)	Live mussels	Live species	Total species	Total	SE	Adult	SE	Juvenile	SE	Corbicula	SE		
SH15A	1	1	3	0.3	--	--	--	--	--	--	--	--	--	--	--	--	--
SH16A	0	0	0	0	--	--	--	--	--	--	--	--	--	--	--	--	--
SH16B	--	--	--	--	0	0	0	0	0	--	--	0.2	0.2	0.4	0.2	--	--
NR03	3	2	7	0.1	--	--	--	--	--	--	--	--	--	--	--	--	--
NRSF	141	7	8	35.3	13	4	4	0.5	0.4	0.5	0.3	0	0.1	0.7	0.5	23.1	0
NR04	18	4	4	3	--	--	--	--	--	--	--	--	--	--	--	--	--
NR05	2	1	1	0.3	--	--	--	--	--	--	--	--	--	--	--	--	--
NR06	32	5	5	5.3	25	6	6	1.2	0.8	0.9	0.7	0.3	0.4	0.1	0.2	8.3	7.7

[1]Letters appended to site identifiers indicates that survey was conducted in one or more locations within the main site (as defined in table 1).

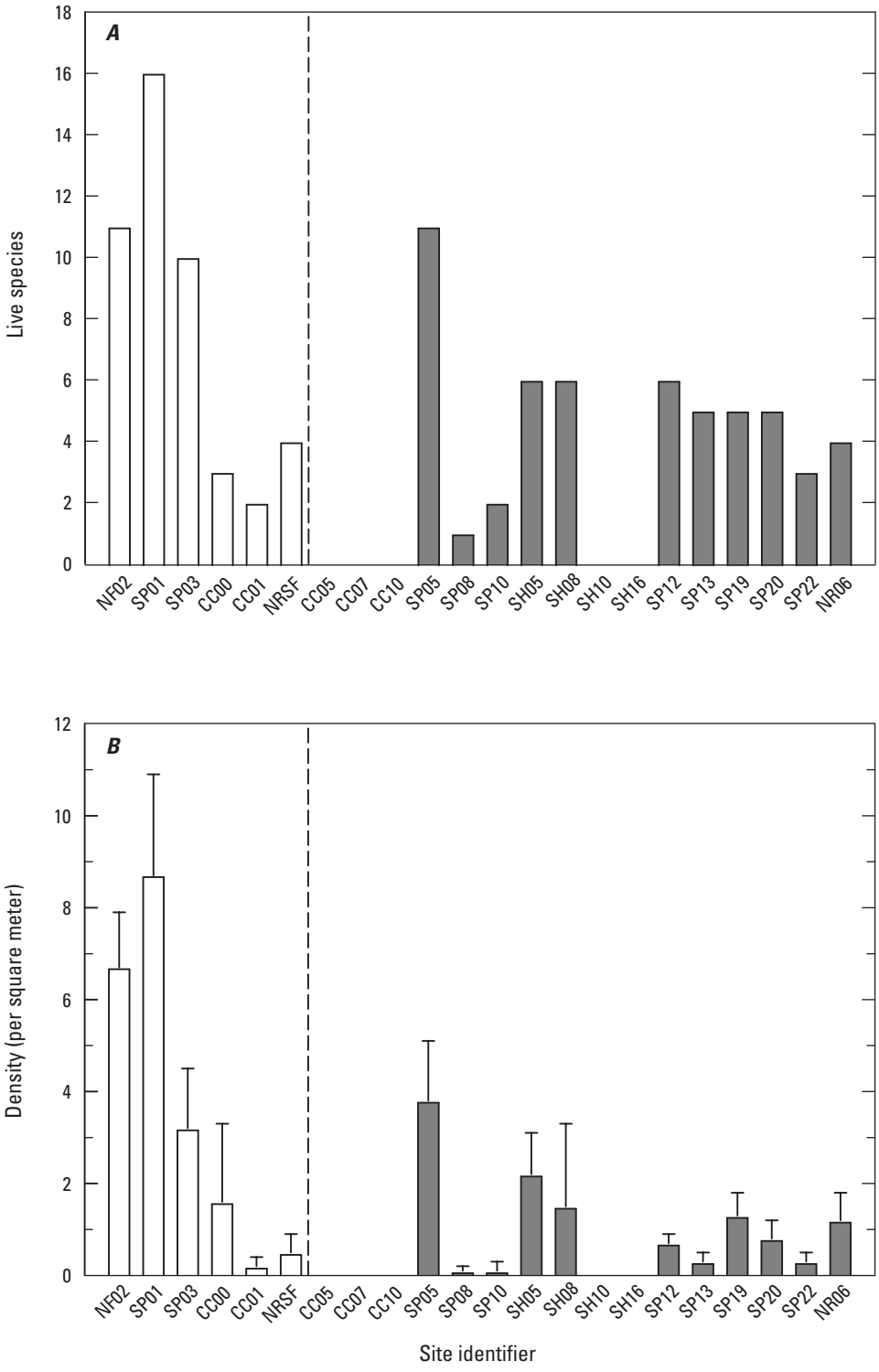

Figure 5. Characteristics of mussel assemblages at reference sites (hollow bars) and test sites (filled bars) from quantitative surveys. *A*, live mussel species; *B*, mussel density (mean and standard error). Data from EcoAnalysts, Inc. (2018).

Metals in Corbicula

Soft tissues of Asian freshwater clams, *C. fluminea* (Corbicula), were collected from mussel survey sites to serve as a potential biomonitor of metal exposure in mussels. Angelo and others (2007) reported significant positive correlations of concentrations of zinc, lead, and cadmium in Corbicula tissues with tissues of unionid mussels from the same sites and indicated that Corbicula and unionids experienced similar levels of metal exposure. In the present study, concentrations of zinc, lead, and cadmium in Corbicula were significantly correlated with concentrations of each of these metals in sediment, although the strength of these associations was stronger for lead and cadmium (probability of type-1 error [p] <0.006) than for zinc (p=0.036; fig. 6). Other studies have had mixed success relating zinc in Corbicula tissue to ambient environmental zinc levels, with weak relations attributed to differences in zinc bioavailability, especially in sediment (Bonnail and others, 2016), or to physiological regulation of internal zinc concentrations (Besser and others, 2007a). Concentrations of lead and cadmium in Corbicula also had significant negative associations with unionid species richness (fig. 6*B*, *C*). Although the weak correlations of zinc in sediment with zinc in Corbicula tissues limit the usefulness of these data for explaining the overall exposure and effects of metals on Tri-State Mining District mussel assemblages, these data support the hypothesis that concentrations of lead and cadmium, although much lower than concentrations of zinc, contribute to overall toxic effects on mussels in Tri-State Mining District streams.

We evaluated the association of trends in mussel surveys with metal concentrations in sediment and water by comparing responses between groups of sites classified as "low metal" and "high metal." Low- and high-metal groupings based on sediment metals were divided at a sum-PEQ index of 1.0, the same benchmark used to evaluate reference sites and a level assumed to be equivalent to the probable effect concentration (MacDonald and others, 2000). Waterborne metal concentrations for each site were estimated from geometric means of zinc concentrations in stream water and push-point samples, with low- and high-metal groups separated by the value of 10 µg/L as zinc, which is the lowest concentration that was always detectable in reconnaissance samples and a concentration that corresponds closely to water-only toxicity thresholds described in the "Toxicity of Waterborne Metals in Long-Term Tests with Juvenile Freshwater Mussels (Spring 2017)" section. Live species richness and CPUE estimated from the qualitative survey and live species richness and mean areal density from the quantitative survey were consistently greater for the low-metal groups, based on either sediment or water (fig. 7), by factors from 1.9 to 8.2. The statistical significance of these differences was evaluated using the Wilcoxon test. For the qualitative survey data, live species richness was significantly greater in the low-metal sites based on zinc in water and sediment (type-1 error p=0.028 for both), and the CPUE was significantly greater in the low-metal sites based on zinc in water (p=0.028). For the quantitative survey data, no significant differences in richness or density were detected between the low- and high-metal sites based on sediment zinc, but richness and density were significantly greater in the low-metal sites based on zinc in water (p=0.027). These analyses indicate that mussel community metrics are negatively associated with increases in metal exposure in streams of the study area. Trends in these data were consistent with data presented by Angelo and others (2007), who reported decreases in mussel abundance and richness downstream from mining activities and significant relations between mussel community characteristics and metal concentrations in sediment.

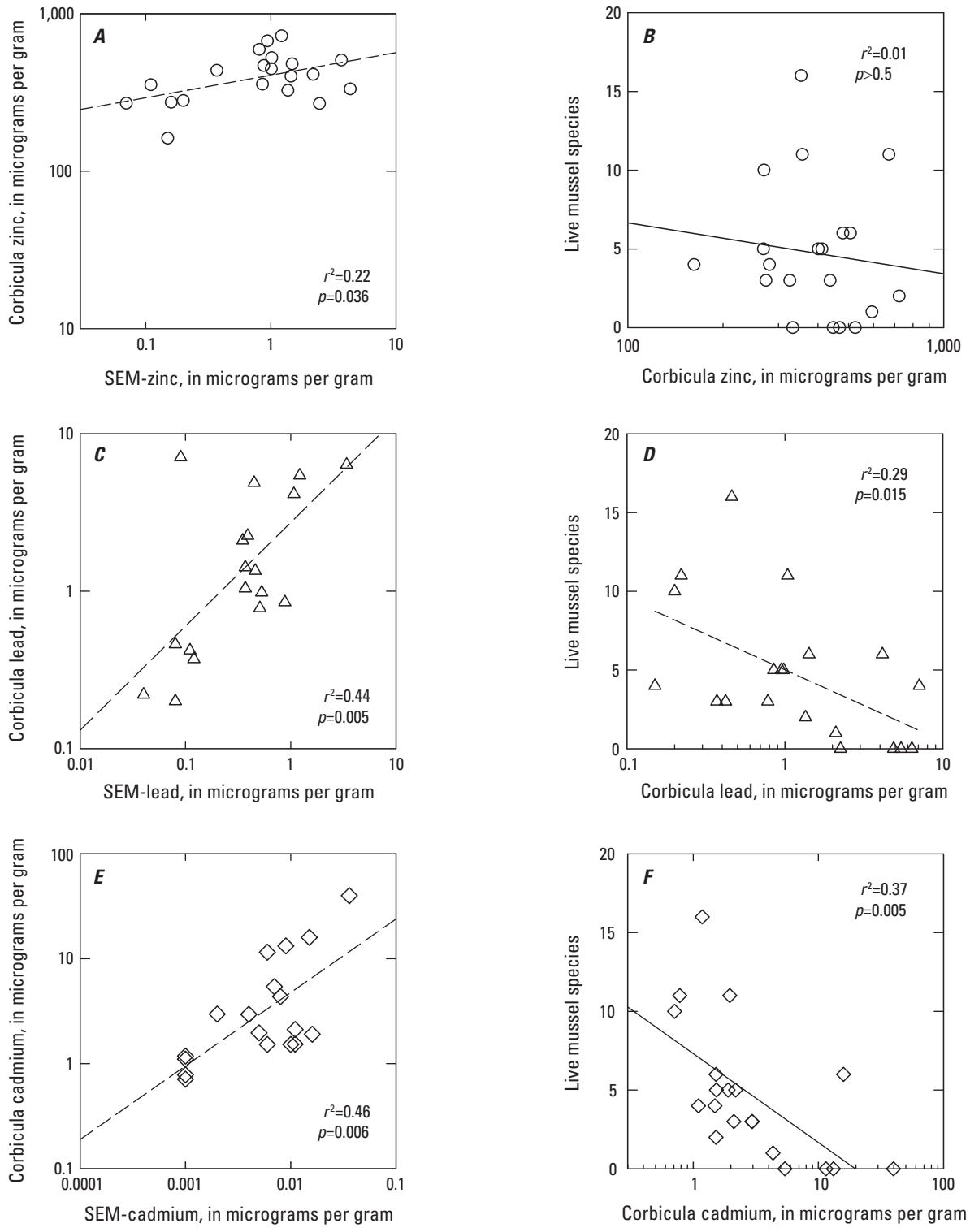

Figure 6. Associations of zinc, lead, and cadmium bioaccumulation by Corbicula with metal concentrations in sediment (plots *A*, *C*, *E*) and with species richness of unionid mussels (plots *B*, *D*, *F*). *A*, Corbicula zinc; *B*, Corbicula zinc in live mussel species; *C*, Corbicula lead; *D*, Corbicula lead in live mussel species; *E*, Corbicula cadmium; *F*, Corbicula cadmium in live mussel species. Hollow shapes are site means. Dashed lines are linear regressions. [SEM, simultaneously extracted metals; r^2, coefficient of determination; *p*, probability; >, greater than]

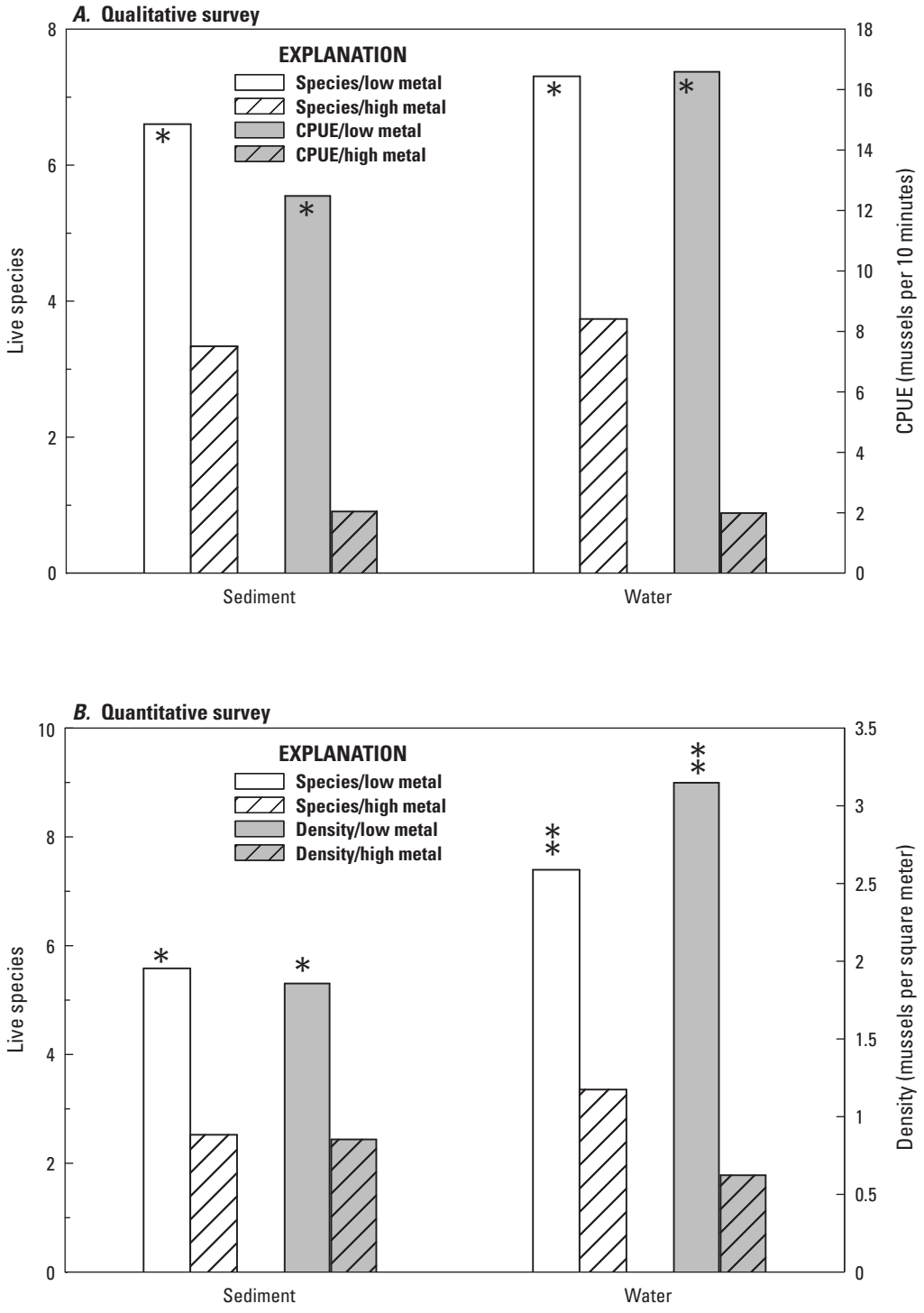

Figure 7. Comparison of mussel survey metrics between low- and high-metal sites. *A*, qualitative survey; *B*, quantitative survey. White bars are mean species richness; gray bars are mean abundance (catch per unit effort [CPUE] or density). For each pair, the hollow bar represents the low-metal range, and the hatched bar represents the high-metal range, defined by benchmarks for zinc in sediment (summed probable effect quotient value=1) or in water (zinc=10 micrograms per liter). A single asterisk indicates significant difference between the pair (probability [*p*] less than [<] 0.05); a double asterisk indicates $0.05<p<0.10$ (Wilcoxon test).

3. Toxicity of Waterborne Metals to Juvenile Freshwater Mussels

The relatively low sensitivity of juvenile mussels in previous laboratory toxicity testing with sediments from the Tri-State Mining District (MacDonald and others, 2009; Besser and others, 2015) is inconsistent with evidence of widespread and severe effects on mussel assemblages (Angelo and others, 2007). These contrasts indicate that laboratory sediment toxicity tests completed to date have not adequately represented the nature and duration of metal exposure experienced by juvenile mussels in the wild. Juvenile mussels primarily live and feed in surficial sediments (Yeager and others, 1994; Cope and others, 2008), but they may be exposed to varying combinations of particulate metals (in sediment and food) and aqueous metals (in pore water and surface water), depending on their burrowing depth and feeding behavior (Kemble and others, 2020). The cumulative metal exposure during the juvenile life stage of freshwater mussels (which may last several years) is greatly underestimated by the standard 28-day sediment toxicity test method (ASTM International, 2020). Recent research has indicated that the length of water-only toxicity tests with juvenile mussels can be extended from 4 weeks to 12 weeks, resulting in greater sensitivity (Wang and others, 2020). Such long-term laboratory exposures may better reflect the responses of juvenile mussels in the wild and thus may better predict changes in mussel assemblages in affected streams (Haag and others, 2019).

Objective

The objective of this study was to determine toxicity thresholds for sensitive responses of juvenile mussels in 12-week water-only toxicity tests with zinc and with a three-metal mixture (zinc+lead+cadmium) at concentrations detected in streams of the study area.

Methods

Mussel Culture

Gravid adult female fatmucket mussels (*L. siliquoidea*) were collected from Silver Fork Creek in Boone County, Mo., in fall 2016 and held in the USGS laboratory at reduced temperatures to delay the release of glochidia. In advance of planned toxicity tests, mussel brood stock was transferred to the laboratory of Chris Barnhart at Missouri State University in Springfield, Mo., for transformation of glochidia to the juvenile stage (Barnhart, 2006). Juvenile mussels were then shipped back to the USGS laboratory and reared for testing. Newly transformed juvenile mussels obtained from several females were reared at the USGS Columbia Environmental Research Center (CERC) to a shell length of about 1.0 mm (about 4 weeks).

Toxicity Tests

Water-only toxicity tests with juvenile mussels were completed after 12 weeks (Wang and others, 2020). Toxicity tests used intermittent-flow proportional diluters, which delivered five test solutions in a 50-percent dilution series, producing a sixteenfold range of nominal concentrations in each test. Filterable zinc concentrations in these tests broadly overlapped with zinc concentrations measured in stream water and surface (push-point) pore water during reconnaissance sampling (fig. 2). Test 1 was a zinc-only exposure and test 2 was an exposure with a mixture of zinc, lead, and cadmium, based on the ratios of these metals in the stream water and pore-water concentrations. Stock solutions with zinc (stock 1) and lead plus cadmium (stock 2) were delivered simultaneously to a diluter by Hamilton syringe pumps (Hamilton, Reno, Nevada). Test 1 had a maximum nominal zinc concentration of 200 µg/L and a low nominal concentration of 12.5 µg/L as zinc. Test 2 included the same series of zinc concentrations plus lead concentrations ranging from 0.125 to 2.0 µg/L and cadmium concentrations ranging from 0.063 to 1.0 µg/L.

Water-only toxicity tests used CERC well water diluted to a hardness of about 166 mg/L as calcium carbonate, consistent with the typical hardness of water measured in reconnaissance samples from the Spring River (table 3). Test temperature was maintained with a temperature-controlled water bath at 23 °C with temperature logged every 15 minutes using a HOBO U22 V2. Exposure chambers were 300-mL tall-form glass beakers with a 2.5-cm overflow hole drilled in the side, such that each screened beaker held 200 mL of water. Overflow holes were covered with a stainless-steel screen (50 mesh/279-µm opening) glued with silicone caulk. Splitters on each of the 6 delivery lines fed 2 boxes, each containing 6 replicate beakers (12 beakers per treatment). After day 28, when 4 replicates from each treatment were destructively sampled, the 8 remaining beakers for each treatment were held in a single splitter box. Between days 0 and 28, the diluter cycled every half hour and the 1.0 L of test solution from each line was split 16 ways to deliver 63 mL per beaker per cycle (3.0 L per day), a replacement rate of 15 volumes per day. After day 28, the diluter cycled every hour, delivering 125 mL per beaker per cycle, to produce the same replacement rate.

At the start of the test, 10 live mussels (visible foot movement) were impartially transferred into each replicate beaker with 5 mL of silica sand (100- to 400-µm particles; Granusil no. 5010; Unimin Corporation, New Canaan, Connecticut), which was washed with well water overnight and rinsed with deionized water before use. Archive samples of 4 replicates of 10 mussels each were collected for measurements of initial lengths and dry weights. Dry weight of mussels was determined after being ovendried for 24 hours at 60 °C. During the tests, mussels were fed 3 mL of mixed algae (510-nanoliter cell volume per milliliter) twice per day. The algae mixture was prepared daily by adding 1 mL of *Nannochloropsis* concentrate and 2 mL of Shellfish Diet concentrate (Reed Mariculture, Campbell, California) into 1.8 L of water. Stocks

of algae were maintained in aerated cont4ainers at 4 °C and changed daily. Every 2 weeks, surviving mussels in each beaker were transferred into 200-mL glass dishes containing about 100 mL of test solution for determination of survival.

Test Endpoints

Test endpoints of survival, growth, and biomass were determined after 4 weeks and 12 weeks. Four of 12 replicates per treatment were destructively sampled on day 28 for determination of length, weight, and biomass. Survival in all replicates was assessed by observing foot movement, either spontaneous or in response to gentle probing. Mussels without foot or shell movement, with an empty shell, or with a gaped shell containing swollen or decomposed tissue were recorded as mortalities and removed from test beakers. Surviving mussels from destructively sampled replicates were preserved in 70 percent ethanol for measurements of shell length and dry weight. Shell length was determined by digital photographic analyses. Growth was calculated as the average dry weight of surviving animals in a replicate (total dry weight per number of survivors), and biomass was the total dry weight of surviving mussels for a replicate. Biomass reflects combined effects of survival and growth and is thus usually more sensitive than survival or growth alone. The biomass endpoint can be considered an estimate of the contribution of a cohort of juvenile mussels to secondary production at a site. Surviving mussels from the other eight replicates were transferred back into their beakers after the beaker was rinsed with test water and clean sand was added. These eight replicates were sampled for the same endpoints after 12 weeks. The test acceptability criterion (TAC) for the chronic mussel test was a minimum survival of 80 percent in the control after 4 weeks (ASTM International, 2015, 2020).

Water Analyses

Samples of water from water-only tests were analyzed for concentrations of filterable (<0.45 µm) zinc, lead, and cadmium on days 0, 28, 56, and 84 and for major ions and DOC on days 28, 70, and 84, as described in section 2. A minimum of three external calibration standards plus a calibration blank were used to calibrate the instrument response, and continuing calibration blanks and standards, laboratory spikes, duplicates, and other laboratory QA/QC procedures were used to verify instrument performance throughout the analyses (appendix 2).

Data Analysis

Toxic effects in the zinc-only test and the metal-mixture test were evaluated relative to measured filtered (0.45-µm) zinc concentrations. Chronic effect concentrations, including the no-observed-effect concentration and lowest-observed-effect concentration based on analysis of variance (ANOVA) were determined using SAS/STAT software (version 9.4). The 20-percent effect concentrations (EC20s) were determined based on concentration-response models (CRMs) generated by Toxicity Relationship Analysis Program software, version 1.30 (Erickson, 2010).

Results and Discussion

Metal Exposure

Based on the data presented in section 2, the maximum nominal zinc concentration in the 12-week water-only toxicity tests was 200 µg/L, and the diluter produced nominal zinc concentrations as low as 12.5 µg/L as zinc. Measured concentrations of zinc, lead, and cadmium in reconnaissance samples were sorted to align samples by percentiles (fig. 3), and these distributions were used to estimate the ratios of the three metals at the concentration ranges where matched percentiles were available. The average zinc:lead ratio was 158 in pore water and 310 in surface water; the average zinc:cadmium ratio was 489 for pore water and 451 for surface water. These ratios were simplified to establish the ratios used in toxicity tests: zinc:lead=200; zinc:cadmium=400.

Metal concentrations in tests were generally close to nominal, and measured zinc concentrations averaged 94 percent of nominal in the zinc-only test and 92 percent of nominal in the metal-mixture test (table 6). Lead concentrations were detectable in the four highest treatments in the mixture study (nominals, 0.25–2.0 µg/L), and measured lead concentrations averaged 51 percent of nominal in these treatments. Cadmium concentrations were only detectable at the highest concentration of the mixture test (nominal cadmium=1.0 µg/L), and measured cadmium in this treatment was 91 percent of nominal. Lead and cadmium concentrations less than analytical detection limits in the water-only test were expected, based on the distribution of measured metal concentrations in field-collected samples, but we added these metals at concentrations less than detection limits under the assumption that the three metals were at the same ratios across the full range of zinc concentrations. These results did not allow us to precisely measure exposure to lead and cadmium in all treatments of the metal-mixture study, but they indicate that mussels in this test were exposed to these metals at concentrations and ratios consistent with those measured in field-collected samples.

Table 6. Toxicity of zinc and a three-metal mixture (zinc, lead, cadmium) in 12-week water-only tests with juvenile mussels.

[20-percent effect concentration values and confidence limits are provided in table 10. Toxicity Relationship Analysis Program software models are provided in appendix 3, figure 3.1. Mean and standard errors (SE) are listed for days 28 and 84; µg/L, microgram per liter; *n*, number of samples; %, percent; mg, milligram; sig, significance; [ ], value less than detection estimated for analysis; ns, mean is not significantly less than the control; *, mean is significantly less than the control (Dunnett's test); ANOVA, analysis of variance; *p*, probability; <, less than]

Zinc (µg/L)		*n*	Survival (%)		Growth (mean dry weight, mg)			Biomass (dry weight, mg)		
Nominal	Measured		Mean	SE	Mean	SE	Sig	Mean	SE	Sig
Zinc test—4 weeks										
Control	[3]	4	95	2.9	0.563	0.065	ns	5.27	0.48	ns
13	13	4	97.5	2.5	0.42	0.032	ns	4.08	0.32	ns
23	23	4	97.5	2.5	0.293	0.04	*	2.87	0.42	*
45	45	4	87.5	2.5	0.243	0.024	*	2.12	0.17	*
95	95	4	97.5	2.5	0.198	0.021	*	1.89	0.14	*
215	215	4	87.5	4.8	0.095	0.013	*	0.87	0.13	*
ANOVA			**(*p*=0.078)**		**(*p*=0.0001)**			***(p=0.0001)***		
Zinc test—12 weeks										
Control	[3]	8	92.5	2.5	9.9	0.55	ns	91.4	5.2	ns
12.5	12	8	98.8	1.3	7.92	0.57	*	78.2	5.8	*
25	22	8	98.8	1.3	7.35	0.44	*	72.7	4.6	*
50	45	8	95	1.9	5.27	0.46	*	50	4.4	*
100	93	8	96.3	1.8	2.36	0.15	*	22.7	1.5	*
200	209	8	86.3	2.6	0.75	0.04	*	6.5	0.4	*
ANOVA			**(*p*=0.0014)**		**(*p*<0.0001)**			**(*p*<0.0001)**		
Mixture test—4 weeks										
Control	[3]	4	100	0	ns 0.503	0.086	ns	5.03	0.87	ns
12.5	13	3	93.3	6.7	ns 0.37	0.067	ns	3.47	0.72	ns
25	22	4	92.5	2.5	ns 0.298	0.033	ns	2.79	0.32	*
50	47	4	97.5	2.5	ns 0.213	0.058	*	2.06	0.48	*
100	87	4	92.5	4.8	ns 0.13	0.011	*	1.21	0.1	*
200	193	4	90	0	* 0.083	0.009	*	0.75	0.09	*
ANOVA			**(*p*=0.1627)**		**(*p* =0.0001)**			**(*p* =0.0001)**		
Mixture test—12 weeks										
Control	[1.2]	8	100	0	ns 22.04	1.91	ns	220.4	19.1	ns
12.5	12	8	97.5	1.6	ns 11.58	0.9	*	113.5	9.7	*
25	22	7	95.7	2	ns 5.16	0.48	*	49.5	5	*
50	47	8	77.5	11.1	* 2.23	0.33	*	19.1	4.6	*

Table 6. Toxicity of zinc and a three-metal mixture (zinc, lead, cadmium) in 12-week water-only tests with juvenile mussels.—Continued

[20-percent effect concentration values and confidence limits are provided in table 10. Toxicity Relationship Analysis Program software models are provided in appendix 3, figure 3.1. Mean and standard errors (SE) are listed for days 28 and 84; µg/L, microgram per liter; n, number of samples; %, percent; mg, milligram; sig, significance; [ ], value less than detection estimated for analysis; ns, mean is not significantly less than the control; *, mean is significantly less than the control (Dunnett's test); ANOVA, analysis of variance; p, probability; <, less than]

Zinc (µg/L)		*n*	Survival (%)		Growth (mean dry weight, mg)			Biomass (dry weight, mg)		
Nominal	Measured		Mean	SE	Mean	SE	Sig	Mean	SE	Sig
Mixture test—12 weeks—Continued										
100	87	6	71.7	10.8	*	0.15	*	9	2	*
					1.25					
200	193	7	37.1	12.3	*	0.07	*	1.6	0.5	*
					0.53					
ANOVA			**(p=0.0001)**		**(p<0.0001)**			**(p<0.0001)**		

Toxicity of Zinc and the Three-Metal Mixture

The 4-week and 12-week toxicity tests with zinc only and with the metal mixture had mean survival in controls ranging from 92.5 to 100 percent, exceeding the minimum TAC of 80 percent (table 6). Growth of mussels in the first 28 days was similar between the two tests (control mean dry weight per survivor: 0.563 milligram [mg] in the zinc test and 0.503 mg in the metal-mixture test), but growth was more rapid in the mixture test between days 28 and 84, and final control growth and biomass were more than twice as great in the mixture test. Statistical analyses of differences among treatments (ANOVA and Dunnett's test on ranked data; table 6) indicated significant reductions in growth and biomass relative to controls after 4 weeks and 12 weeks in the zinc-only test and the metal-mixture test. Survival was not significantly reduced in the zinc-only test on either sampling date or in the metal-mixture test after 4 weeks, but survival at week 12 was significantly reduced in three treatments from the metal-mixture test.

Concentration-response plots indicated clear negative trends for growth and biomass, with increased metal concentrations in both tests on both sampling dates, but fitting models to the data was difficult because decreased growth and biomass were frequently evident at the lowest metal concentration greater than the control. Without one or more low test concentrations with responses close to the control, it is difficult to reliably estimate low effect concentrations, and regression models may predict low-level effects at concentrations less than the control. To avoid underestimating EC20s, growth and biomass data were fitted to two-parameter sigmoid regression models (SigmaPlot, version 14.0). These models estimate only the 50-percent effect concentration and the slope, while fixing the baseline response at the mean control response.

Toxic effects of metals on juvenile *L. siliquoidea* differed between 4-week and 12-week exposures, as well as between zinc-only exposures and exposures to the three-metal mixture. Mussel survival was largely unaffected in the zinc-only test, with differences of 10 percent or less between high-zinc treatments and controls or low-zinc treatments (table 6). Because effects on survival were minimal, thresholds for effects on growth (mean dry weight of individual) and biomass (total dry weight per replicate) were similar. Toxicity thresholds for growth and biomass on day 28 were similar for the zinc-only test (EC20s of 8.3 and 9.0 µg/L as zinc) and the metal-mixture test (EC20s of 9.7 and 8.3 µg/L as zinc; fig. 8). However, toxicity thresholds for the two tests diverged between week 4 and week 12, and EC20s for growth and biomass increased in the zinc-only test (to 16 and 20 µg/L as zinc, respectively) and decreased in the mixture test. By day 84, survival in the metal-mixture test had decreased significantly in the three highest treatments, with an EC20 for survival of 48 µg/L as zinc, and EC20s for growth and biomass had converged at 6.4–6.5 µg/L as zinc (fig. 8). These trends are consistent with the predominance of acute toxicity from zinc. The current EPA water quality criteria for zinc, based on extensive toxicity testing, established a chronic criterion equal to the acute criterion. Based on this evidence, we would expect similar effect concentrations for the zinc-only test after 4 or 12 weeks, but toxicity in the zinc-only test decreased between 4 and 12 weeks. The unusual length of these studies introduces the possibility that surviving animals at the end of the 12-week test had become more tolerant of aqueous zinc than they were during the first 4 weeks of the test. The greater toxicity of the metal mixture after 12 weeks was presumably not caused by zinc alone but included some contributions from lead and cadmium, even though they were present at low concentrations in water. This hypothesis is supported by the differences in the chronic toxicity thresholds for these metals. When nominal concentrations of each of the three metals from the mixture test were converted to toxic units based on chronic water quality criteria (for example, 90 µg/L for zinc, 2.5 µg/L for lead, and 0.72 gram per liter for cadmium at a hardness of 85 mg/L; EPA, 2021b), summed toxic units for lead and cadmium were about equal to those for zinc.

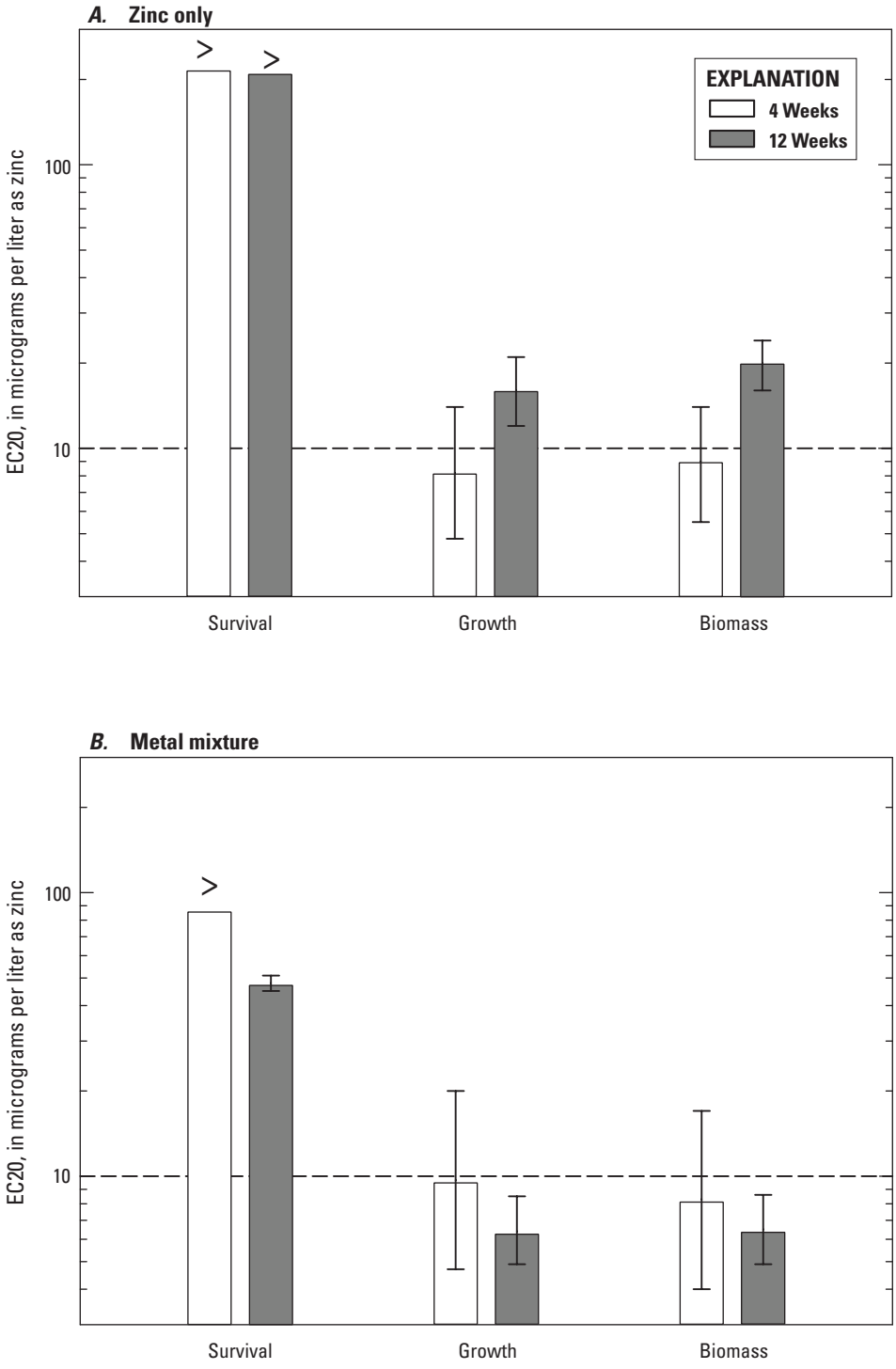

Figure 8. Effect concentrations for water-only tests. *A*, with zinc only; *B*, with zinc in a three-metal mixture (zinc, cadmium, and lead). The 20-percent effect concentrations (EC20s) and 95-percent confidence intervals (error bars) for 4-week and 12-week exposures were estimated with Toxicity Relationship Analysis Program software (appendix 3, fig. 3.1). Symbol (>) indicates the unbounded lower estimate of EC20. Dashed lines indicate zinc concentration of 10 µg/L.

Results of this study differed in some respects from results of recent zinc chronic tests completed with *L. siliquoidea* in our laboratory (Wang and others 2020). The most direct comparisons are between the 12-week zinc-only test in the present study and these recent tests, which were not part of the Tri-State Mining District mussel project but were based on the same 12-week water-only test method used for the present study. Wang and others (2020) reported that *L. siliquoidea* were less sensitive to zinc early in the test, with EC20s of 66 µg/L for growth and biomass after 28 days—about eight-fold greater (less toxic) than EC20s from the zinc-only test in the present study. However, 12-week EC20s for growth and biomass reported by Wang and others (2020) had decreased to 21–22 µg/L, close to 12-week EC20s from the present zinc-only test (16–20 µg/L as zinc). The primary differences in methodology between the two studies were that the test waters were different dilutions of CERC well water (hardness of 100 mg/L for Wang and others [2020] versus 166 mg/L in the present study) and that the previous test was stocked with larger juvenile *L. siliquoidea*, with a mean shell diameter of 1.55 mm at the start of the study, compared to 0.62 mm in the present test. The greater toxicity observed in the first 28 days of our zinc-only test may indicate greater sensitivity of the smaller/younger cohort of juvenile mussels to zinc, whereas the convergence of growth and biomass EC20s for the two studies over 12 weeks may represent convergence of zinc tolerance of the two test groups as they became older/larger. In a similar result, Wang and others (2010) reported that 96-hour median lethal concentrations for zinc to juvenile *L. siliquoidea* increased by a factor of 10 with an increase in the age of juveniles from 5 days to 6 months at the start of the test.

Implications for Toxic Effects on Mussels

These results indicate that the presence of waterborne lead and cadmium in the metal-mixture test resulted in greater chronic toxicity to juvenile mussels compared to the test with zinc alone. In 28-day tests, EC20s were similar for zinc alone and for the zinc-lead-cadmium mixture, with an overall range of EC20s from 8.3 to 9.7 µg/L as zinc. By day 84, sensitivity to zinc alone had decreased but sensitivity to the mixture had increased, as indicated by the onset of significant effects on survival and lower EC20s for growth and biomass (6.4–6.5 µg/L as zinc). These differences are not surprising because many studies have demonstrated that zinc is primarily toxic in short-term exposures (for example, up to 28 days in our tests) and that internal regulation of zinc tends to ameliorate longer-term toxic effects (Besser and others, 2007b; Mebane and others, 2007; Wang and others, 2010). The increased sensitivity of mussels to the metal mixture in the 12-week study presumably reflects contributions of lead and (or) cadmium to chronic toxicity, even though these metals were present at concentrations two orders of magnitude lower than zinc concentrations.

Samples of surface water and pore water collected during summer 2016 frequently exceeded toxicity thresholds for filtered zinc from either the zinc-only test or for the metal-mixture test. The frequency of exceedances of these zinc-based thresholds was about the same for surface-water and pore-water samples and about the same for tributaries and main stem sites. Overall, the frequency of exceedance of zinc thresholds was more than 75 percent for the more sensitive growth thresholds from the metal-mixture test and about 50 percent for thresholds from the zinc-only test. Thresholds for survival—only estimated for the mixture test—were exceeded by an average of more than 25 percent of samples. The frequency of toxicity predicted by exposure to the metal mixture detected in waters of Tri-State Mining District streams was greater than the observed frequency of toxic effects on survival (9 percent of samples) and biomass (12 percent of samples) of juvenile *L. siliquoidea* in previous 4-week toxicity tests with Tri-State Mining District sediment (Besser and others, 2015). Analyses of metals in Tri-State Mining District sediments and pore waters from the previous tests indicated that ratios of zinc, lead, and cadmium were similar to those in waters analyzed for the present study. The greater frequency of toxicity at relatively low metal concentrations in the water-only tests may be explained by increased chronic effects in the 12-week exposure, compared to the previous 4-week sediment exposures. However, the lower frequency of toxicity in the previous sediment tests could also reflect low bioavailability of zinc and other metals in sediment pore waters. EC20s estimated from pore-water metal concentrations during 12-week sediment toxicity tests (see section 4) were 4–40 times greater than water-only EC20s, indicating lower metal bioavailability in pore waters.

The low thresholds for toxicity of waterborne zinc to juvenile mussels derived from our water-only toxicity tests were consistent with effects on wild mussels in Tri-State Mining District streams. Reconnaissance sampling at 31 sites in 2016 produced qualitative data on mussel assemblages and metal concentrations in stream water and shallow pore water, and 20 of these sites were sampled again in 2017 using quantitative methods (EcoAnalysts, Inc., 2018; table 4). Effects of waterborne metals on mussel assemblages were evaluated by comparing sites with low- and high-metal concentrations in water, with the threshold set at 10 µg/L, a concentration slightly greater than the EC20 for effects on growth in 12-week water-only tests. Sites with average zinc concentrations of 10 µg/L or less had greater average live species richness and greater abundance with density estimated by the CPUE (mussels per time) in the qualitative survey and from density (mussels per area) in the quantitative survey. Averages of these variables were consistently greater for sites in the low-metal group, and means for low-metal sites ranged from 51 to 500 percent greater than high-metal sites. Means for live species richness and CPUE from low-metal sites were significantly greater than means for high-metal sites (fig. 7; Wilcoxon test, p=0.01). Exceptions to this trend included sites SP05 and SP06, in Spring River downstream from Center

Creek, and SH05, in upper Shoal Creek, which had greater richness and abundance of live mussels than other sites in the high-metal group. The relatively robust mussel communities at these high-metal sites may reflect differences in concentrations of waterborne and sediment metals among these sites, for example, relatively low waterborne zinc at SH05 (16 µg/L) and relatively low sediment metals at SP05 (sum-PEQ=1.44). Differences in mussel assemblages among these three sites may also reflect other local conditions, such as differences in the accessibility of these sites for mussel recolonization; for example, sites SP05 and SP06 are a short distance downstream from high-quality communities at reference sites in the upper Spring River.

The concordance of results from field sampling and laboratory testing indicates that exposure of freshwater mussels to waterborne metals contributes to adverse effects on freshwater mussel communities in Tri-State Mining District streams. The toxicity predicted by EC20s from water-only tests is greater than that observed in previous exposures with metal-contaminated sediments, but these thresholds are consistent with trends in species richness and density of mussel assemblages. It also is possible that effects observed in previous short-term sediment toxicity tests did not adequately represent longer term effects of exposure to metal mixtures. Another factor that may have affected the relative sensitivity of juvenile mussels in our water-only tests compared to previous sediment tests was the difference in the age/size of juvenile mussels at the start of the tests. Juvenile mussels stocked in the previous sediment tests were larger (mean starting shell lengths of 1.5 and 2.1 mm) than the juveniles stocked into the present water-only tests (mean starting shell length of 0.62 mm).

4. Chronic Toxicity of Metals in Sediments to Juvenile Mussels

In 2007, the USGS characterized sediment toxicity and sediment chemistry at 70 sites across the Tri-State Mining District. This USGS study included laboratory toxicity tests with mussels (*L. siliquoidea*) and amphipods (*H. azteca*; Ingersoll and others, 2008; Besser and others, 2015). Toxic effects on both species increased with increasing metal concentrations in sediments, but mussels were less sensitive than amphipods in these tests. Results of mussel community surveys (Angelo and others, 2007) and sediment toxicity tests (Ingersoll and others, 2008) indicated that metals in surface water, sediment, and pore water likely affected mussels in the Tri-State Mining District (MacDonald and others, 2009). At the time of the previous study, options for characterizing toxicity of sediment and water to juvenile freshwater mussels were limited to short-term (28-day) exposures, based on standard methods for water-only tests (for example, Wang and others, 2013; ASTM International, 2015). The 28-day exposure period limited the range of starting sizes of juvenile mussels that could be used for sediment tests because small juveniles do not grow in 28 days to a size that can be readily recovered from sediment, especially if test sediments contain large amounts of coarse particles (that is, medium and coarse sand-sized particles as many as 2 mm in diameter). Recently, our laboratory has successfully completed several water-only toxicity tests for durations of as many as 12 weeks (Wang and others, 2020). The development of this 12-week sediment toxicity method for juvenile mussels provided an opportunity to evaluate the longer term effects of exposure to sediments. Such exposures may be necessary to reliably characterize sensitivity of freshwater mussels to contaminated sediments during their juvenile stage, which may last for many months or years. Effects detected in long-term laboratory exposures of juvenile mussels to contaminants in sediment or water may explain changes observed in wild mussel communities. Recent studies reported that growth of juvenile mussels during 96-day in situ exposures in Kentucky streams was negatively associated with chronic chemical stressors and that growth was consistently low in streams that had experienced declines of their mussel faunas (Haag and others, 2019).

Objective

The objective of this study was to document the frequency and severity of toxic effects experienced by juvenile mussels during 12-week exposures to sediments representing the range of metal pollution in streams of the study area. Specific questions to be addressed with data from this study included the following:

1. Are responses observed using the 12-week mussel bioassays comparable to those observed in 6-week chronic bioassays with the amphipod *H. azteca*, the most sensitive species in previous sediment toxicity tests with sediments?

2. Does addition of low levels of metals (zinc, lead, and cadmium) that correspond to ambient concentrations in stream water and pore water result in changes in the responses of mussels that are concurrently exposed to metal-contaminated sediments?

3. What levels of metal exposure in sediments—with and without water column metal exposure—represent thresholds for toxicity to mussels?

Methods

Sediment Toxicity Tests

Two sediment toxicity tests were completed to determine the effects of sediment alone and sediment plus metals added to overlying water. Tests with amphipods and mussels were completed concurrently in fall 2017, and a second mussel test (with metals added to overlying water) was completed in spring 2018. Juvenile mussels were obtained from gravid

L. siliquoidea collected from Silver Fork Creek in Boone County, Mo., in spring 2017. Glochidia were transformed to juveniles at Missouri State University (Barnhart, 2006), and newly transformed juveniles were reared at the USGS laboratory to the appropriate age for testing. Amphipods (*H. azteca*) were obtained from continuous cultures at the USGS laboratory. Cohorts of neonate amphipods produced on the same day were reared to 6–7 days of age before the start of the test.

One week before the start of each test, each sediment sample was homogenized and added to exposure beakers, clean overlying water was added, and test chambers were held under static conditions to allow the sediment to equilibrate with oxygenated overlying water (Ingersoll and others, 2008; Wang and others, 2013). Water renewal was started on day 1. On day 0, groups of 10 test organisms were impartially transferred to each replicate exposure beaker. During stocking, 4 replicate groups of 10 organisms were set aside as archive samples to characterize the average dry weight of starting size for each test. In addition, all replicate groups of mussels were photographed before stocking, and shell lengths were determined by digital image analysis. Mean lengths of juvenile mussels stocked in the two sediment tests were close to the recommended starting size of 1.5 mm, although the mussels stocked in the 2017 test were smaller (1.22 mm average) than those stocked for the 2018 test (1.41 mm). Stocking was not consistent with the random order recommended by ASTM International (2020). Treatments were stocked in order based on laboratory-assigned sample numbers (table 4), with the control (sample 25) stocked first, followed by samples 1–24 in order.

The sediment test completed in fall 2017 consisted of whole-sediment toxicity tests with juvenile mussels, *L. siliquoidea* (84 days), and with the amphipod *H. azteca* (42 days; ASTM International, 2020). This test used sediments (<2 mm) from 25 primary sampling sites. Clean overlying water, consisting of well water diluted to a hardness of 166 mg/L as calcium carbonate, was added at a rate of four volume replacements per day using a proportional diluter system but without any added metals. In the second test in spring 2018, juvenile mussels produced by a different set of females were exposed to the same sediments and overlying water, except that the water added to the system contained a mixture of three metals at nominal concentrations of 10 µg/L for zinc, 0.08 µg/L for lead, and 0.025 µg/L for cadmium. This mixture was intended to represent a typical exposure regime for mussels in streams of the study area, based on results of field sampling of stream water and pore water in 2016 (fig. 1) and on results of water-only toxicity tests. The mixture was expected to produce about a 10–20-percent reduction in the mussel biomass endpoint, based on 12-week water-only tests with zinc (see section 3) and based on similar tests reported by Wang and others (2020). The metal mixture was prepared in a 700-L reservoir and delivered via diluter delivery lines so that all test chambers would receive the same metal concentration during water renewals.

Test conditions for sediment toxicity tests were based on standard and published methods (for example, Wang and others, 2013, 2020; ASTM International, 2015, 2020; Besser and others, 2015). The exposure system was maintained at 23 °C, with ambient laboratory light (about 500 lux) and a 16-hour light:8-hour dark photoperiod. Test chambers were 300-mL tall-form beakers with screened overflow windows, which contained 100 mL of sediment and 175 mL of overlying water. The duration of sediment tests with freshwater mussels was extended from 28 days to 84 days (12 weeks), based on methods used for 28-day sediment tests (ASTM International, 2020) and 12-week water-only tests (Wang and others, 2020). Each test included a control sediment from the Spring River (SP02; Spring River at Waco, Mo.; Besser and others, 2015) and clean quartz sand (ASTM International, 2020).

Mussels were fed 3 mL of algae mixture (510-nanoliter cell volume per milliliter) two times per day. Algae mixtures were prepared daily by adding 1 mL of *Nannochloropsis* concentrate and 2 mL of Shellfish Diet concentrate (Reed Mariculture, Campbell, Calif.) into 1.8 L of test water. Algae mixtures were kept in a refrigerator at 4 °C in aerated containers. Algae concentrations in the stock suspension and in selected treatments were measured using an algal cell counter before and after feeding. Amphipods were fed once daily with diatoms (*Thalassiosira weissflogii* [(Grunow) Fryxell & Hasle], 1200TM; Reed Mariculture, Campbell, Calif.) and with a suspension of flake fish food (Tetramin; Tetra Werke), and feeding rates were increased over time (ASTM International, 2020).

Test Endpoints

Mussel tests lasted 12 weeks, with endpoints of survival, growth, and biomass. After 4 weeks, mussels in each replicate beaker were separated from sediment by wet sieving, the number of survivors was recorded, and digital images of surviving mussels were obtained for determination of shell length. Survival of juveniles was assessed by observing foot movement, either spontaneous or in response to gentle probing. Growth was calculated as the average dry weight (after drying at 60 °C for 24 hours) of surviving animals in a replicate (total dry weight per number of survivors), and biomass was the total dry weight of surviving mussels for a replicate. Surviving mussels were then transferred to clean replicate beakers with fresh 100-mL parts of each sediment, which had been equilibrated for 1 week before this transfer as described for the beginning of the test. Mussels were transferred to clean sediment in beakers again after 8 weeks following the same procedure. After 12 weeks, remaining replicates were photographed for length determination and then destructively sampled for determination of survival, growth, and biomass as described previously for the 4-week samples.

Amphipod tests were started with eight replicate test chambers for each sediment. After 28 days, sediments from all replicates were sieved to determine survival; survivors from four replicates were sampled destructively and weighed at

60 °C to determine growth (mean dry weight per individual) and biomass (total dry weight per replicate). Survivors in the remaining four replicates were transferred into clean beakers with 5 mL of quartz sand substrate and clean test water and returned to the water delivery system. Starting on day 28, neonates in remaining replicates were counted and removed daily until the test was ended on day 42. On day 42, surviving amphipods from the remaining replicates were processed as described previously for day-28 samples, except surviving adults were preserved in formalin to allow determination of the number of males and females in each replicate before determination of dry weights. Reproduction data were used to calculate the total number of young produced per replicate and the total number of young per surviving female.

Sampling and Analysis of Water and Sediment

During toxicity tests, overlying water was sampled from all 24 sediments and the control during weeks 1, 4, 9, and 12 (2017 test) and weeks 1, 6, and 12 (2018 test) for analysis of zinc, lead, and cadmium. Monitoring of overlying waters also included temperature (daily); dissolved oxygen, pH, and conductivity (weekly); and ammonia, hardness, and alkalinity (expressed as milligrams per liter as calcium carbonate) at the beginning and end of each test. Samples of whole sediment and pore water were collected during mussel toxicity tests from extra replicate chemistry beakers for each sediment, which were treated the same as other replicates. At about day 60, small (2.9-mL) peeper samplers were deployed in all 96 chemistry beakers (2 tests×24 sediments×2 replicates) to sample pore-water metals. After a 10-day equilibration period, peepers were gently removed from sediments, and the contents of two replicate peepers were composited without dilution for metals analysis. After peepers were removed, sediments from chemistry beakers were sampled for analysis of SEM and AVS. Samples collected during tests were analyzed using the same methods for the pretest sampling described in section 2.

Quality Assurance for Toxicity Tests

Quality assurance in sediment toxicity tests was judged based on performance of the control sediment and the sand control (ASTM International, 2020, annex 5). The TAC for mussel survival after 12 weeks was assumed to be 80 percent, the same TAC established for the 28-day sediment test. No TAC has been established for mussel growth in sediment tests. The sand control was used to assess the quality of test water and the adequacy of diets fed to mussels, independent of nutrients supplied by the control sediment. For tests using a reference envelope, growth for reference samples should not greatly exceed that in the sand controls to avoid setting too high a standard for performance of test sediments. Test acceptability for the amphipod test was judged by performance of the site SR control sediment with the following TACs: survival greater than or equal to (≥) 80 percent on days 28 and 42; mean dry weight ≥0.35 gram on day 28 and ≥0.5 gram on day 42; and reproduction ≥6 young per female (ASTM International, 2020, annex 2).

Data Analysis

Statistical analyses of survival, weight, biomass, and reproduction data were completed in accordance with requirements outlined by the EPA (2000) and ASTM International (2015, 2020). Responses of animals in test sediments were evaluated by comparison to the range of responses in reference sediments using a "reference envelope" approach (for example, Wang and others, 2013; Besser and others, 2015; Steevens and others, 2020). Although various statistical methods have been used to compare data to a reference envelope (for example, Hunt and others, 2001), we analyzed our sediment toxicity data by ANOVA with rank-transformed data, and we used Fisher's least significant difference test to determine which test means were significantly less than the lowest reference mean.

Relations of metal concentrations in sediment and water to responses of mussels in laboratory tests or in the field were examined using Toxicity Relationship Analysis Program (version 1.30; Erickson, 2010) or SigmaPlot (version 14; SPSS Inc.). These models were used to estimate concentrations of metals or metal mixtures associated with various levels of biological effects (appendix 3). We used the EC20, the concentration associated with a 20-percent reduction of a specified endpoint, as the primary basis for comparing sensitivity among species and endpoints with the rationale that 20 percent represents a biologically meaningful level of effect that should be statistically separable from control or reference responses.

Results and Discussion

Characterization of Sediments and Pore Water

Sediments collected from primary sampling sites in summer 2017 were characterized for physical and chemical characteristics that could affect the toxicity and bioavailability of metals, and most of these analyses were repeated with sediments from chemistry beakers sampled during each test (table 7). Most characteristics of sediments tended to indicate high metal bioavailability and high toxicity risks. Zinc concentrations in the SEM fraction exceeded the zinc probable effect concentration (that is, PEQ index exceeded 1) in almost all test sediments, and the sum-PEQ index for zinc+lead+cadmium exceeded 5.0 in six of the test sites. Sediment characteristics that tend to ameliorate metal toxicity hazards were generally low, and most samples had low TOC (median of 0.77 percent) and had concentrations of SEM that exceeded the molar AVS binding capacity (median [ΣSEM–AVS] of 7.7 micromoles per gram). Sediment texture was dominated by sand-sized particles that tend to have low metal-binding capacity. Given these conditions, it is not surprising that zinc concentrations in pore water were high, and 8 of 19 test sediments

Table 7. Physical and chemical characteristics of sediment from the Tri-State Mining District during toxicity tests, 2017 and 2018.

[ID, identifier; SEM, simultaneously extracted metals; µg/g, microgram per gram; Zn, zinc; Cd, cadmium; Pb, lead; PEQ, probable effect quotient (=SEM concentration/probable effect concentration); sum-PEQ, summed probable effect quotient values; ΣSEM, sum of simultaneously extracted metals; µmol/g, micromole per gram; AVS, acid-volatile sulfide; [ΣSEM–AVS], summed molar concentrations of simultaneously extracted metals relative to acid-volatile sulfide; µg/L, microgram per liter; --, no data or not applicable; <, less than]

Site	SEM (µg/g)			PEQ			Sum-PEQ	ΣSEM (µmol/g)	AVS (µmol/g)	[ΣSEM–AVS] (µmol/g)	Peeper metals (µg/L)		
	Zn	Cd	Pb	Zn	Cd	Pb					Zn	Cd	Pb
						2017 test							
NF02	120	0.38	7.39	0.26	0.00	0.0016	0.32	1.9	0.5	--	2.2	<0.1	0.07
SP01A	48.6	0.46	19.1	0.11	0.00	0.15	0.26	0.9	0.2	0.8	8.3	<0.1	0.05
SP03	31.5	0.31	11.6	0.07	0.00	0.09	0.16	0.6	0.2	0.5	3.0	<0.1	0.05
CC00	176	1.53	15.0	0.38	0.00	0.12	0.50	2.9	2.0	0.9	7.4	<0.1	0.08
CC01G2	73.4	0.71	14.2	0.16	0.00	0.11	0.27	1.3	0.6	0.7	0.50	<0.1	0.07
CC05D	470	3.67	138	1.02	0.01	1.08	2.11	8.0	1.3	6.7	57	<0.1	2.51
CC07	2,060	13.7	474	4.49	0.03	3.70	8.23	34.1	7.8	26.3	275	0.24	4.19
CC09	1,430	5.94	232	3.12	0.02	1.81	4.94	23.2	2.7	20.5	34	<0.1	0.28
CC10	1,550	7.89	219	3.38	0.02	1.71	5.11	25.0	0.9	24.1	358	0.79	1.07
SP05	462	2.17	50.1	1.01	0.01	0.39	1.40	7.4	2.0	5.4	19	<0.1	0.08
SP08	339	2.70	40.6	0.74	0.01	0.32	1.06	5.5	0.9	4.6	51	<0.1	0.14
SP10	570	4.48	59.3	1.24	0.01	0.46	1.72	9.2	5.3	3.9	13	<0.1	0.27
SH02	1,400	4.43	134	3.05	0.01	1.05	4.11	22.3	5.4	16.9	49	<0.1	0.51
SH05	1,670	6.26	135	3.64	0.02	1.05	4.71	26.4	9.7	16.7	24	<0.1	0.17
SH06	1,100	4.03	115	2.40	0.01	0.90	3.31	17.6	6.6	11.0	12	<0.1	0.18
SH07	760	4.23	81.9	1.66	0.01	0.64	2.31	12.2	3.4	8.8	14	<0.1	0.20
SH10	461	2.77	58.1	1.00	0.01	0.45	1.47	7.5	4.7	2.8	9.2	<0.1	0.12
SH16A	372	2.04	42.8	0.81	0.01	0.33	1.15	6.0	0.4	5.6	75	0.16	0.20
SP12B	713	2.65	54.1	1.55	0.01	0.42	1.98	11.4	0.9	10.5	76	<0.1	0.13
SP13	595	3.42	55.0	1.30	0.01	0.43	1.73	9.5	1.7	7.8	88	<0.1	0.10
SP20	1,100	6.59	110	2.40	0.02	0.86	3.27	17.6	2.7	14.9	268	<0.1	0.34
SP22	747	4.04	66.1	1.63	0.01	0.52	2.15	12.0	3.1	8.9	32	<0.1	0.17
TC01	3,460	10.9	188	7.54	0.03	1.47	9.03	54.7	9.0	45.7	180	<0.1	0.13
NR06	92.3	0.40	10.6	0.20	0.00	0.08	0.28	1.6	1.3	0.3	2.3	<0.1	0.08
SP02	--	--	--	--	--	--	--	--	--	--	5.7	<0.1	0.06
Median				1.3			1.9			7.8	28.0		

Table 7. Physical and chemical characteristics of sediment from the Tri-State Mining District during toxicity tests, 2017 and 2018.—Continued

[ID, identifier; SEM, simultaneously extracted metals; µg/g, microgram per gram; Zn, zinc; Cd, cadmium; Pb, lead; PEQ, probable effect quotient (=SEM concentration/probable effect concentration); sum-PEQ, summed probable effect quotient values; ΣSEM, sum of simultaneously extracted metals; µmol/g, micromole per gram; AVS, acid-volatile sulfide; [ΣSEM–AVS], summed molar concentrations of simultaneously extracted metals relative to acid-volatile sulfide; µg/L, microgram per liter; --, no data or not applicable; <, less than]

Site	SEM (µg/g)			PEQ			Sum-PEQ	ΣSEM (µmol/g)	AVS (µmol/g)	[ΣSEM–AVS] (µmol/g)	Peeper metals (µg/L)		
	Zn	Cd	Pb	Zn	Cd	Pb					Zn	Cd	Pb
2018 test													
NF02	930	0.4	7.2	2.03	0.08	0.06	2.2	14.27	1.00	13.3	3.5	<0.1	<0.2
SP01A	50	0.4	9.9	0.11	0.08	0.08	0.27	0.82	0.20	0.6	5.9	<0.1	<0.2
SP03	30	0.3	12	0.07	0.06	0.09	0.22	0.52	0.10	0.4	3.0	<0.1	<0.2
CC00	160	1.3	15	0.35	0.26	0.12	0.73	2.53	1.90	0.6	2.6	<0.1	<0.2
CC01G2	73	0.7	14	0.16	0.14	0.11	0.41	1.19	0.70	0.5	2.3	<0.1	<0.2
CC05D	450	2.8	140	0.98	0.56	1.09	2.6	7.58	0.90	6.7	21	<0.1	0.2
CC07	2,100	13	430	4.58	2.61	3.36	11	34.32	5.70	28.6	249	0.33	1.25
CC09	1,100	3.9	180	2.40	0.78	1.41	4.6	17.73	1.80	15.9	50	<0.1	<0.2
CC10	1,500	6.9	230	3.27	1.39	1.80	6.5	24.12	0.80	23.3	189	1.03	0.29
SP05	420	1.8	45	0.92	0.36	0.35	1.6	6.66	1.70	5.0	25	<0.1	<0.2
SP08	350	2.5	41	0.76	0.50	0.32	1.6	5.57	0.80	4.8	5.2	<0.1	<0.2
SP10	550	3.7	60	1.20	0.74	0.47	2.4	8.74	5.10	3.6	18	<0.1	<0.2
SH02	1,500	3.9	130	3.27	0.78	1.02	5.1	23.61	4.30	19.3	34	<0.1	0.31
SH05	1,700	5.9	140	3.70	1.18	1.09	6.0	26.73	8.50	18.2	43	<0.1	0.32
SH06	1,000	3.9	110	2.18	0.78	0.86	3.8	15.86	6.00	9.9	19	<0.1	0.24
SH07	780	3.7	84	1.70	0.74	0.66	3.1	12.37	3.20	9.2	29	<0.1	0.34
SH10	480	2.7	64	1.05	0.54	0.50	2.1	7.68	4.50	3.2	14	<0.1	<0.2
SH16A	420	2	46	0.92	0.40	0.36	1.7	6.66	0.40	6.3	13	<0.1	<0.2
SP12B	720	2.7	55	1.57	0.54	0.43	2.5	11.30	1.10	10.2	62	<0.1	<0.2
SP13	670	3.5	69	1.46	0.70	0.54	2.7	10.61	2.10	8.5	83	<0.1	<0.2
SP20	1,100	5.9	110	2.40	1.18	0.86	4.4	17.41	2.90	14.5	158	<0.1	<0.2
SP22	400	2	37	0.87	0.40	0.29	1.6	6.32	1.80	4.5	49	<0.1	0.29
TC01	3,100	8.4	170	6.75	1.69	1.33	9.8	48.32	6.90	41.4	179	<0.1	<0.2
NR06	89	0.4	12	0.19	0.08	0.09	0.37	1.42	1.40	0.0	9.7	<0.1	<0.2
SR	--	--	--	--	--	--	--	--	--	--	3.2	<0.1	<0.2
Median				1.3			2.5			7.6	**20.9**		

had pore-water zinc concentrations greater than 50 µg/L. Concentrations of zinc or metal mixtures in sediment (zinc-PEQ, sum-PEQ, and [ΣSEM–AVS]) did not differ consistently in test sediments between 2017 and 2018, but concentrations of PWP-zinc were less in the 2018 test than in the 2017 test. A total of 11 test sediments demonstrated decreases in PWP-zinc in the 2018 test (median decrease of 36 µg/L), and 8 sediments demonstrated increases (median increase of 19 µg/L; table 7).

Mussel Tests

The overall average length of mussels stocked in the 2017 test was 1.22 mm; however, when digitized images of the mussels stocked for these tests were measured, we observed a trend for decreasing mean lengths of mussels across the 25 sediments. Sediments stocked last had mean lengths that averaged 15–20 percent less than those stocked first (fig. 9*A*). Because the control and reference sediments were the first sediments stocked, this gradient in starting length could have affected the comparisons made to evaluate toxicity of test sediments, especially for sediments that were stocked with smaller mussels late in the sequence. The 2018 mussel test used a cohort of larger test organisms (mean day-0 length of 1.41 mm). This test was stocked in two cycles in an attempt to reduce selection bias. First, all test chambers were stocked with five mussels, then all chambers were stocked with a second group of five mussels. Despite this change, we observed a similar decreasing trend for day-0 shell lengths in the order the sediments were stocked. Mean day-0 lengths in the 2018 test ranged from about 1.6 mm in the first sediment stocked to about 1.3 mm in the last sediments stocked.

Controls in 2017 and 2018 mussel tests had a mean 12-week survival of 82.5 percent, exceeding the TAC minimum of 80 percent. Growth of mussels at 12 weeks in the 2017 and 2018 tests was similar for the controls (SR; 3.9- and 3.8-mg mean dry weight, respectively), but mean growth in the sand control was lower in 2017 than in 2018 (2.7 mg versus 3.8 mg). Poor growth in the sand control could indicate that mussels were underfed, but this hypothesis is not supported by the consistency of mussel growth in the reference sites (lowest reference mean of 3.6 mg for both tests).

Sediment Toxicity

Survival and growth of juvenile mussels in the 2017 whole-sediment test differed substantially among reference and test sites. After 12 weeks, 14 of 19 test sites had a mean survival less than the lowest reference mean, and 4 test sites (CC07, CC09, CC10, SH16A) had a mean survival significantly less than all means in the reference envelope (ANOVA/Fisher's least significant difference; table 8). Growth in both length and dry weight indicated fewer differences among sediments than survival, but most test sites had growth means at day 83 that were less than the range for the reference envelope (table 8). Overall, mean growth in dry weight was less than the reference envelope in 13 of 19 test sediments and means for 2 sediments (CC07 and SP20) were significantly reduced relative to all reference means (table 8). All but 1 of the 19 test sites had a mean biomass less than the reference envelope, and 12 test sites had means significantly less than all reference means.

Results of the 2017 mussel sediment test may have been affected by variation in the starting size of juvenile mussels because of nonrandom stocking. A significant trend for decreased shell lengths with increased stocking order was observed on day 0 (fig. 9*A*) and was still significant on day 28 (fig. 9*C*), so differences in growth among treatments during this exposure period cannot be attributed solely to metal exposure. However, growth expressed as length increments (net increase in length) for the 12-week exposure period was not significantly correlated with stocking order (fig. 9*D*), indicating that effects of nonrandom stocking on mussel size were transient. Stocking treatments with smaller mussels could also have led to reduced survival because of either inherent greater sensitivity of smaller mussels or the technical difficulty of recovering smaller mussels at day 28; however, survival at day 28 was not significantly correlated with stocking order (fig. 9*B*), indicating no effect of starting size of mussels on survival. These results indicate that a significant bias in starting size because of nonrandom stocking skewed treatments that started with smaller mussels toward lesser growth on day 28 but not at the end of the 12-week test. At the end of the 12-week exposure, sediments with reduced survival, growth, and biomass were clustered in several stream reaches most affected by mining activities: Center Creek (CC05D to CC10), Middle Spring River (SP05 and SP08), lower Shoal Creek (SH10 TO SH18A), lower Spring River (SP12B and SP13), and Tar Creek (TC01; fig. 10; table 8).

Results of the 2018 mussel test indicated less variation of endpoints among test sediments (table 8). This difference was most noticeable for survival, and no 2018 test sediments had a mean survival less than the reference envelope. Mussel growth in dry weight differed among sediments, and the mean for 8 of 19 test sites was less than the reference envelope; the range of these differences in dry weight was relatively narrow, and none of the means for test sites were significantly less than all the reference means. Seven of the eight sediments with reduced growth in dry weight in the 2018 test also had reduced growth in dry weight in the 2017 test.

The lesser toxicity observed in the 2018 mussel test was unexpected, considering the addition of the metal mixture to the overlying water of this test. We expected that addition of aqueous metals would produce an increase in toxicity relative to the sediment-only tests completed in 2017; however, metal analyses of overlying waters indicated that we had underestimated the potential for loss of dissolved metals to the reservoir, to the water delivery system, and (or) to the test chambers and sediments. Although nominal zinc concentrations of 10 µg/L were automatically added to overlying water, measured zinc concentrations in overlying water were no greater than those measured in the 2017 sediment test (median

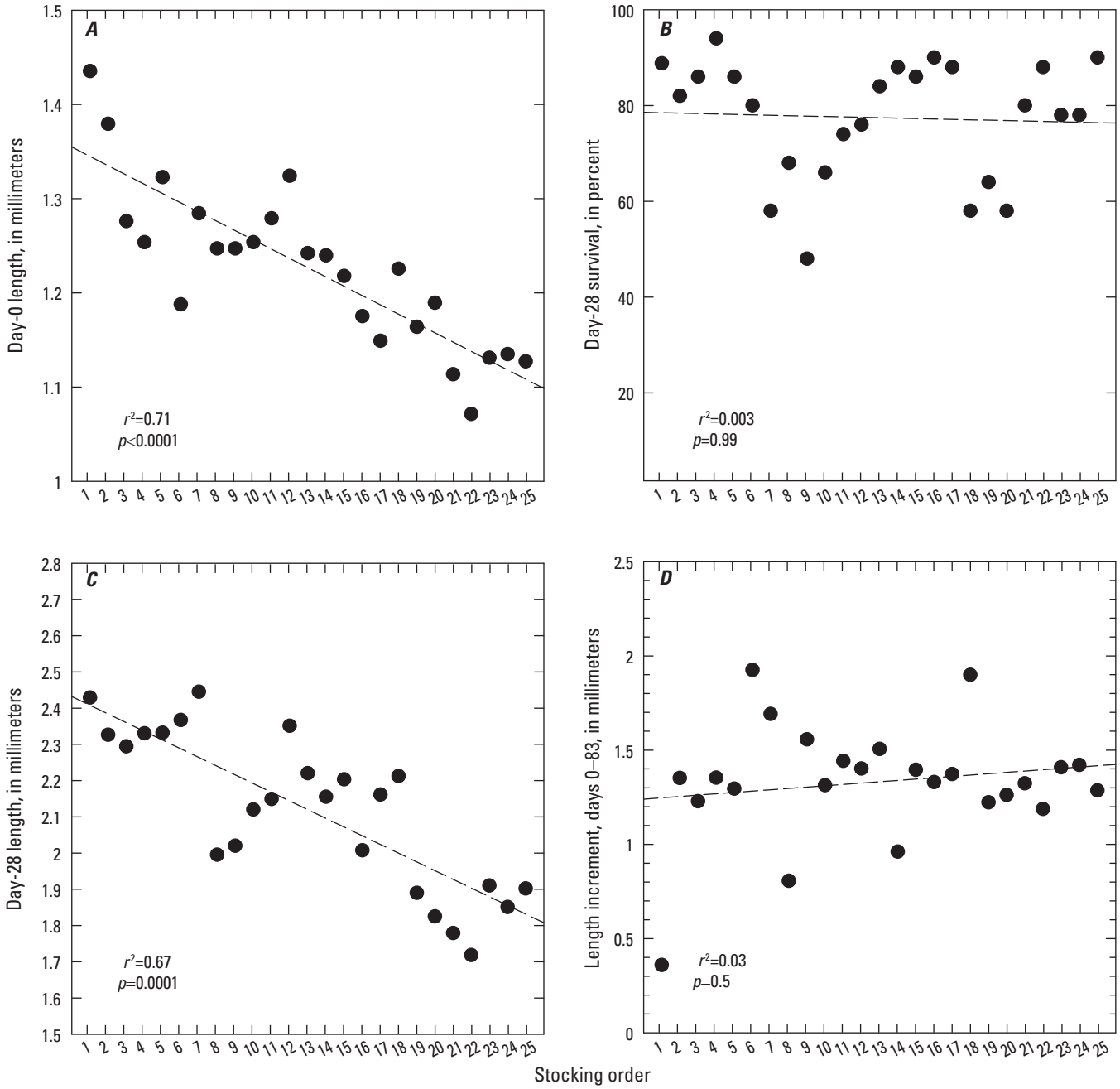

Figure 9. Effect of stocking order (1=first stocked, 25=last stocked) on mussel endpoints in 2017 sediment test. *A*, length of mussels at stocking; *B*, survival of mussels at day 28; *C*, length of mussels at day 28; *D*, increment of length increase between days 0 and 83. Means (number of samples=4) are shown with linear regressions (dashed lines). r^2, coefficient of determination; *p*, probability; <, less than]

zinc concentration of 4.6 µg/L in 2017 versus 3.2 µg/L in 2018). As a result, the 2018 study did not provide a test of our hypothesis about the contribution of metals in overlying water to toxic effects on juvenile mussels; however, the similar pattern of growth results between the two tests indicated that the responses of mussels in the two tests were not simply random.

The amphipod test met the TAC for survival (≥80 percent) in the control and in all reference sediments on days 28 and 42. Amphipod growth did not meet the 28-day TAC of 0.35-mg dry weight per survivor in either the control or the reference sediments, but growth in the controls recovered to approach the 42-day TAC (0.47 mg versus 0.50 mg).

Table 8. Toxicity of sediments to juvenile mussels in 12-week toxicity tests for sediment (fall 2017) and sediment-plus-water (spring 2018) tests.

[Means with standard error (SE) and results of reference envelope (RE) analysis. Fisher's least significant difference test (on ranks) was used for comparisons between test and reference sites. n, number of samples; mg; milligram; --, no comparison with reference; <, mean less than all reference means; nd, mean not different from reference; x, mean significantly less than all reference means; p, probability of type-1 error]

Site	Whole-sediment tests—Fall 2017										Site	Sediment-plus-water test—Spring 2018								
	n	Survival (of 10)			Growth (mean dry weight, mg)			Biomass (total dry weight, mg)				*n*	Survival (of 10)			Mean dry weight (mg)			Biomass (dry weight, mg)		
		Mean	SE	RE	Mean	SE	RE	Mean	SE	RE			Mean	SE	RE	Mean	SE	RE	Mean	SE	RE
CTRL	8	8.25	--	--	3.85	0.23	--	31.6	2.7	--	CTRL	8	8.25	0.45	--	3.803	0.157	--	31.29	1.87	--
NF02	5	7.60	1.36	--	4.75	1.34	--	29.43	1.7	--	NF02	5	6.60	0.51	--	3.628	0.220	--	23.54	0.85	--
SP01A	5	8.60	0.51	--	3.83	0.24	--	33.1	3.1	--	SP01	5	6.40	0.60	--	4.098	0.420	--	25.98	3.28	--
SP03	5	9.40	0.24	--	3.64	0.20	--	34.2	2.1	--	SP03	5	8.20	0.37	--	3.956	0.368	--	32.42	3.24	--
CC00	5	8.00	0.84	--	3.82	0.70	--	29.7	4.1	--	CC00	5	7.40	0.24	--	3.879	0.418	--	28.92	3.82	--
CC01G2	5	7.20	0.58	--	4.59	0.41	--	32.2	1.2	--	CC01	5	7.10	0.60	--	3.759	0.247	--	27.98	3.09	--
CC05D	5	4.60	0.93	<	5.01	0.86	nd	22.4	4.4	<x	CC05D	5	8.20	0.58	nd	3.934	0.254	nd	31.88	1.75	nd
CC07	5	4.80	0.66	<x	2.43	0.35	<x	11.4	1.8	<x	CC07	5	9.20	0.58	nd	3.355	0.174	<	31.03	3.00	nd
CC09	5	2.20	0.37	<x	3.78	0.57	nd	8.6	2.2	<x	CC09	5	9.00	0.55	nd	4.529	0.238	nd	40.60	2.74	nd
CC10	5	5.00	0.45	<x	3.48	0.32	<	17.4	2.1	<x	CC10	5	8.40	0.51	nd	3.211	0.093	<	26.82	1.07	nd
SP05	5	6.60	0.87	<	4.12	0.26	nd	26.3	2.2	<	SP05	5	7.80	0.20	nd	3.435	0.122	<	26.81	1.29	nd
SP08	5	5.80	0.49	<	4.22	0.22	nd	24.8	3.0	<	SP08	5	7.60	0.51	nd	4.172	0.324	nd	32.02	3.92	nd
SP10	5	8.20	0.73	nd	3.78	0.27	nd	30.4	1.6		SP10	5	7.40	0.81	nd	4.341	0.623	nd	31.39	4.46	nd
SHO2	5	7.80	0.58	nd	2.71	0.25	<	21.2	2.5	<	SH02	5	9.60	0.40	nd	3.369	0.140	<	32.37	2.05	nd
SHO5	5	7.60	0.51	nd	3.51	0.32	<	26.0	1.1	<	SH05	5	7.20	0.66	nd	4.265	0.230	nd	30.93	3.54	nd
SH06	5	8.60	0.40	nd	3.09	0.38	<	26.3	2.8	<	SH06	5	9.00	0.63	nd	4.026	0.357	nd	35.86	3.12	nd
SH07	5	6.80	0.37	<	3.34	0.26	<	22.6	1.7	<x	SH07	5	8.60	0.75	nd	4.285	0.223	nd	36.42	2.66	nd
SH10	5	6.20	1.11	<	4.77	0.72	nd	26.9	1.9	<	SH10	5	8.80	0.37	nd	3.985	0.276	nd	35.01	2.69	nd
SH16A	5	5.00	0.89	<	2.99	0.22	<	14.6	2.5	<x	SH16	5	8.20	0.73	nd	3.711	0.291	<	31.07	4.82	nd
SP12B	5	4.60	0.60	<x	3.41	0.51	<	15.2	2.0	<x	SP12	5	8.80	0.37	nd	3.997	0.168	nd	35.30	2.58	nd
SP13	5	6.80	0.80	<	2.85	0.10	<	19.1	1.8	<x	SP13	5	8.80	0.37	nd	3.431	0.097	<	30.19	1.47	nd
SP20	5	6.80	0.73	<	2.41	0.15	<x	16.2	1.5	<x	SP20	5	9.60	0.24	nd	3.189	0.262	<	30.48	2.22	nd
SP22	5	6.00	0.63	<	3.08	0.25	<	17.9	1.2	<x	SP22	5	9.40	0.24	nd	3.532	0.397	<	32.91	3.27	nd
TC01	5	4.80	1.07	<	3.22	0.20	<	15.4	3.6	<x	TC01	5	8.00	0.55	nd	4.349	0.333	nd	35.04	3.82	nd
NR06	5	7.60	0.81	nd	3.00	0.56	<	21.5	2.5	<x	TC06	5	6.40	0.81	nd	2.327	0.233	<	15.35	3.00	<
		***p*<0.00001**			***p*<0.00001**			***p*<0.00001**					***p*<0.00001**			***p*=0.0002**			***p*=0.0026**		

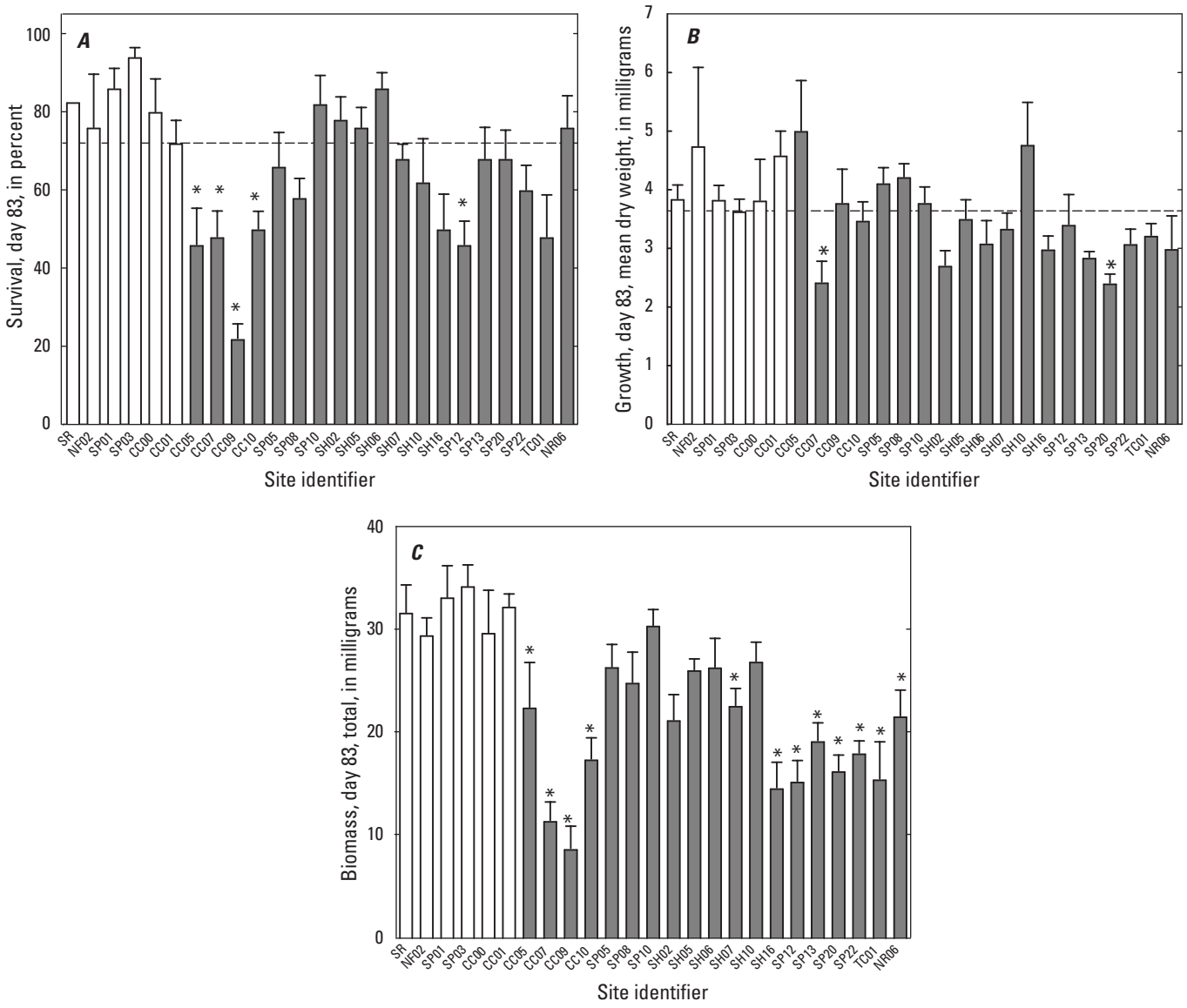

Figure 10. Mussel endpoints in reference sediments (hollow bars) and test sediments (filled bars) during sediment toxicity tests in 2017. *A*, survival; *B*, growth expressed as mean dry weight, in milligrams; *C*, biomass, in milligrams. Bars are means and error bars are standard errors (number of samples=4). Dashed horizontal line indicates lowest reference mean. Asterisk indicates mean is significantly less than the lowest reference mean (rank analysis of variance with Fisher's least significant difference test, probability of type-1 error less than 0.05).

Reproduction in the control sediment met the TAC of 6.0 young per female, but two of the five reference sediments had reproduction less than the TAC.

Toxicity of sediments to *H. azteca* (table 9) followed trends similar to those in the 2017 mussel test. Sediments from the three sites with highest metal concentrations (Center Creek sites CC07 and CC09 and Tar Creek site TC01) indicated statistically significant decreases from reference sites for amphipod survival on days 28 and (or) 42, and sediments from sites in middle Spring River (SP05, SP08) and Shoal Creek (SH07 and SH10) also had one or more endpoints below the reference envelope. All these sites also had reductions in one or more endpoints in the 2017 mussel test. Amphipod endpoints with the greatest number of reductions relative to the reference envelope were day-28 biomass (7 of 19 test sites) and day-42 survival (6 sites). Day-42 growth and biomass indicated minimal effects of sediments. Reproduction was low in several of the same sediments that had low day-42 survival, but only one site (CC07, which had no reproduction) fell to less than the wide reference range for reproduction. Reference sediment SP03 had unexplained low values for all day-42 sublethal endpoints, but even if this sample was excluded from the reference envelope, the (slightly) narrower reference range would identify toxic effects only in a few additional test sediments.

Table 9. Toxicity of Tri-State Mining District sediments in 6-week exposures with amphipods, *Hyalella azteca*, fall 2017.

[Means with standard error (SE) and results of reference envelope (RE) analysis. Fisher's least significant difference test (on ranks) was used for comparisons between test and reference sites. ID, identifier; *n*, number of samples; %, percent; mg; milligram; --, no comparison with reference; ns, not significant; <, mean less than all reference means; x, mean significantly less than all reference means ANOVA, analysis of variance; *p*, probability of type-1 error]

Site	4-week endpoints										6-week endpoints											
	n	Day-28 survival (%)			Growth (mean dry weight, mg)			Biomass (total dry weight, mg)			*n*	Day-42 survival (%)			Growth (mean dry weight, mg)			Biomass (total dry weight, mg)			Young per female		
		Mean	SE	RE	Mean	SE	RE	Mean	SE	RE		Mean	SE	RE	Mean	SE	RE	Mean	SE	RE	Mean	SE	RE
CTRL	16	92.5	7.07	--	0.320	0.03	--	2.97	0.44	--	4	92.5	2.5	--	0.466	0.40	--	4.41	0.55	--	9.20	2.03	--
NF02	8	90.0	7.56	--	0.223	0.06	--	2.05	0.70	--	4	87.5	4.8	--	0.530	0.20	--	0.47	0.71	--	8.20	4.93	--
SP01A	8	95.0	7.56	--	0.208	0.03	--	1.97	0.23	--	4	92.5	4.8	--	0.548	0.10	--	5.20	0.55	--	2.43	1.00	--
SP03	8	93.8	7.44	--	0.263	0.05	--	2.34	0.39	--	4	95.0	2.9	--	0.553	0.05	--	5.34	0.14	--	9.63	2.33	--
CC00	8	92.5	10.35	--	0.195	0.08	--	1.82	0.63	--	4	87.5	7.5	--	0.200	0.07	--	1.860	0.45	--	0.280	0.10	--
CC01G2	8	85.00	10.69	--	0.263	0.07	--	2.22	0.65	--	4	82.5	4.8	--	0.548	0.12	--	4.64	0.59	--	7.45	1.94	--
CC05D	8	86.3	13.02	ns	0.290	0.07	ns	2.55	0.93	ns	4	82.8	7.6	ns	0.535	0.02	ns	4.41	0.38	ns	6.88	2.09	ns
CC07	8	60.0	19.27	<x	0.183	0.02	<	1.04	0.37	<	4	32.5	4.8	<x	0.458	0.03	ns	2.82	0.42	ns	0.50	0.50	ns
CC09	8	86.3	13.02	ns	0.215	0.02	<	1.94	0.30	ns	4	77.5	4.8	x	0.403	0.07	ns	3.21	0.44	ns	6.83	2.50	ns
CC10	8	23.8	14.08	<x	0.187	0.04	ns	0.33	0.24	<x	4	22.5	2.9	<x	0.440	0.05	ns	1.42	0.53	<	0.00	0.00	<
SP05	8	83.8	23.26	<	0.220	0.04	ns	1.54	0.72	<	4	95.0	6.5	ns	0.525	0.04	ns	5.11	0.20	ns	6.88	1.55	ns
SP08	8	82.5	8.86	<	0.295	0.14	ns	2.56	1.16	ns	4	75.0	4.8	<	0.525	0.11	ns	4.21	1.12	ns	4.83	2.07	ns
SP10	8	98.8	3.54	ns	0.263	0.02	ns	2.55	0.14	ns	4	92.5	4.8	ns	0.640	0.20	ns	5.93	1.74	ns	7.78	1.23	ns
SH02	8	97.5	7.07	ns	0.285	0.03	ns	2.70	0.35	ns	4	92.5	4.8	ns	0.540	0.01	ns	5.40	0.21	ns	5.80	1.78	ns
SH05	8	88.8	9.91	ns	0.300	0.11	ns	2.61	0.92	ns	4	90.0	7.1	ns	0.535	0.07	ns	4.84	0.63	ns	8.55	3.28	ns
SH06	8	92.5	7.07	ns	0.340	0.05	ns	2.93	0.28	ns	4	90.0	5.8	ns	0.598	0.03	ns	5.80	0.07	ns	5.80	1.21	ns
SH07	8	90.0	19.27	ns	0.233	0.08	ns	1.70	0.30	<	4	97.5	2.5	ns	0.460	0.04	ns	4.54	0.79	ns	3.40	1.47	ns
SH10	8	91.3	11.26	ns	0.293	0.03	ns	2.93	0.33	ns	4	75.0	6.5	<	0.555	0.03	ns	4.54	0.36	ns	11.75	3.15	ns
SH16A	8	87.5	14.88	ns	0.345	0.01	ns	2.76	0.63	ns	4	95.0	2.9	ns	0.500	0.02	ns	4.74	0.42	ns	9.38	2.77	ns
SP12B	8	96.3	5.18	ns	0.310	0.07	ns	3.11	0.64	ns	4	90.0	4.1	ns	0.495	0.06	ns	4.57	1.10	ns	9.70	6.32	ns
SP13	8	88.8	12.46	ns	0.268	0.03	ns	2.42	0.51	ns	4	85.0	6.5	ns	0.463	0.03	ns	3.96	0.48	ns	7.68	1.66	ns
SP20	8	91.3	9.91	ns	0.228	0.06	ns	2.13	0.64	ns	4	87.5	7.5	ns	0.543	0.01	ns	4.90	0.40	ns	3.20	0.65	ns
SP22	8	92.5	11.65	ns	0.178	0.05	<	1.72	0.49	<	4	85.0	6.5	ns	0.712	0.02	ns	6.24	0.67	ns	7.00	1.45	ns
TC01	8	68.8	15.53	<	0.163	0.10	<	1.04	0.39	<	4	65.0	5.0	<x	0.625	0.06	ns	4.74	1.39	ns	2.00	0.71	ns
NR06	8	81.3	22.32	ns	0.173	0.08	<	1.2	0.57	<	4	82.5	7.5	ns	0.615	0.04	ns	5.32	0.14	ns	5.88	2.39	ns
ANOVA (*p*)		***p*<0.0001**			***p*<0.0001**			***p*<0.0001**				***p*<0.0001**			***p*=0.06**			***p*=0.0007**			***p*=0.0005**		

Results of the amphipod test are consistent with the trends seen in the other tests completed for the present study, with relatively severe short-term responses followed by moderating effects during longer exposures.

Sediment Toxicity Thresholds

We estimated EC20s for multiple endpoints from 12-week mussel tests and 6-week amphipod tests from CRMs based on several metrics of metal exposure. Survival, growth (mean dry weight), and biomass of juvenile mussels in the 2017 sediment test indicated decreasing trends with increasing metal concentrations in sediment or pore water. Concentration-response plots and EC20 estimates for 2017 test endpoints based on SEM-zinc concentrations are presented in figure 11. Survival and growth EC20s had relatively weak regression fits (r^2=0.22), and biomass had a better fit (*r compile*^{2}=0.48) and greater sensitivity. Regression models for the 2017 test that focused on endpoint responses over days 28–83 (weeks 4–12), to minimize effects of nonrandom stocking, had small differences in model fit (better for survival, worse for growth) and endpoint sensitivity (better for survival, worse for growth; appendix 3, fig. 3.3). Across all endpoints, the geometric mean of EC20s (GM–EC20) for SEM-zinc were 2.47 PEQ

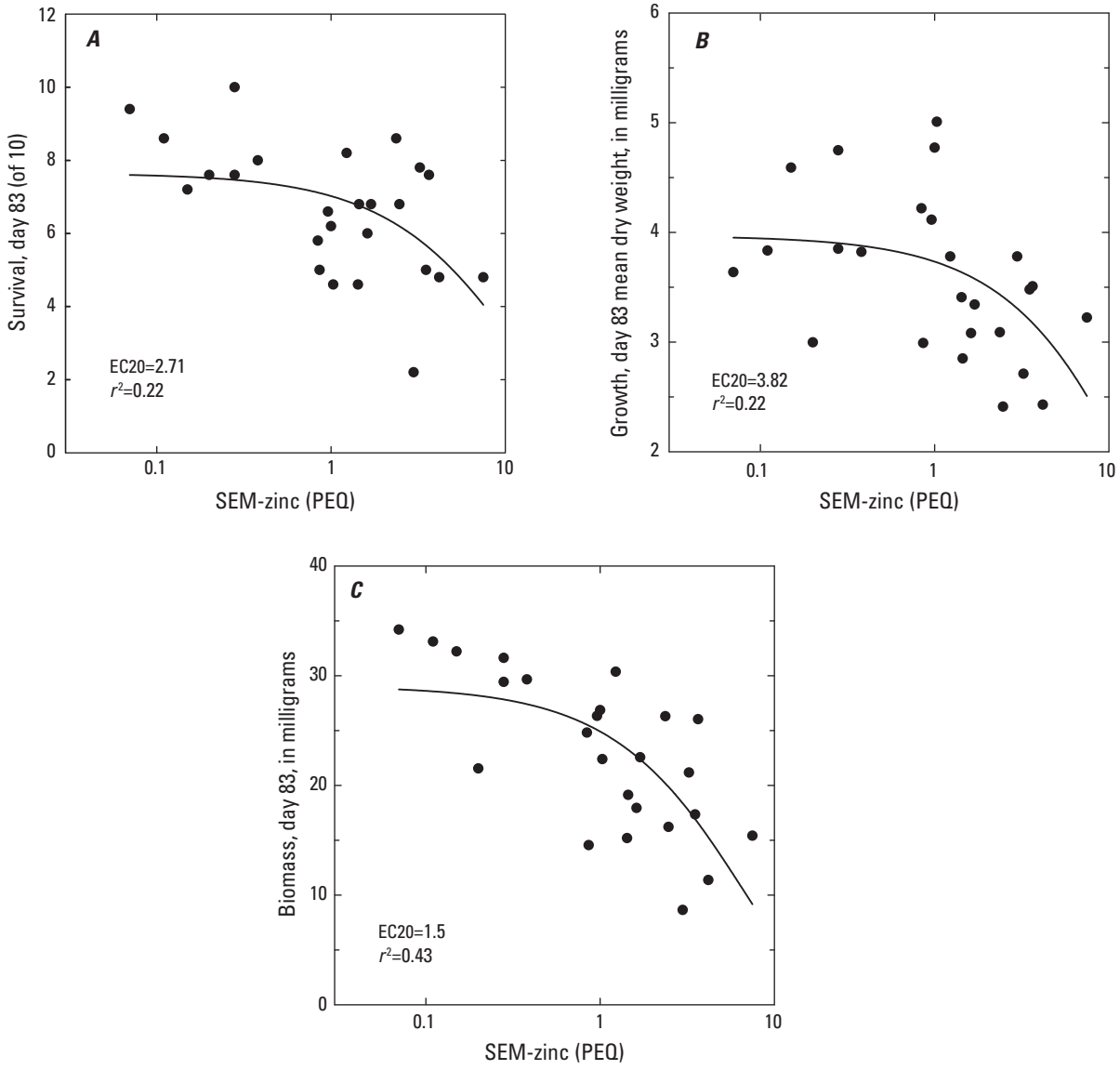

Figure 11. Concentration-response plots and 20-percent effect concentrations (EC20s) for effects of sediment zinc on mussel endpoints in 12-week sediment toxicity tests in 2017. *A*, survival; *B*, growth expressed as mean dry weight, in milligrams; *C*, biomass, in milligrams. EC20s were estimated from three-parameter sigmoid regressions (SigmaPlot; appendix 3). Circles are site means. Solid lines are regressions. [SEM, simultaneously extracted metals; PEQ, probable effect quotient; r^2, coefficient of determination]

Table 10. Comparison of toxicity thresholds for *Hyalella azteca* and *Lampsilis siliquoidea* estimated from tests with Tri-State Mining District sediment, 2007 and 2017.

[EC20, 20-percent effect concentration; r^2, coefficient of determination; %, percent; GM–EC20, geometric mean of EC20s for 2017 sediment tests; SEM, simultaneously extracted metals; Zn, zinc; PEQ, probable effect quotient (sediment metal concentration/probable effect concentration); --, no data or not applicable; [SEM–AVS], simultaneously extracted metals normalized to acid-volatile sulfide; µmol/g, micromole per gram; PWP–Zn, zinc in pore-water peepers; µg/L, microgram per liter; OW–Zn, zinc on overlying water; >, greater than; values in parentheses indicate r^2; values in brackets indicate 95% confidence limits]

Exposure	Units	2017 EC20 with (r^2) or [95% confidence limits]				2017 threshold (GM–EC20)	[1]2007 threshold	2007/2017 ratio
		Survival (12 week)	Growth (12 week)	Biomass (12 week)	Reproduction			
12-week mussel test (2017)								
SEM–Zn (0–12 week)	PEQ	2.7 (0.22)	3.7 (0.22)	1.5 (0.43)	--	2.47	64	26
SEM–Zn (4–12 week)	PEQ	3.6 (0.39)	5.1 (0.10)	1.7 (0.43)	--	3.15	64	20
SEM-mix	Sum-PEQ	3.9 −0.32	5.6 (0.26)	2.2 (0.48)	--	3.64	52	7.8
[SEM–AVS]	µmol/g	6.1 (0.44)	11.2 (0.24)	4.3 (0.68	--	6.75	73	8.3
PWP–Zn	µg/L	181 (0.18)	215 (0.25)	86 (0.46)	--	150	53	0.53
OW–Zn	µg/L	6.1 (0.44)	11.2 (0.25)	3.71	--	6.33	--	--
Water Zn (Zn only)	µg/L	>215 --	16.0 [12–21]	20	--	41.0	--	--
Water Zn (metal mix)	µg/L	48 [45–51]	6.4 [5–8]	6.5	--	12.6	--	--
6-week amphipod test (2017)								
SEM–Zn	PEQ	[2]3.6	[3]6.0 **[3](0.13)**	[4]2.9 **[4](0.02)**	[5]4.3 **[5](0.68)**	4.05	6.4	[6]1.6

[1]T20=20-percent toxicity threshold from MacDonald and others (2009).

[2]Denotes 4-week period for survival.

[3]Denotes 4-week period for growth.

[4]Denotes 6-week period for biomass.

[5]Denotes 6-week period for reproduction.

[6]Denotes 2007/2017 ratio.

(1,134 µg/g) for the full 12-week test models and 3.15 PEQ (1,446 µg/g) for the interval between 4 and 12 weeks (table 10), indicating that the sensitivity of the mussels was less during the later stages of the exposure but that the association of responses with metals remained equally strong. CRMs based on SEM-zinc for the 42-day tests with *H. azteca* in 2017 are presented in figure 12 (for zinc PEQs) and appendix 3, figure 3.8 (for sum-PEQ, [ΣSEM–AVS], and PWP-zinc).

Mussel growth, expressed as shell length, indicated a narrow range of differences among sediments in the 2017 test, and these data did not produce well-defined CRMs (results not shown). Additional CRMs for mussel endpoints in the 2017 test were based on other measures of sediment metal exposure: (1) the mixture of zinc, lead, and cadmium in the SEM fraction, expressed as sum-PEQ, and (2) SEM normalized to AVS ([SEM–AVS]; table 10; appendix 3, fig. 3.7). These models indicated consistent patterns across the different endpoints, with the lowest EC20s for biomass and highest EC20s for growth (fig. 13*A*). Although CRMs for sediment metals indicate low sensitivity of the growth endpoint, the consistently lower EC20s for biomass compared to survival indicate that reduced growth contributed to the sensitive biomass endpoint.

Previous mussel sediment toxicity tests with sediments from the Tri-State Mining District in 2007 (Ingersoll and others, 2008) used sieved fine (<0.25-mm) sediments, which tend to have greater metal concentrations (and may therefore

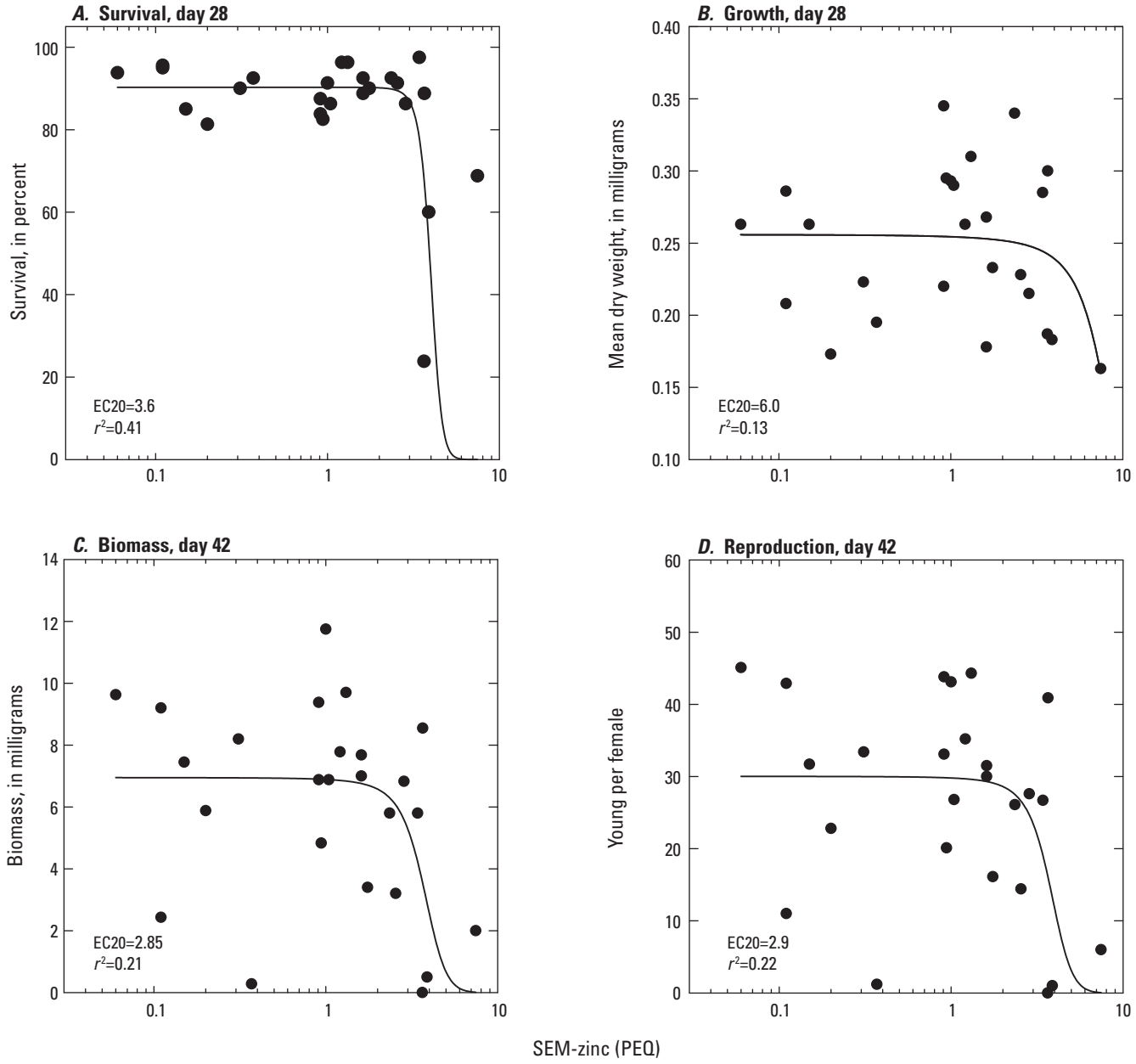

Figure 12. Concentration-response plots and 20-percent effect concentration (EC20) estimates of EC20s for effects of sediment zinc on amphipod, *Hyalella azteca*, endpoints in 2017 test. *A*, day-28 survival; *B*, day-28 growth expressed as dry weight, in milligrams; *C*, day-42 biomass, in milligrams; *D*, day-42 reproduction. Circles indicate site means. Curved line is three-parameter sigmoid regression (SigmaPlot; appendix 3). [SEM, simultaneously extracted metals; PEQ, probable effect quotient; r^2, coefficient of determination]

tend to be more toxic) than the bulk (<2-mm) sediment fraction used for 2007 amphipod tests and for tests with species in 2017 and 2018. Thresholds estimated from the 2007 tests indicate that the sensitivity of juvenile mussels in fine sediments was an order of magnitude less than the sensitivity of amphipods in bulk sediments (6.4 PEQ for amphipods versus 64 PEQ for mussels; table 10). Sediments in the <0.25-mm fraction had greater average metal concentrations than those in the <2-mm fraction, but these differences were not great, with bulk sediments having concentrations of cadmium, lead, and zinc that averaged 75–84 percent of the concentrations in the fine fraction (Ingersoll and others, 2008). Mussel effect thresholds (20-percent effect concentrations for survival [T20s]) [ΣSEM–AVS] in 2007 tests were calculated separately for the <0.25- and <2-mm sediment fractions, and toxicity thresholds estimated from these data also were similar for fine and bulk sediments (53 and 64 micromoles per gram, respectively). Given the relatively small differences in metal concentrations

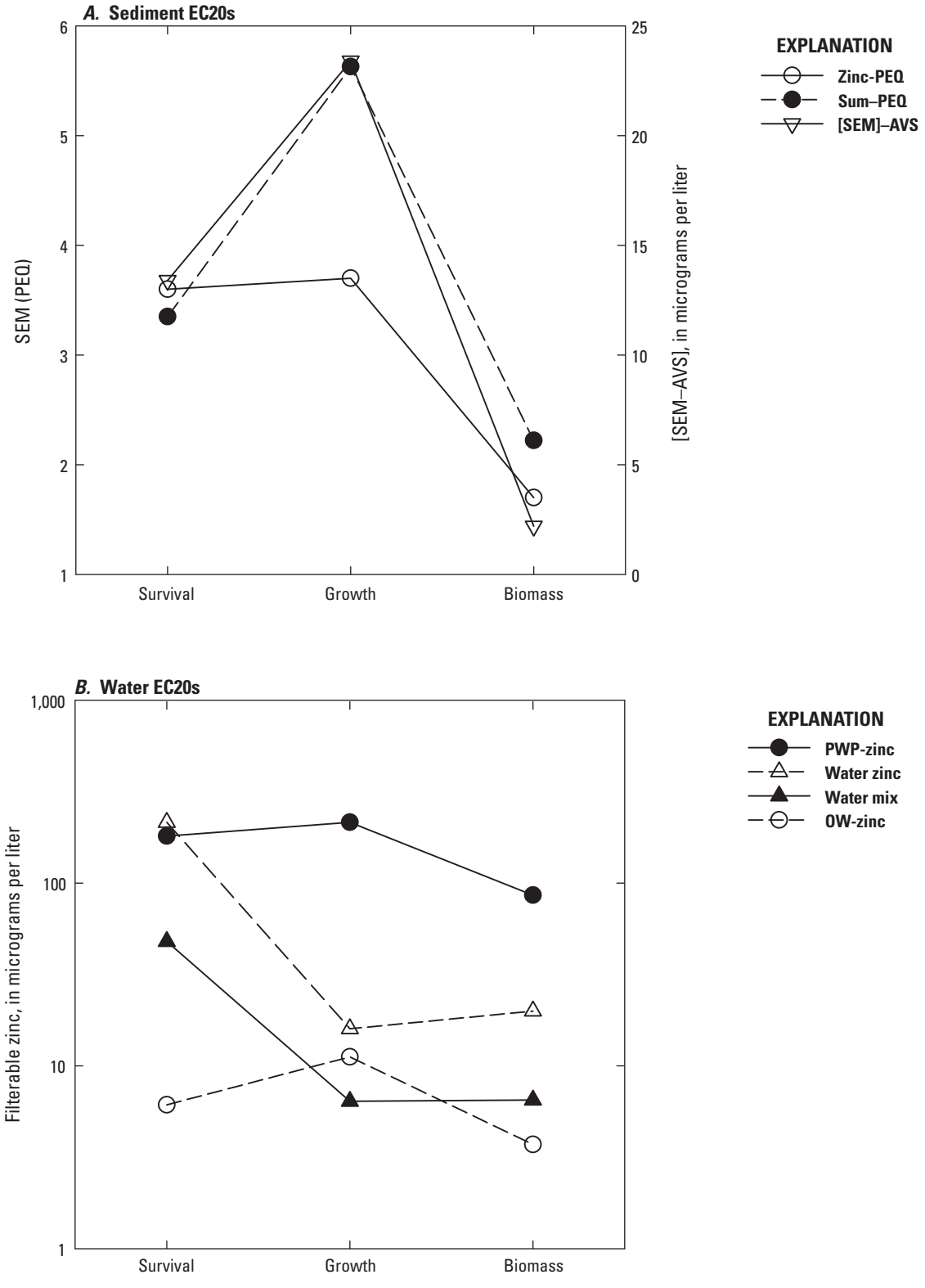

Figure 13. Comparison of mussel 20-percent effect concentrations (EC20) among toxicity endpoints and among exposure metrics. *A*, in sediment; *B*, in water. [SEM, simultaneously extracted metals; PEQ, probable effect quotient; [SEM–AVS], simultaneously extracted metals normalized to acid-volatile sulfide; sum-PEQ, summed PEQ values for zinc, lead, and cadmium; PWP-zinc, zinc in pore-water peepers; water zinc, zinc in water-only test; water mix, zinc in water-only test with three-metal mixture; OW-zinc, zinc in overlying water]

in these different sediment size fractions, it is likely that the greater effect concentrations for mussels in 2007 tests reflected lesser sensitivity of mussels to metals in shorter sediment exposures rather than differences in metal concentrations between sediment size fractions.

Toxicity thresholds derived from measured zinc concentrations in pore water or overlying water indicated similarities and differences with sediment thresholds (table 10; fig. 13*B*). In general, thresholds for zinc in water, like sediment thresholds, were greatest for survival and lowest for biomass; however, this pattern was sometimes obscured by differences among sample types, notably the roughly twentyfold differences in thresholds for pore waters (peepers) and overlying waters in 2017 sediment tests. These large differences indicate that one or both sample types did not accurately represent metal exposure by juvenile mussels. Differences in aqueous metal exposure may be mediated by burrowing behavior, which can either increase or decrease exposure to aqueous metals. Different sample types may also contain different mixtures of toxic metals, and results of the two water-only tests indicate lower thresholds for the three-metal mixture than for zinc alone, indicating a meaningful contribution of lead and cadmium to toxicity of the mixture.

The 2018 mussel sediment-plus-water test had consistent baseline responses for survival, growth, and biomass endpoints after 12 weeks, but 2018 endpoints did not indicate consistent decreasing trends at higher metal concentrations. Growth data from the 2018 test (table 8) indicated decreases in nine test sites relative to the reference envelope, and several sites approached 20-percent reductions in growth from the reference mean. All these sites also demonstrated growth less than the reference envelope in the 2017 test, but several sites with high metal exposure demonstrated greater growth in the 2018 test, preventing us from formulating a useful CRM.

Toxicity thresholds from long-term tests with mussels and amphipods were generally lower than those estimated by MacDonald and others (2009) from 28-day toxicity tests. Thresholds for SEM-zinc are compared in table 10 among species, endpoints, and years. The GM–EC20 for SEM-zinc toxicity to mussel endpoints in 12-week tests in 2017 was more than twentyfold lower than the T20 values for 4-week mussel tests in 2007 (MacDonald and others, 2009), and the mussel GM–EC20 for SEM-zinc also was less than the GM–EC20 for amphipods (2.6 PEQ and 3.9 PEQ, respectively) in the 2017 tests. MacDonald and others (2009) recommended using thresholds based on toxicity of sediment zinc to *H. azteca* for ecological risk assessment of metals in Tri-State Mining District sediments, but results of our 12-week mussel tests produced GM–EC20s for mussels that were lower than either T20s for amphipods from 28-day tests in 2007 or GM–EC20s for amphipod 6-week tests in 2017 (table 10). These results support our hypothesis that thresholds for toxicity to *L. siliquoidea* estimated from 12-week exposures are lower than those estimated from short-term tests and that juvenile mussels are equally sensitive or more sensitive than amphipods to toxic effects of metals in sediments.

Differences Between 2017 and 2018 Mussel Tests

The two sediment tests demonstrated unexpected differences in sensitivity despite testing the same sediment samples and the same test organism. These differences could be due to differences in the quality or sensitivity of test organisms or to differences in metal exposure between the two tests. The use of smaller mussels to stock the 2017 test also could have contributed to the greater sensitivity observed in that test. Differences in starting size of mussels among treatments because of nonrandom stocking could have affected differences in growth early in the 2017 study, but trends for reduced survival, growth, and biomass among treatments after the full 12-week exposure were not closely correlated with the starting size of mussels. Differences in the starting size of juvenile mussels among 2018 treatments were similar to those observed in the 2017 test, but these differences did not have any effect on toxic effects in long-term tests. Mussels exposed to sediments in the 2017 and 2018 tests indicated reductions in growth, relative to reference sediments, in many sediments in the same range of sediment metal exposure. Many of the same sediments that were toxic in the 2017 mussel test also were the most toxic sediments in the amphipod test, indicating that differences in toxicity among treatments in the 2017 tests were predominantly related to metal exposure, not to effects of stocking bias.

The absence of clear concentration-response trends in the 2018 study may reflect differences in metal exposure, notably decreases in PWP-zinc during the 6-month storage period between tests for several sediments that had indicated high toxicity in the 2017 test. The apparent differences in sensitivity of mussels between the 2017 and 2018 tests did not correspond to differences in sediment metal concentrations because the sum-PEQ index averaged about 25 percent greater for sediment from the 2018 test compared to 2017 and the [ΣSEM–AVS] value was nearly identical across the two tests. However, PWP-zinc averaged about 30 percent lower in the 2018 test. This difference was strongly affected by several sites (CC07, CC10, and SP20) that had high PWP-zinc concentrations and had significant reductions in growth and (or) survival in the 2017 test. Thus, it seems that the lack of sensitivity to sediments in the 2018 test may reflect reduced metal sensitivity because of the greater starting size of the mussels and (or) changes in bioavailability of sediment metals during storage.

Another factor that could have affected differences in sensitivity between the 2017 and 2018 tests was the addition of metals into overlying water during the 2018 test. Our expectation was that these added metals would be physically and biologically available, for example, as free dissolved metals that would be bioavailable to juvenile mussels via the gill or other body surfaces. However, it is possible that these added metals were either not physically available (for example, if juvenile mussels remained buried in sediment and were not exposed to additional metals in overlying water) or not bioavailable

(for example, because of sorption to surfaces of sediments or test chamber surface or partitioning to resistant phases such as metal sulfides). Either of these scenarios could account for the reduced toxicity observed in the 2018 test, although the amount of metals added to the overlying water (nominal, 10 µg/L as zinc) seems too small to stimulate changes in mussel burrowing behavior or to substantially shift chemical equilibria. The amount of metals added to overlying water in the 2018 test was much less than the added metal concentrations (20–80 µg/L as zinc) associated with reversals of toxicity in the 2019 supplemental test (see section 5).

Toxicity of Metals in Water and Sediment

Results of whole-sediment toxicity tests with and water-only toxicity tests that simulated exposure to waterborne metals in Tri-State Mining District streams (see section 3) were consistent with the hypothesis that mussels in Tri-State Mining District streams are adversely affected by long-term exposures to metals. Our attempts to better understand the relative contributions of exposure to metals in sediment versus overlying water to adverse effects on juvenile mussels were unsuccessful, and we have only indirect evidence of the relative contributions of metal exposure via sediment and water exposure routes. However, the nature of the effects we observed in separate tests with metals in water and sediment give us some clues about the similarity of the exposure routes and mechanisms of toxicity. Exposure to waterborne zinc alone produced short-term effects on growth that decreased in severity between 4-week and 12-week exposures. Water-only exposures that combined zinc with low levels of lead and cadmium, based on concentrations measured in streams, also produced short-term effects on mussel growth, but these effects became more severe during the 12-week tests; mussels in the metal-mixture tests also experienced reduced survival and more severe reductions in biomass than those observed in the zinc-only test. Toxic effects in our 12-week sediment tests consisted of reduced survival, especially during the first 4 weeks of exposure, and reduced growth that persisted over the duration of the study. The limited severity of the toxic effects observed in sediment tests may reflect our intentional selection of sampling sites that supported mussel assemblages and tended to have relatively low sediment metal concentrations.

We were unable to document increased toxicity because of added metals in overlying water in the 2018 sediment test, but zinc concentrations in overlying water of sediment tests exceeded EC20s for mussel growth and biomass (6.4–6.5 µg/L as zinc) determined from the water-only test with the three-metal mixture. The lowest threshold based on zinc in pore water peepers (80 µg/L as zinc for effects on biomass) was an order of magnitude greater than the water-only thresholds, but the frequency of exceedance of this threshold was the same as for the water-only threshold (5 of 19 test sites). These similar patterns of exceedance of water-based thresholds, despite differences in the zinc concentration in pore water versus overlying water, indicate that contaminated sediments are the dominant exposure route and that water-based threshold tests simply reflect aqueous concentrations in equilibrium with sediment thresholds.

Sensitivity of Different Sizes of Juvenile Mussels

Differences in sensitivity of different cohorts of juvenile *L. siliquoidea* to metal toxicity may be attributable to differences in starting age/size. Decreasing sensitivity with increasing age/size is a common phenomenon in ecotoxicology, and Wang and others (2010) reported tenfold increases in acute zinc median lethal concentrations for *L. siliquoidea* between tests with newly transformed (5-day-old; about 0.4 mm) juveniles and tests with 6-month-old (2.07 mm) juveniles. The water-only tests described in section 3 were started with smaller juvenile mussels than are currently recommended for sediment testing because these tests included only a clean sand substrate, and small size did not cause difficulties for recovering either live mussels or shells of dead mussels. The EC20s estimated from these tests were as low as any previous 12-week water-only tests with juvenile mussels (Wang and others, 2020). Also, differences in sensitivity between the 2017 and 2018 sediment tests may reflect the use of two size ranges of juvenile mussels for these tests. It also is possible that unintentional stocking of different-sized mussels across different treatments of the sediment tests affected their response to sediment metals, but we see little evidence of this phenomenon.

The unanswered questions that remained after these tests led us to design a supplemental study to address more directly the question of water-plus-sediment exposures (see section 5). These studies provided another opportunity to address the question of differences in sensitivity with size and age of juvenile mussels. Finally, the selection of sediments for the supplemental studies allowed us to evaluate if other characteristics of sediments other than toxic metals might affect the survival and growth of juvenile mussels.

5. Toxicity of Metals in Sediment and Water to Size Classes of Juvenile Mussels

Background

This objective of this supplemental study was to investigate the relative contribution of waterborne and sediment metals to toxic effects on juvenile mussels, which was one of the original research questions of this project that was left unanswered because of an unsuccessful attempt to enrich overlying waters with metals during the 2018 sediment toxicity tests. The design for the supplemental experiments included 5 levels of an aqueous metal mixture and 4 sediment treatments. Waterborne metals were added in a 50-percent dilution series (plus a control) that delivered a sixteenfold range of nominal

metal concentrations. Sediments were prepared by compositing parts of Tri-State Mining District sediments remaining after about 2 years of storage. Sediment composites included sediments with low, medium, and high sediment metal concentrations in combination with low and high percentages of coarse sand (particle diameters of 0.5–2.0 mm).

Objectives

The supplemental studies were designed to address the following research questions raised by results of sediment toxicity tests in 2017 and 2018.

1. What levels of metals added to overlying water will substantially increase toxicity in combination with metals in sediment and pore water?
2. Does the starting age/size of juvenile mussels affect their sensitivity to metals in sediment and overlying water?
3. Do juvenile mussels respond to sediment physical characteristics (for example, high coarse sand content) in addition to high metal concentrations?

Methods

Experimental Design

In fall 2019, four sediment composites were prepared from stored sediments collected in summer/fall 2017 (tables 11 and 12). Sediment 1 was a reference low-metal sediment dominated by fine sediment particles (sum-PEQ=1.4; coarse sand=4.3 percent). Sediments 2 and 3 had intermediate metals concentrations (sum-PEQ of about 3) but differed in sediment texture (sediment 2, 9.3 percent coarse sand; sediment 3, 58 percent coarse sand). Sediment 4 had high metals (sum-PEQ=7.8) and a coarse texture (43 percent coarse sand). Two tests with these sediments were completed in fall 2019: test A was stocked with juvenile mussels with average starting shell lengths of 0.91 mm (about 1 month old), and test B was stocked with larger juveniles from the same maternal cohort with average starting lengths of 1.68 mm (about 2 months old). Diluters delivered water containing five levels of a metals mixture (zinc/lead/cadmium) in the same ratios used in the water-only test (see section. 2) plus a control water. Waterborne metal treatments 1 (low) to 5 (high) were separated by factors of 0.5, and the nominal high zinc concentration was 80 µg/L for test A and 160 µg/L for test B (table 12). These solutions were automatically prepared in diluted well water (hardness of 166 mg/L) and delivered to test chambers at the rate of four volume replacements per day.

Toxicity and Chemistry Methods

Short-term (28-day) tests were based on ASTM International (2015) and the methods from previous studies of effects of mining-derived metals on juvenile mussels (MacDonald and others, 2009; Besser and others, 2015). This approach is similar to terminating the 12-week sediment tests described in section 4 after 4 weeks. Longer exposures could not be completed because they would have required replacement of sediment in test beakers, and the supply of sediments was limited. Samples of sediment and pore water were collected from separate chemistry beakers during the test and were analyzed for SEM, AVS, and pore-water (peeper) zinc as described in section 4.

Data Analysis

Data from tests A and B were analyzed by separate two-way ANOVAs using rank-transformed data. These analyses evaluated the significance of overall differences among the 4 sediment treatments and among the 6 water treatments, as well as the interactive effects of sediment and water treatments. Means for toxicity endpoints in sediment treatments were compared with sediment 1 and means of water treatments were compared with the water control.

Results and Discussion

Chemistry

Metal concentrations in sediment composites and overlying waters of 2019 tests were close to targets (tables 11 and 12). Sediment 4 had concentrations of zinc-PEQ, sum-PEQ, and [ΣSEM–AVS] about 2.5 times greater than those in sediments 2 and 3. Zinc concentrations in pore waters corresponded closely to concentrations in sediment, with little or no increase of pore-water metals in treatments with high metal concentrations added to overlying water. Zinc concentrations in pore water of sediments 2 and 3 varied by more than a factor of two among water treatments, but this variation was independent of overlying water treatments. In contrast to our previous attempts to control metal concentrations in overlying water, overlying water in test chambers remained close to nominal concentrations delivered by the diluters (fig. 14). Zinc concentrations in overlying water indicated only a minor effect of pore-water zinc, which was noticeable only in treatments with high pore-water zinc and low zinc in overlying water.

Table 11. Physical and chemical characteristics of sediment and pore water in supplemental tests, fall 2019, for pretest chemistry.

[TOC, total organic carbon; %, percent; SEM, simultaneously extracted metals; Zn, zinc; Pb, lead; PEQ, probable effect quotient (sediment metal concentration/probable effect concentration); Cd, cadmium; sum-PEQ, summed probable effect quotient values; ΣSEM, sum of simultaneously extracted metals; µmol/g, micromole per gram; AVS, acid-volatile sulfide; [ΣSEM–AVS], summed molar concentrations of simultaneously extracted metals relative to acid-volatile sulfide; DOC, dissolved organic carbon; mg/L, milligram per liter; Na, sodium; Mg, magnesium; K, potassium; Ca, calcium; Mn, manganese; Cl, chlorine; SO_4, sulfate]

Sediment characteristics						Sediment metals							Pore-water chemistry								
Sediment	TOC (%)	Clay (%)	Silt (%)	Total sand (%)	Coarse sand (%)	SEM–Zn	SEM–Pb (PEQ)	SEM–Cd (PEQ)	Sum-PEQ (SEM)	ΣSEM (µmol/g)	AVS (µmol/g)	[ΣSEM–AVS] (µmol/g)	DOC	Hardness (mg/L)	Na (mg/L)	Mg (mg/L)	K (mg/L)	Ca (mg/L)	Mn (mg/L)	Cl (mg/L)	SO_4 (mg/L)
1	0.98	9.0	23	68	4.3	0.74	0.17	0.5	1.4	5.2	4.6	0.6	2.20	82	3.7	2.1	1.5	29	0.8	5.2	1.6
2	0.87	9.3	13	78	9.3	2.0	0.38	1.0	3.3	14	3.1	11	2.70	102	3.7	3.3	1.7	35	2.7	13	8
3	0.57	4.7	11	85	58	1.6	0.39	0.8	2.7	11	2.4	8.6	1.70	98	3.0	2.4	1.4	35	0.4	5.4	4.0
4	0.69	6.6	13	80	43	4.4	1.3	2.2	7.8	31	5.6	25.1	1.70	96	2.8	2.0	1.3	35	2.0	4.1	22.0

Table 12. Physical and chemical characteristics of sediment and pore water in supplemental tests, fall 2019, for midtest chemistry.

[Zn, zinc; µg/L, microgram per liter; nom., nominal; sed., sediment; <, less than; >, greater than]

Water	Zn in pore water (µg/L)					Zn in overlying water (µg/L)									
	Test A					Test A					Test B				
	Nom.	Sed. 1	Sed. 2	Sed. 3	Sed. 4	Nom.	Sed. 1	Sed. 2	Sed. 3	Sed. 4	Nom.	Sed. 1	Sed. 2	Sed. 3	Sed. 4
0	0	4.2	81	53	216	0	4.6	14	11	25	0	<4	6.3	5.6	16
1	5	7	102	71	207	5	8.0	16	13	28	10	7.4	11.0	9.6	18
2	10	>3	58	114	247	10	14	23	23	31	20	14	18	17	26
3	20	19	200	83	217	20	16	27	19	35	40	25	31	30	39
4	40	<3	80	61	291	40	32	43	42	58	80	54	58	53	67
5	80	6	79	82	238	80	74	80	70	88	160	92	124	101	110

Toxicity

Survival of small juvenile mussels in test A was unaffected by sediment or water treatments (fig. 15*A*). In test B, survival in sediment 2 (medium metals/fine texture) was significantly reduced relative to the reference sediment (sediment 1); this reduction was most evident in water treatment 1 (nominal, 10 µg/L as zinc), and survival gradually increased at higher water treatments. Growth in test A was significantly reduced in sediment 2 compared to the reference sediment across all six water treatments (fig. 15*C*). Growth also was reduced in sediment 4 (high metals, coarse texture) at the three lowest water treatments of test A, but growth increased at the two highest water treatments, reaching levels similar to sediments 1 and 3. Growth effects were less affected in test B, but overall growth in sediment 2 was significantly less than growth in the reference sediment. Growth in test B followed similar complex patterns among all sediment treatments: level or decreasing growth in water treatments as much as 40 µg/L, increased growth in the 80-µg/L treatment, and sharply decreased growth in the highest water treatment (160 µg/L).

Concentration-Response Relations

These results indicate that metals in sediment and overlying water affected toxicity to juvenile mussels. The clearest example of the effect of sediment metals was the growth data for sediment 2 in test A, where growth was reduced relative to most other sediments but indicated no differences across the six water treatments (0–80 µg/L as zinc; fig. 15*C*). Variation of toxicity with metal concentrations in water did not follow simple concentration-response trends. Data for all endpoints that indicated toxicity also indicated "reversals"—decreases in toxicity with increasing metal concentrations in water. This pattern was most prominent in test A for effects of growth in sediment 4, which were as low as sediment 2 at low water concentrations but increased to near the reference sediment at higher water metals treatments (fig. 15*C*). Similar reversals of toxicity were evident for growth data from both tests and for survival data for sediment 2 in test B. These reversals were across similar ranges of waterborne metals concentrations (20–40 µg/L as zinc in test A; 40–80 µg/L as zinc in test B), and toxicity (especially decreased growth) resumed at concentrations greater than these concentration ranges.

These unusual reversals of concentration-response trends indicate that exposure to increasing metal concentrations in overlying water elicited a protective response in the juvenile mussels. One protective mechanism known to be active in freshwater mussels is the induction of metallothionein (MT), a low molecular weight protein that has a strong affinity for binding divalent metals. Induction of MT can exist in unionid mussels and a wide range of other aquatic taxa, consistent with its role in regulation of essential metals (Amiard and others, 2006). Studies with the unionid *Anodonta grandis* (Say, 1829) documented that MT can be induced in mussels by exposure to toxic metals (Couillard and others, 1993). Conditions leading to MT induction vary widely among published studies, but induction of MT has often been associated with cadmium exposure (Amiard and others, 2006). Once MT is induced, its high cysteine content can bind strongly to cationic metals including cadmium, zinc, and lead, a process that can lead to reduced toxicity (Couillard and others, 1993). The rapidity of MT induction varies widely among published laboratory and field studies, but induction over periods as short as several days has been reported (Amiard and others, 2006). In studies with *C. fluminea* (Correia and others, 2002), MT was induced by exposure to waterborne copper, but not by copper-spiked sediments, resulting in concentration-response trends like those observed in the present study. Aqueous copper was toxic at low concentrations, with no induction of MT. At intermediate copper concentrations, toxicity was detected within 1 day but decreased to control levels after several days, concurrent with maximum induction of MT. At the highest copper concentrations tested, toxicity was detected without increases in MT. This response at high metal exposure levels has been described as "spillover" because MT binding capacity and biosynthetic capacity are exceeded and toxic effects ensue (Baudrimont and others, 1999, Correia and others,

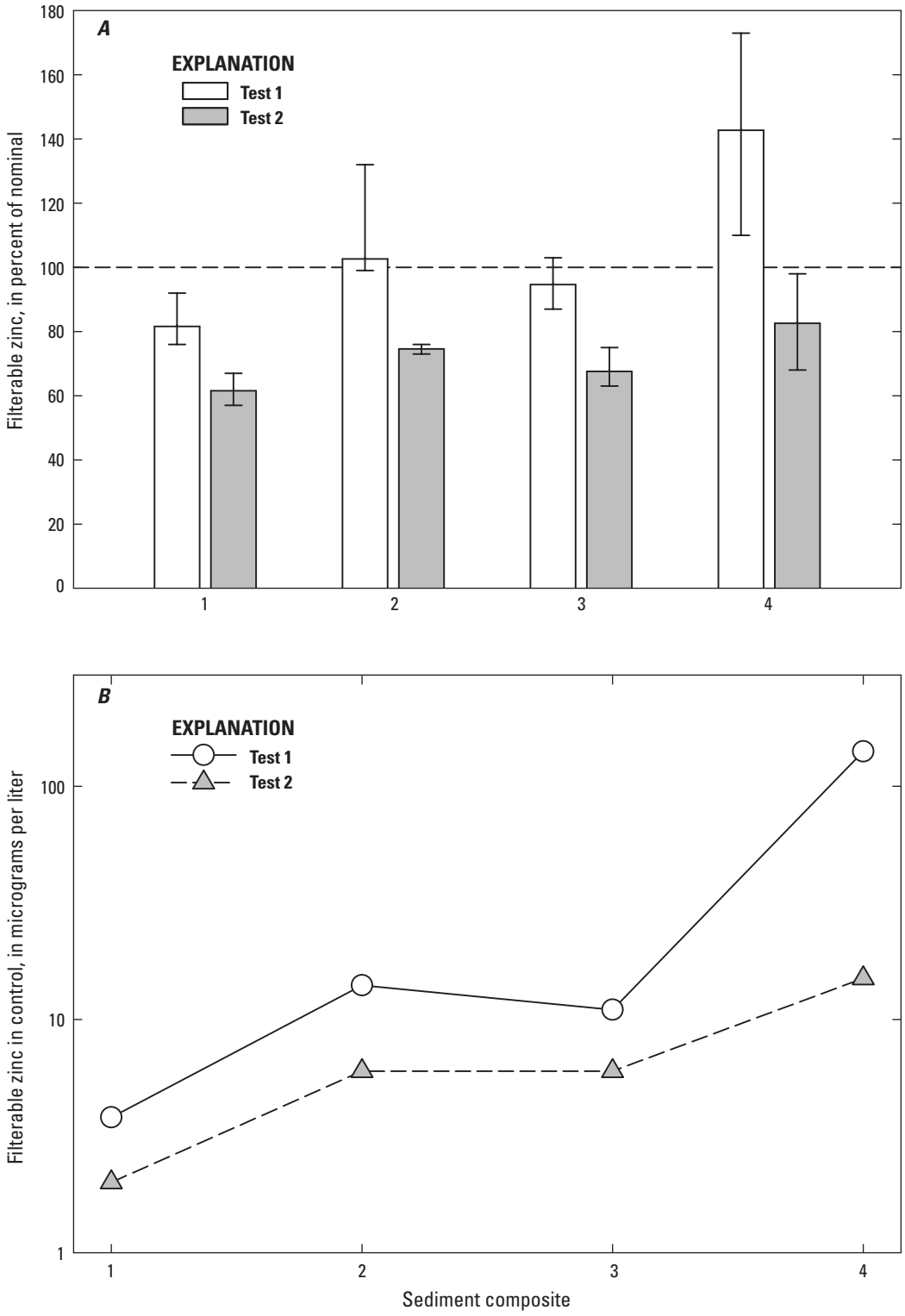

Figure 14. Filterable zinc in overlying water of supplemental (sediment-plus-water) toxicity test, 2019. *A*, zinc as a percentage of nominal (bars = means and error bars = standard deviations for the three highest water treatments); *B*, mean zinc concentrations in water controls (no waterborne zinc added) of four sediment treatments.

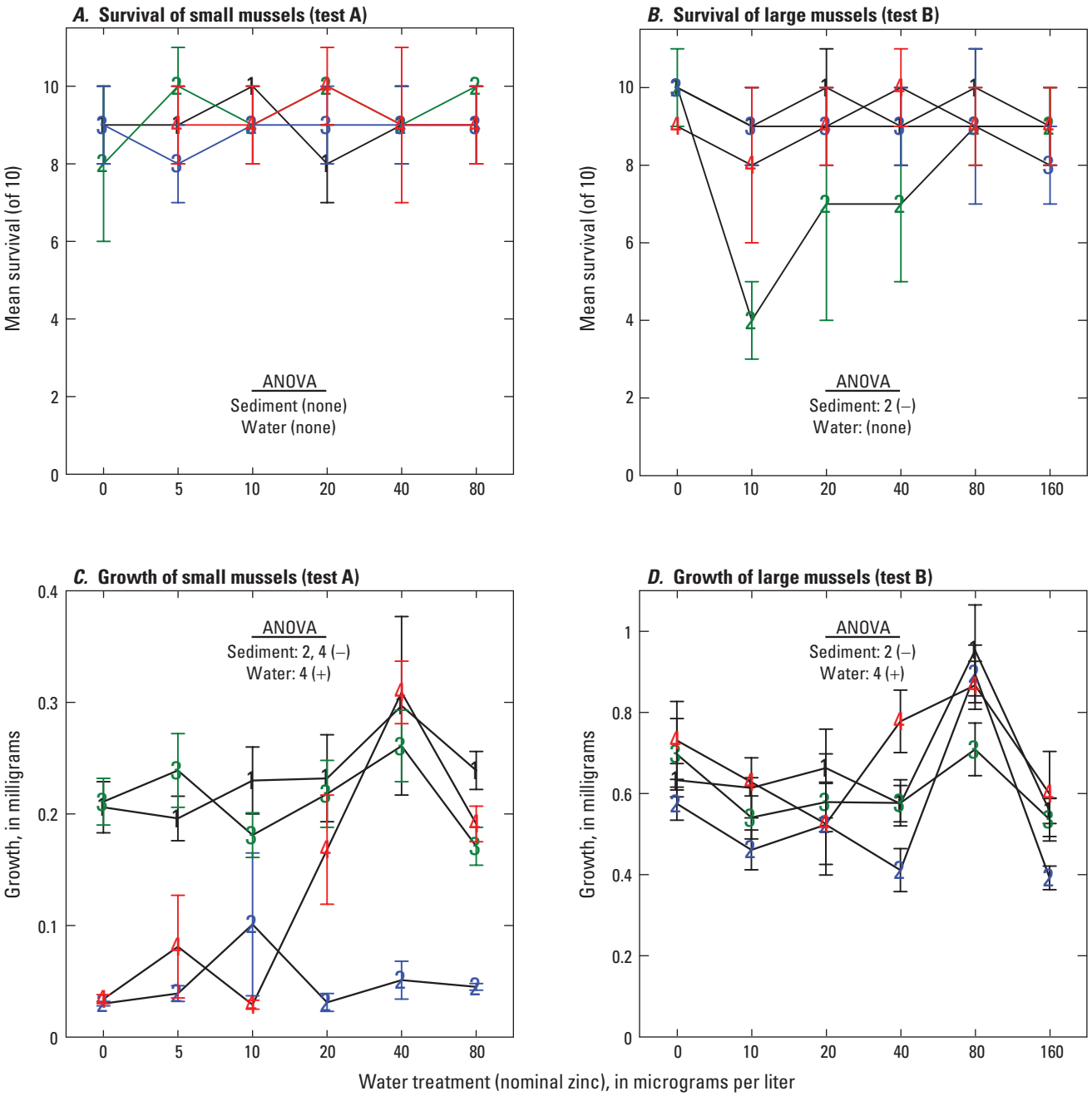

Figure 15. Survival and growth of two age/size classes of juvenile mussels in 28-day exposures to 4 composite sediments (shown as numbered and colored symbols) and 6 metal treatments in overlying water, fall 2019. *A*, survival of small mussels; *B*, survival of large mussels; *C*, growth of small mussels; *D*, growth of large mussels. Colored numbers and error bars indicate means and standard error for water treatments (4 samples per treatment). Text boxes indicate which responses in sediment treatments were significantly different from reference sediment 1 and (or) which water treatments differed significantly from the control (two-way analysis of variance [ANOVA] with mean comparisons by Fisher's least significant difference). [–, decrease; +, increase]

2002). Although we did not attempt to verify induction of MT analytically, the sequence of responses observed in these published studies was consistent with the sequence of events observed in tests A and B: (1) initial toxicity, (2) reversal/reduction of toxicity, and (3) spillover toxicity observed in tests A and B. Trends observed in toxicity data from the 2019 tests are presented in tables 13 and 14 in a simplified matrix of color-coded cells that indicate which treatments seem to have experienced toxicity, reversals, and spillover.

Mussel Age/Size and Behavior

Physiological responses to metal exposure may depend on several aspects of the juvenile mussel and the environment. The sensitivity of mussels to metal toxicity may depend on their size and age, as indicated by the greater growth response of the smaller (test A) mussels in sediment 2, compared to the larger (test B) mussels. This differential sensitivity also is reflected in the thresholds for reversal and restoration of growth effects—about 40–80 µg/L as zinc in test A and 80–160 µg/L as zinc in test B.

The results of tests A and B also indicate that behavioral responses to environmental conditions may modify the metal exposure of juvenile mussels. The uniformly low growth of

Table 13. Summary of mussel toxicity trends in 2019 test A: sediment-plus-water toxicity test with small mussels.

[Data for this table are shown in figure 15. Sediments: 1, low metals/fine (reference); 2, medium metals/fine; 3, medium metals/coarse; 4, high metals/coarse. Responses: NT (no color), not toxic; T (yellow), toxic; R (green), reversal; TT (orange), spillover (toxicity increase). µg/L as zinc, microgram per liter as zinc]

Sediment	Water (µg/L as zinc)					
	0	5	10	20	40	80
			Survival			
1	NT	NT	NT	NT	NT	NT
2	NT	NT	NT	NT	NT	NT
3	NT	NT	NT	NT	NT	NT
4	NT	NT	NT	NT	NT	NT
			Growth			
1	NT	NT	NT	NT	R	TT
2	T	T	T	T	T	T
3	NT	NT	NT	NT	R	TT
4	T	T	T	R	R	TT

Table 14. Summary of mussel toxicity trends in 2019 test B: sediment-plus-water toxicity test with large mussels.

[Data for this table are shown in figure 15. Sediments: 1, low metals/fine (reference); 2, medium metals/fine; 3, medium metals/coarse; 4, high metals/coarse. Responses: NT (no color), not toxic; T (yellow), toxic; R (green), reversal; TT (orange), spillover (toxicity increase). µg/L as zinc, microgram per liter as zinc]

Sediment	Water (µg/L as zinc)					
	0	10	20	40	80	160
			Survival			
1	NT	NT	NT	NT	NT	NT
2	NT	T	T	T	R	NT
3	NT	NT	NT	NT	NT	NT
4	NT	NT	NT	NT	NT	NT
			Growth			
1	NT	NT	NT	NT	R	TT
2	NT	T	T	T	R	TT
3	NT	NT	NT	NT	R	TT
4	NT	NT	NT	R	R	TT

mussels in sediment 2 in test A included low growth in the water control treatment, which can only be attributed to exposure to metals in sediments. In addition, the lack of any reversal and restoration of toxicity in this treatment indicates that these mussels were not substantially affected by levels of metals in overlying waters. One plausible explanation for these results is that mussels in this treatment remained burrowed in sediment 2 throughout the test. Such burrowing behavior is consistent with the consensus that juvenile mussels live and feed within the sediment for the first few years of their life (Yeager and others, 1994; Cope and others, 2008). However, a recent study by Kemble and others (2020) reported deeper burrowing of *L. siliquoidea* in soft, fine-textured sediment compared to coarse sand, indicating that burrowing behavior may vary depending on habitat conditions. Unlike fine-textured sediments 1 and 2, the predominance of coarse sand in sediments 3 and 4 may have limited the ability of small juvenile mussels to burrow to avoid high metal concentrations in overlying water. The resulting exposure to waterborne metals could have induced MT-mediated resistance to physiologic detoxification at low levels of metals in overlying water, reversal of toxic effects at intermediate levels of water metals, and increased toxicity at the highest waterborne exposure concentrations.

Toxicity of Sediment Versus Overlying Water

The strong effect of sediment metals on toxicity made it difficult to isolate the effect of metals in overlying water. Treatments that indicated toxicity at control or low metal concentrations in water (for example, sediments 2 and 4 in test A) provide strong evidence of toxicity of sediment metals. At the other extreme, only the toxicity in the water treatments with the highest metals, with nominal zinc concentrations of 80–160 µg/L as zinc, could be confidently attributed to toxic effects of overlying water. Overall, toxicity in these tests was consistent with sediment toxicity thresholds estimated from the 2017 sediment tests. Composite sediment 2 caused severe reductions in growth of the small (1-mm) mussels in test A at SEM-zinc of 2.0 PEQ and sum-PEQ of 3.3 PEQ. This same sediment was associated with toxic effects on survival and growth of larger (2-mm) mussels in test B. In contrast, no toxicity was observed in sediment 3 in either test, despite sediment metal concentrations (zinc-PEQ=1.6; sum-PEQ=2.7) that were close to those in sediment 2.

The contrast between the consistent toxicity of low-sand sediment 2 and the minimal toxicity in high-sand sediment 3 indicates that sediment texture affected sediment toxicity indirectly by forcing alternative routes of exposure. Juvenile mussels may have been able to burrow more deeply into fine sediments (Kemble and others, 2020), where they would have been primarily exposed to sediment metals, whereas mussels in coarse sediment may have been unable to borrow and thus had greater exposure to waterborne metals. The combination of coarse sand and high metals in sediment 4 indicated a mixture of these responses, with severe toxicity (presumably because of sediment metals) in the control water and low water treatments, reversal of growth effects at intermediate water treatments, and resumption of growth effects at the highest water treatment. Based on this limited dataset, it seems that coarse sediments may limit burrowing behavior of juvenile mussels—perhaps only the smallest size classes—and leave juvenile mussels more vulnerable to toxicity of overlying water. However, the evidence that waterborne metals may stimulate tolerance to metal exposure indicates that toxicity thresholds estimated from our water-only tests may overestimate the sensitivity of juvenile mussels to metals in overlying water.

6. Summary and Conclusions

Species richness and abundance of mussel assemblages were negatively associated with metal exposure—Long-term exposure to metals in sediment, water, or diet has been proposed as a plausible cause for reduced mussel abundance and reduced mussel species richness in streams of the study area (Angelo and others, 2007). In the present study, analyses of metals in tissues of Corbicula collected during mussel surveys demonstrated that metals were biologically available for uptake by bivalve mollusks (fig. 6) and that metal concentrations in sediment and water were negatively associated with mussel community metrics (fig. 16). In the qualitative mussel survey, CPUE (mussels/time) and species richness differed significantly between sites with low versus high metals in water or sediment ($p<0.02$). In the quantitative survey, species richness and density (mussels/area) also differed significantly between low- and high-metal sediments ($p<0.03$), but associations between these metrics and metal concentrations in water were less significant ($p<0.10$). Although mussel community metrics did not generally follow the clear concentration-response trends typical of controlled laboratory tests, species richness (expressed as a percentage of maximum species detected in each stream reach) was reduced by 50 percent at sites with SEM-zinc concentrations of 0.65 PEQ (298 µg/g) or greater (fig. 17).

Water-only toxicity tests and whole-sediment toxicity tests documented significant toxic effects on survival, growth, and biomass of juvenile mussels.—Effect concentrations for juvenile mussels exposed to waterborne zinc in 12-week tests were consistent with results of other recent chronic zinc tests with *L. siliquoidea* (Wang and others, 2020). In tests with waterborne zinc only, toxic effects were limited to reduced growth and biomass, and thresholds for these endpoints were greater (indicating lesser sensitivity) after 12 weeks than after 4 weeks. Despite this reduced toxicity with increasing age/size, chronic effects of zinc only on juvenile mussels in these 12-week tests were detected at filtered zinc concentrations far less than the hardness-based water quality criteria for zinc, for example, 180 µg/L at the hardness of the water tested (165 mg/L as calcium carbonate; EPA, 2021b).

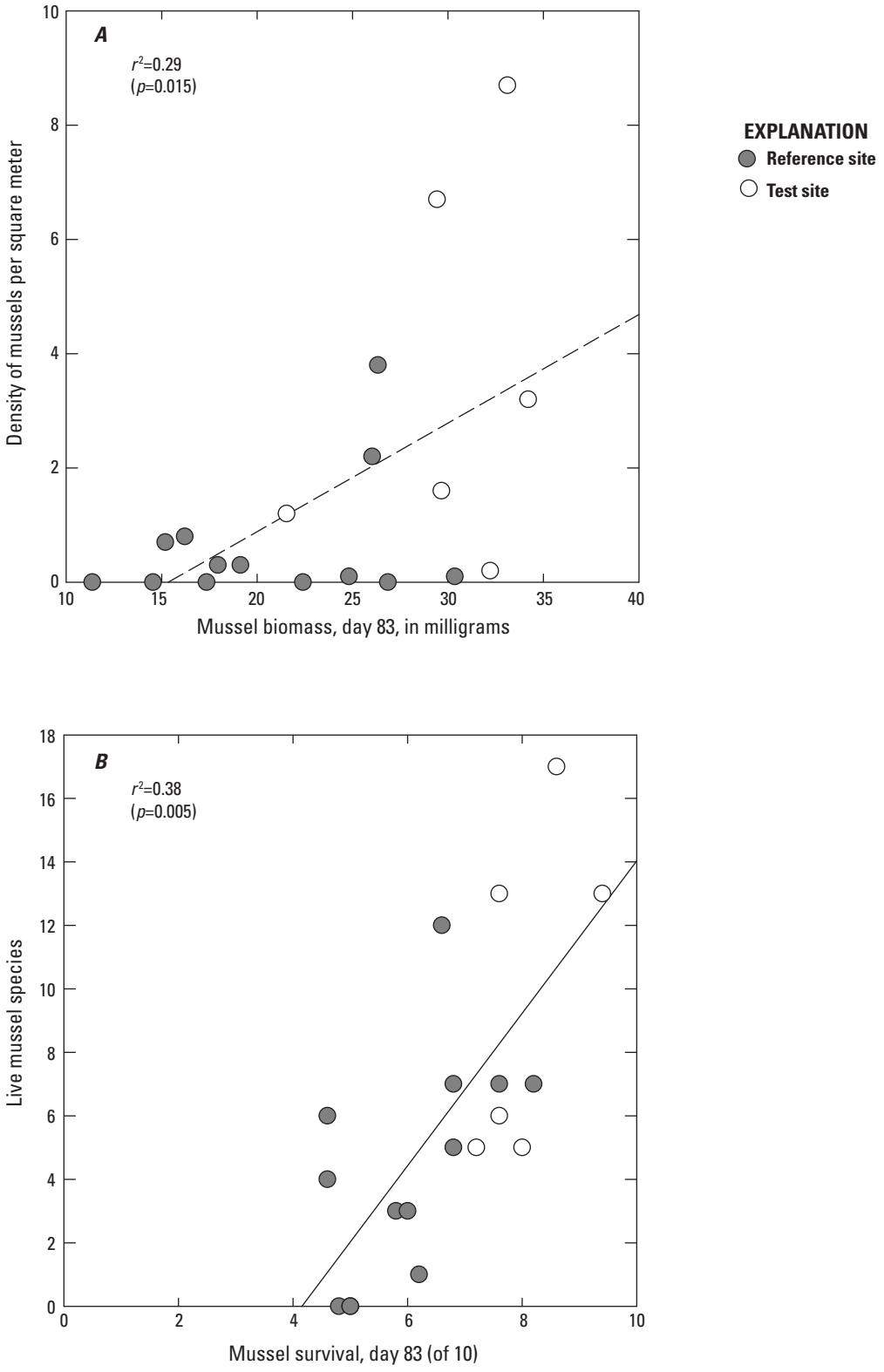

Figure 16. Relations between mussel endpoints from 2017 sediment toxicity tests and community metrics from quantitative mussel surveys, with linear regressions. *A*, mussel biomass versus density; *B*, mussel survival versus species richness. Hollow symbols are reference sites; solid symbols are test sites. Solid line is linear regression. [r^2, coefficient of determination; *p*, probability]

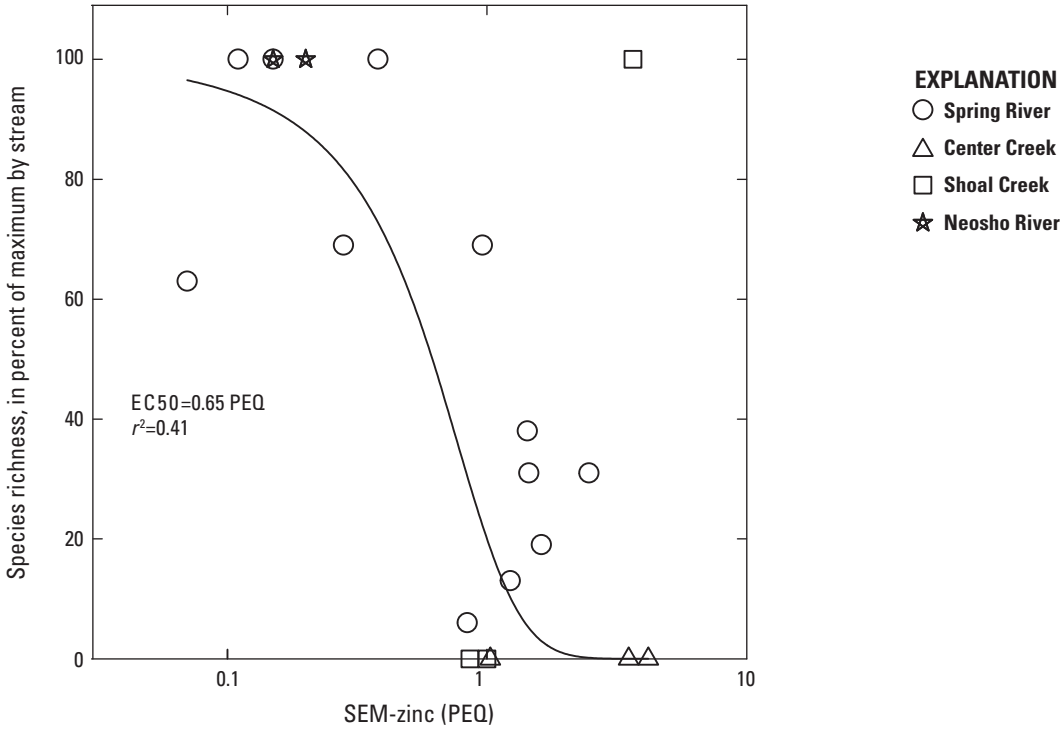

Figure 17. Estimated 50-percent effect concentration (EC50) for effects of sediment zinc on mussel species richness. Species richness values are expressed as a percentage of the maximum number of species for each stream. Curved line is three-parameter sigmoid regression. [PEQ, probable effect quotient, r^2, coefficient of determination; SEM, simultaneously extracted metals]

Mussels exposed to mixtures of zinc, lead, and cadmium in water for 12 weeks not only indicated greater sensitivity of effects on growth and biomass but also indicated significant effects on survival that were not observed after 4 weeks. The range of water-only EC20s for effects of the metal mixture on growth and biomass of juvenile mussels (6.4–20 µg/L as zinc) was similar to waterborne zinc concentrations in stream water (>10 µg/L) that were associated with decreases in abundance and species richness in mussel assemblages (fig. 16).

Whole-sediment toxicity tests completed in fall 2017 also demonstrated significant toxic effects on juvenile mussels. After 12 weeks, mussels in most of the 19 test sediments had mean survival, growth, and biomass the ranges of means for reference sediments, and two or more test sediments had means for each endpoint that were significantly less than all reference means (table 4). Toxicity thresholds for juvenile mussels estimated from several metrics of metal exposure (SEM-zinc, sum-PEQ for the zinc/lead/cadmium mixture, [ΣSEM–AVS], and pore-water zinc) in the 12-week sediment test were less than thresholds from 4-week mussel tests with sediments sampled in 2007 (MacDonald and others, 2009; table 10). For example, the GM–EC20 for SEM-zinc was 2.49 PEQ or 1,143 µg/g, which is much lower than the T20 of 23,700 µg/g determined for sediment zinc (table 10). In contrast, toxicity thresholds for amphipods (*H. azteca*) in 6-week tests in 2017 were within a factor of two of thresholds from 4-week tests in 2007.

Concurrent exposure of juvenile mussels to metals in water and sediment did not always cause increased toxic effects.—The first sediment-plus-water test (spring 2018) indicated little toxicity, even though this test included exposure to sediments that were toxic in the 2017 whole-sediment test plus a water-phase exposure with a nominal zinc concentration of 10 µg/L (in a mixture with lead and cadmium) that was consistent with EC20s derived from the water-only tests (fig. 8). The reduced toxicity observed in the 2018 test was contrary to our expectations that addition of metals to overlying water would increase toxicity, relative to sediments without added metals in overlying water. Several factors could help explain the lack of toxicity in the 2018 water-plus-sediment test: (1) the mean starting size of mussels in the 2018 test was greater than the 2017 test (1.4 mm versus 1.2 mm; these older, larger mussels could have been more tolerant to the metal exposure); (2) PWP-zinc concentrations averaged lower in the 2018 test, consistent with a possible decrease in metal bioavailability; and (3) addition of low levels of waterborne metals in the 2018 test could have induced metal tolerance as we observed in the 2019 study.

Results of the 2019 water-plus-sediment test indicate that mussels developed tolerance to metal toxicity.—This test used composite sediment samples, which were selected to produce four sediment treatments with combinations of low and high metal concentrations and low and high coarse sand content. The water-phase exposure was delivered by a proportional

diluter to produce ranges of the three-metal mixture of as much as 80 µg/L as zinc (test A) and 160 µg/L as zinc (test B). Results of this test indicated that significant toxic effects at low waterborne metal concentrations (as much as 20–40 µg/L) were reversed at higher waterborne metal concentrations (tables 13 and 14). We hypothesize that these reversals of toxicity at zinc concentrations greater than water-only toxicity thresholds (20–80 µg/L as zinc) were attributable to induction of metal-binding protein(s), such as MT (Amiard and others, 2006). The pattern of toxicity we observed—toxicity at low waterborne metal concentrations, reduced toxicity with increasing metal concentrations, and a return to toxicity at the highest concentrations tested—is consistent with the pattern of MT effects in published studies (for example, Correia and others, 2002).

Burrowing behavior of juvenile mussels may have affected their development of tolerance to metal toxicity.—It is widely accepted that juvenile mussels in the wild remain buried in fine-textured sediments (Yeager and others, 1994). In 2019 tests with sediments dominated by coarse sand (composites 3 and 4), toxic effects corresponded more closely to metal concentrations in overlying water than to metals in sediment, perhaps because juvenile mussels could not burrow into these coarse sediments to avoid metals in overlying water (Kemble and others, 2020). Reversals of toxicity were most evident in sediment 2 (tables 11, 12, 13, and 14), which had metal concentrations close to sediment effects thresholds, and at low added zinc concentrations in water. At higher added metal concentrations in water, we hypothesize that toxicity was reduced because of induction of MT, but this protection was overwhelmed at the highest added metal concentrations (typically >40 µg/L).

Toxic effects on mussels in the 2017 study were detected at sediment metal concentrations less than thresholds for toxicity in 2007 tests.—EC20s estimated from the 2017 sediment test were not as robust as the T20s derived by MacDonald and others (2009) because the previous thresholds were derived from larger sample sets (70 sediments tested with amphipods and 43 with mussels, compared to 25 sediments tested in 2017) and at wider ranges of sediment metal concentrations. Maximum zinc, lead, and cadmium concentrations were 3–5 times greater in 2007 samples (Ingersoll and others, 2008). To reduce the influence of individual EC20s estimated from the 2017 test, we calculated composite EC20s for mussels and amphipods based on GM–EC20s for multiple endpoints. The zinc GM–EC20s for mussels and amphipods were 2.8 PEQ and 3.4 PEQ, respectively. These 2017 results are lower than the T20s for both species estimated by MacDonald and others (2009), consistent with longer exposures (12 weeks for mussels, 6 weeks for amphipods) in the 2017 tests. The GM–EC20s from 2017 tests were less than the T20s by factors from 5 to 23 for mussels and by factors of about 0.7 to 2.4 for amphipods. The GM–EC20s from the 2017 mussel test (for example, the EC20 for SEM-zinc of 2.5 PEQ (or 1,148 µg/g) were more protective than either 2007 mussel thresholds (T20 for SEM-zinc=6.4 PEQ; MacDonald and others, 2009) or 2017 amphipod thresholds (EC20 for SEM-zinc=4.0 PEQ). Thresholds based on other metrics of metal exposure, including sum-PEQ and [ΣSEM–AVS], indicated similar trends between species and years, and GM–EC20s for these variables from 2017 tests were less than corresponding T20 values (table 10).

Adverse effects on mussel community metrics and toxic effects in water-only toxicity tests were detected at waterborne metal concentrations typical of Tri-State Mining District streams.—Waterborne zinc concentrations seemed to explain the similarity of toxic effects on juvenile mussels across different exposure types. Similar pore water (push-point) zinc concentrations were associated with thresholds for effects in the mussel survey and in the water-only toxicity tests. The zinc EC20s for the water-only (metal-mixture) test ranged from 6.4 µg/L (growth and biomass) to 48 µg/L (survival; table 6). This range is consistent with the significant reductions in community metrics at sites with zinc in surface water and push-point pore water greater than 10 µg/L (fig. 7). In addition, sediment 2 in the 2019 water-plus-sediment tests (tests A and B) indicated toxic effects on survival and growth associated with overlying water at zinc concentrations in a similar range, 14–43 µg/L (tables 11 and 12). Both of these results indicate that waterborne zinc concentrations in the range of 6.3 to 43 µg/L, co-occurring with proportional levels of lead and Cd, were associated with effects on juvenile mussels. However, toxicity thresholds based on Zn in peeper samples (EC20s from 86 to 215 µg/L; table 10) were greater than those for the water-only tests. Toxic effects in the 2019 water-plus-sediment test (sediment 2) were also associated with higher Zn concentrations in peeper samples (58–200 µg/L). These greater thresholds for peeper metals may indicate lower bioavailability of pore water in contact with deep sediment, or they may indicate that mussels inhabiting surface sediments are exposed to lower metal concentrations, for example, concentrations closer to EC20s for overlying water or water-only tests.

Toxic effects on mussels in sediment toxicity tests were consistent with effects on mussel community metrics.—Survival and biomass of mussels in the 2017 sediment toxicity test were significantly correlated with density and species richness of mussel assemblages (fig. 16). These associations indicate that reductions in survival and (or) growth could contribute to reduced abundance of mussels in the field. These associations also are consistent with a recent study of mussel assemblages in defaunated Tennessee streams, which concluded that long-term reduction in mussel growth was associated with failure of juvenile recruitment and loss of mussel species (Haag and others, 2019). Community metrics indicated significant differences between high- and low-metal sites (fig. 7), but these metrics were more variable than endpoints of laboratory tests and they generally did not produce good fits to CRMs. However, we used a sigmoidal model to estimate that a 50-percent effect concentration for reduction in mussel species richness—normalized to the maximum richness for each stream segment—was 0.64 PEQ (or 294 µg/g as SEM-zinc (fig. 17).

Table 15. Frequency of mussel community effects and toxic effects in laboratory tests.

[qual., qualitative; CPUE, catch per unit effort; quant., quantitative; X, less than reference; O, not less than reference; --, no data]

Site	Sites with responses less than reference				Percentage of sites with effect			
	Qual. species	Qual. CPUE	Quant. species	Quant. density	Quant. survey	Survey mean	Toxicity	Toxicity mean
Center Creek								
CC01G2	X	X	O	X	50	88	--	67
CC03	X	X	--	--	--		--	
CC05D	X	X	X	X	100		33	
CC07	X	X	X	X	100		100	
CC09	--	--	--	--	--		67	
CC10B	X	X	X	X	100		67	
Middle Spring River								
SP05	--	--	O	O	0	33	0	0
SP06	--	--	O	O	0		--	
SP08A	O	X	--	--	--		0	
SP08B	X	X	X	X	100		--	
SP10C	X	X	O	X	50		0	
Shoal Creek								
SH02A	X	X	--	--	--	50	0	11
SH05A	--	--	O	O	0		0	
SH06A	X	X	--	--	--		0	
SH07	X	X	--	--	--		33	
SH08	X	O	O	O	0		--	
SH10B	X	X	X	X	100		0	
SH15A	X	X	--	--	--		--	
SH16A	X	X	--	--	--		33	
SH16B	--	—	X	X	100		--	
Lower Spring River								
SP12B	X	X	--	--	--	20	67	40
SP12C	X	X	O	O	0		0	
SP13	X	X	O	X	50		33	
SP19	O	O	O	O	0		--	
SP20	--	—	O	O	0		67	
SP22	X	X	O	X	50		33	
Neosho River								
NR03	X	X	--	--	--	0	--	33
NR04	X	X	--	--	--		--	
NR05	X	X	--	--	--		--	
NR06	X	O	O	O	0		33	

Toxicity tests and mussel surveys identified similar stream reaches with effects on mussel communities.—Rankings of severity of effects based on toxicity tests and mussel surveys were generally in agreement, but some responses differed in severity. Results of the qualitative mussel surveys indicated severe effects relative to reference conditions across almost all test sites. Richness and CPUE for 78 percent of test sites had responses less than the 5th percentile of the reference envelope (table 15). Results of quantitative surveys and sediment toxicity tests had wider ranges of sensitivity, and effects were detected at 35–58 percent of sites for the quantitative survey and at 4–48 percent of sites for sediment toxicity. These graded responses made quantitative survey data and toxicity data more useful for distinguishing different levels of effects among sites (table 15). The quantitative survey and sediment toxicity tests indicated that the greatest effects of metals toxicity were detected in Center Creek, where 67 percent of test sediments were toxic and 88 percent of sites had reduced quantitative survey metrics. Upper Shoal Creek indicated little or no toxicity and no effects in either quantitative surveys or toxicity tests, despite two sites that exceeded sediment toxicity thresholds (SH02 and SH05); however, toxic effects were detected at several sites in the downstream reach (SH07 through SH16B). The middle Spring River (downstream from Center Creek; sites SP08 and SP10) indicated no toxicity but indicated effects in mussel surveys. Several sites in lower Spring River below Empire Lake (SP12B, SP13, and SP22) had a high frequency of toxicity and moderate effects in the quantitative survey. We did not have enough colocated survey and toxicity data to evaluate the severity of effects in the Neosho River drainage.

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Appendixes 1–3

Appendix 1. Methods for Mussel Surveys

Reconnaissance Site Selection

Reconnaissance sites were selected based on the following criteria: accessibility, published sediment chemistry and toxicity data, available unionid community data, and geographic information system data. Geographic information system data were used to (1) delineate historically stable stream reaches; (2) complete a broad-scale geographic overview for identification of habitat features generally associated with freshwater mussel communities such as gravel bars, riffles, and shoals; and (3) identify access points.

Because unionids generally require permanent, flowing water above stable, gravel-dominated substrates with a component of finer grained particles such as sand, sites were chosen with these criteria in mind and pool habitats were avoided. Areas where the overlay of 1963 topographic maps and current maps indicated shifts in the channel also were avoided because unionid communities are generally detected in stable river channels.

Reconnaissance sites were chosen to represent the range of potential contamination (that is, reference, low contamination, medium contamination, and high contamination) and were distributed as evenly as possible across States, basins, and streams while meeting other site selection criteria. Sites also were placed upstream and downstream from mining input streams to document any change in sediment/water contamination and unionid community characteristics. As parts of the Spring River, Center Creek, Shoal Creek, and Neosho River were traversed, additional sites were investigated based on field observations such as the presence of shell material on the banks or in the shallows and (or) seemingly suitable unionid habitat (stable but permeable substrate with some current velocity). Because these conditions vary with the river system, reference reaches were investigated first to facilitate understanding of mussel habitat within the Spring and Neosho River systems.

An initial 56 sites were selected in 2016, with additional sites added during the 2016 reconnaissance survey. Additional reconnaissance surveys were completed in 2017 and 2018 to fill in geographic gaps and to further investigate river reaches of interest. A total of 102 sites were investigated: 2 on the North Fork Spring River, 34 on the Spring River, 27 on Center Creek, 30 on Shoal Creek, 1 on Lost Creek, 2 on Tar Creek, and 6 on the Neosho River (EcoAnalysts, Inc., 2018).

2016–17 Reconnaissance Survey

Accessibility varied by site and by State and included streamside, boat, and canoe access. Long stretches of river were accessible in Missouri, whereas only point access was available in much of Kansas and Oklahoma. In Missouri, most sites were accessed through bridge right-of-ways or other public lands, and sites upstream and downstream from the access were investigated while walking in the stream or from a canoe or boat. Because Kansas and Oklahoma required landowner permission to access and sample sites, only sites with landowner access permission to the river were investigated. Permissions were coordinated before fieldwork when possible and in the field when needed.

A unionid habitat checklist was developed by EcoAnalysts, Inc.; the U.S. Fish and Wildlife Service; and the U.S. Geological Survey Columbia Environmental Research Center to incorporate unionid habitat factors that might be qualitatively assessed. Unionid habitat requirements can vary by species and by stream, and efforts to describe suitable unionid habitat have ranged from simple variables such as substrate composition and current velocity to more complex hydraulic parameters such as shear stress, boundary Reynold's number, and relative substrate stability (Ecological Specialists, Inc., 2014). For the reconnaissance survey, simple attributes that could be qualitatively assessed were used. Habitat attributes that allow unionids to persist include current velocities slow enough to allow juveniles to settle to the substrate; substrate that is sufficiently stable so that it is not scoured during high flow events; available food, dissolved oxygen, and minerals; favorable water temperatures and water quality; and habitat for host fish (Strayer, 2008; Haag, 2012). Because the Asian clam, *Corbicula fluminea* (O.F. Müller, 1774), has been used as a unionid surrogate in the past (Angelo and others, 2007), the presence of *C. fluminea* also was documented, indicating that sediment and water quality were at least sufficient for this tolerant species.

A map was created at each site, and habitat observations recorded included the following information:

- Geomorphic stream features (riffle, pool, run),
- Bank stability,
- Stream stability,
- Substrate composition (Wentworth scale; Wentworth, 1922),
- Substrate stability/embeddedness/burrowing capacity,
- Instream depositional features, and
- Distance to tributaries.

Each site was subjected to an initial reconnaissance search consisting of field team members dispersing throughout the area searching for the presence of unionids, *C. fluminea*, and (or) suitable unionid habitat. Methods included a shoreline search for shell material, snorkeling throughout the area and fanning the substrate, and scuba diving in deeper areas. If a unionid community was discovered and (or) field biologists agreed that the site harbored suitable unionid habitat, the area was delineated with a survey-grade Trimble Global Positioning System and was qualitatively sampled with timed searches to determine unionid distribution throughout the area, unionid community composition, and catch per unit effort (qualitative sites). Qualitative search effort per site ranged from 30 to 360 person-minutes and averaged 76 minutes.

All unionid data were recorded on standard data sheets, and habitat characterizations were logged within field notes and within the habitat checklist. All data sheets were scanned upon return from the field, and data were entered into a database and underwent a line-by-line quality assurance/quality control procedure. All Global Positioning System points and features were downloaded and placed into an ArcGIS catalog. Digital photographs taken in the field were stored on a project-specific memory card with a unique identifier, and photographs were compiled into an archive set. This archive is unedited and will not be opened. The project-specific memory card was tracked through a chain of custody form and is currently housed at EcoAnalysts, Inc.

2017 Quantitative Survey

After the reconnaissance survey, qualitative unionid data, habitat characteristics, and results of sediment and pore-water sampling were analyzed for unionid community characteristics (number of live species, total number of species, catch per unit effort); habitat suitability; and level of lead, zinc, and cadmium contamination. Using this information, 22 sites were selected for quantitative unionid sampling (EcoAnalysts, Inc., 2018). Sites were distributed over a range of contamination levels on the North Fork Spring River, Spring River, Center Creek, Shoal Creek, and Neosho River. At each site, as many as 100 randomly distributed 0.25-square meter quantitative quadrat samples were collected within the previously delineated unionid habitat area/unionid community to characterize species richness, density, and age distribution. The number of quantitative samples depended on the size of the delineated area and ranged from 15 to 100 (EcoAnalysts, Inc., 2018). Each quantitative sample was excavated to a depth of about 15 centimeters, and material was placed into an attached mesh bag. Each sample was washed through a 6-millimeter sieve and searched for unionids and *C. fluminea*. All live unionids were identified to species, measured (length in millimeters), and aged (external annuli count). Fresh dead shells were identified to species and counted. Weathered dead and subfossil shells were identified to species and noted as present. All live unionids were returned to the river near their collection point, and at least one individual of each species was photographed. Live *C. fluminea* detected during quantitative sampling were enumerated and retained to obtain soft tissue as unionid surrogates for determination of tissue metals concentration. Live *C. fluminea* were placed in a plastic sample bag (one composite sample per site) filled with water and allowed to expel gut contents over a 24-hour period, after which they were transferred to a clean plastic jar, placed in a cooler filled with dry ice, and frozen. All *C. fluminea* samples were transferred to the U.S. Geological Survey Columbia Environmental Research Center after field sampling.

The following quantitative metrics were used to compare sites within each stream:

- Total number of live unionids,
- Total number of live species (species richness),
- Total number of species (live and dead),
- Mean total unionid density (per square meter) and standard error (SE),
- Mean adult unionid density (per square meter) and SE,
- Mean juvenile unionid density (per square meter) and SE,
- Percentage of juvenile unionids,
- Percentage of unionid mortality, and
- Mean *C. fluminea* density (per square meter) and SE.

Quantitative microscale habitat features (depth [in centimeters], current velocity [in feet per second], and substrate constituents) were recorded at each quantitative sample point to compare physical habitat characteristics between sites in each stream. For each quantitative sample, a Gravelometer was used to characterize particle sizes (in millimeters) in each of the four corners and the middle of the quadrat frame (modified pebble count). Pebble count data were entered into a program (Reference Reach Spreadsheet, version 4.2, Ohio Department of Natural Resources) to calculate the size greater than 50 percent and 84 percent of particles, respectively (known as the D50 and D84, respectively).

Additionally, qualitative habitat characteristics, including the following, were recorded:

- Dominant land use (forest/natural, agriculture, and so on),
- General site habitat (primary or secondary channel; run, riffle, and [or] pool),
- Riparian corridor size (less than or equal to 10 meters or greater than 10 meters),
- Canopy cover (shaded, mostly shaded, mostly open, open),

- Substrate stability (loose, somewhat loose, somewhat stable, stable), and
- Instream depositional features (gravel bars, detritus).

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Appendix 2. Quality Assurance/Quality Control for Chemical Analyses

This appendix describes quality assurance and quality control measures, which were not described in the main text for the purpose of conciseness, used during the quantification of elements (zinc, cadmium, lead, nickel, copper), major ions (fluoride, chloride, sulfate, dissolved organic carbon (DOC), sodium, magnesium, potassium, calcium, iron), and acid-volatile sulfide as appropriate in surface and pore water, sediment, and *Corbicula fluminea* (O.F. Müller, 1774) tissues. As described in the main text, analyses of elements and major cations (sodium, magnesium, potassium, calcium, iron) were completed using inductively coupled plasma-mass spectrometry (ICP–MS). Anions were quantified using ion exchange chromatography with suppressed conductivity detection, and concentrations of DOC were quantified using a combustion analyzer. The total number of samples and the ranges of detection or quantification limits are provided in table 2.1.

Quality control measures for ICP–MS analyses included the use of at least three National Institute of Standards and Technology-traceable calibration standards and a calibration blank, laboratory control standards (LCSs), interference check solutions, analysis duplicates, analysis spikes, and dilution analyses. Recoveries of the analytes in the calibration standards were 90 to 110 percent, and the internal standard recoveries were 60 to 120 percent throughout the analyses. Second source initial calibration verification standards (ICVS) and continuing calibration verification standards (CCVS) and blanks were analyzed every 10 samples; recoveries of the analytes from the ICVS and CCVS were 90 to 110 percent for nickel, copper, lead, zinc, and cadmium and 80 to 120 percent for the major cations. Analyte recoveries from the LCS and interference check solution and recoveries of the analysis spikes are summarized by analyte in table 2.2 below for ICP–MS analyses. The relative percentage differences between analysis duplicates and the percentage difference between dilution analyses also are listed in table 2.2. Additional quality control samples for sediment and *C. fluminea* tissues were the analysis of certified reference materials, method replicates, and method spikes (table 2.2). The quality control results indicated that the ICP–MS measurements were generally in control.

Quality control measures for anion and DOC analyses included the use of at least three National Institute of Standards and Technology-traceable calibration standards, analysis duplicates, and analysis spikes. A laboratory control solution was included in the anion analyses for some, but not all, analytical sequences. Recoveries of the analytes in the calibration standards for anions and DOC were generally 80 to 120 percent. ICVS and continuing calibration standards (CCVS and blanks) were analyzed every 10 samples; recoveries of the analytes from the ICVS and CCVS were 80 to 120 percent. Analyte recoveries from the LCSs and recoveries of the analysis spikes are summarized by analyte in table 2.2 for the anion and DOC analyses. The relative percentage differences between analysis duplicates and the percentage difference between dilution analyses also are listed in table 2.2. The quality control results indicated that the ICP–MS measurements were generally within control ranges.

Table 2.1. Summary of reporting limits, limits of quantification, or limits of detection; sample numbers; and numbers of censored sample results.

[Concentration results for major anions and cations were censored at a predetermined reporting limit (RL); dissolved organic carbon results were censored at the limit of detection (LOD), which was estimated on a batch-by-batch basis. Results for lead, cadmium, zinc, nickel, and copper were censored at the limit of quantification (LOQ); the LOQs also were estimated on a batch-by-batch basis. Results for sediments and *Corbicula fluminea* tissues are reported on a dry-weight basis. The total number of samples analyzed for the analyte of interest, and the number of samples that were censored (percentage of total number of samples that were censored) are provided. TR, total recoverable; SEM, simultaneously extracted metals]

Matrix, analyte	Unit of measurement	Range of RLs, LODs, or LOQs	Total number of samples	Number of samples censored (percentage of total)
		Surface water		
Fluoride	Milligrams per liter	0.2	55	35 (64)
Chloride	Milligrams per liter	0.3	55	0 (0)
Sulfate	Milligrams per liter	1.5	55	0 (0)
Dissolved organic carbon	Milligrams per liter	0.1–0.6	56	2 (4)
Zinc	Micrograms per liter	1–40	555	188 (34)
Cadmium	Micrograms per liter	0.02–2	436	313 (72)
Lead	Micrograms per liter	0.03–1	436	304 (70)
Nickel	Micrograms per liter	0.2–0.3	7	0 (0)
Copper	Micrograms per liter	0.4–0.6	7	0 (0)
Sodium	Milligrams per liter	0.1	55	0 (0)
Magnesium	Milligrams per liter	0.1	55	0 (0)
Potassium	Milligrams per liter	0.1	55	0 (0)
Calcium	Milligrams per liter	0.1	55	0 (0)
Iron	Milligrams per liter	0.5	55	49 (89)
		Pore water		
Fluoride	Milligrams per liter	0.2	98	80 (82)
Chloride	Milligrams per liter	0.3	98	1 (1)
Sulfate	Milligrams per liter	1.5	98	31 (32)
Dissolved organic carbon	Milligrams per liter	0.1–0.6	98	0 (0)
Zinc	Micrograms per liter	1–40	181	23 (13)
Cadmium	Micrograms per liter	0.02–2	181	145 (80)
Lead	Micrograms per liter	0.03–1	181	64 (35)
Nickel	Micrograms per liter	0.2–0.3	25	0 (0)
Copper	Micrograms per liter	0.4–0.6	25	21 (84)
Sodium	Milligrams per liter	0.1	97	0 (0)
Magnesium	Milligrams per liter	0.1	97	0 (0)
Potassium	Milligrams per liter	0.1	97	0 (0)
Calcium	Milligrams per liter	0.1	97	0 (0)
Iron	Milligrams per liter	0.5	97	41 (42)
		Corbicula fluminea tissue		
Zinc	Micrograms per gram	1–4	22	0 (0)
Cadmium	Micrograms per gram	0.1	22	0 (0)
Lead	Micrograms per gram	0.1	22	0 (0)
		Sediment		
TR-nickel	Micrograms per gram	0.2	25	0 (0)
TR-copper	Micrograms per gram	1	25	0 (0)
TR-zinc	Micrograms per gram	1	29	0 (0)

Table 2.1. Summary of reporting limits, limits of quantification, or limits of detection; sample numbers; and numbers of censored sample results.—Continued

[Concentration results for major anions and cations were censored at a predetermined reporting limit (RL); dissolved organic carbon results were censored at the limit of detection (LOD), which was estimated on a batch-by-batch basis. Results for lead, cadmium, zinc, nickel, and copper were censored at the limit of quantification (LOQ); the LOQs also were estimated on a batch-by-batch basis. Results for sediments and *Corbicula fluminea* tissues are reported on a dry-weight basis. The total number of samples analyzed for the analyte of interest, and the number of samples that were censored (percentage of total number of samples that were censored) are provided. TR, total recoverable; SEM, simultaneously extracted metals]

Matrix, analyte	Unit of measurement	Range of RLs, LODs, or LOQs	Total number of samples	Number of samples censored (percentage of total)
		Sediment—Continued		
TR-cadmium	Micrograms per gram	0.02–0.03	29	0 (0)
TR-lead	Micrograms per gram	0.1–0.3	29	0 (0)
Acid-volatile sulfide	Micromoles per gram	0.1–0.7	79	5 (6)
SEM-nickel	Micrograms per gram	0.5–3	49	1 (2)
SEM-copper	Micrograms per gram	0.2–0.3	49	0 (0)
SEM-zinc	Micrograms per gram	1–5	83	0 (0)
SEM-cadmium	Micrograms per gram	0.1–0.2	83	0 (0)
SEM-lead	Micrograms per gram	1	83	0 (0)

Table 2.2. Summary of quality control results.

[Target recoveries for quality control samples are generally 90 to 110 percent for analytes in laboratory control standard (LCS), analysis spikes, and method spikes, with the exception of major ions and dissolved organic carbon, which have a target recovery range of 80 to 120 percent. The target recovery range for metals in the interference check solution is 80 to 120 percent, and recoveries of analytes from certified reference materials (CRMs) depend on the quantification method. For example, a total recoverable metal in a tissue will have a much more comparable recovery (80 to 120 percent) relative to a sediment material. Sediments are certified based on total extractions rather than total recoverable or simultaneous extractions with acid-volatile sulfide; therefore, it is reasonable to expect a much lower recovery of analytes from sediment CRMs. Target differences between analysis duplicates, method replicates, and dilution analysis samples (undiluted versus 5x diluted) are generally less than a 10-percent relative standard deviation or relative percentage difference. Few outliers from the target ranges were detected; outliers are not included in the ranges below (see footnotes). %, percent; RPD, relative percentage difference; RSD, relative standard deviation; NA, not applicable, this type of quality control (QC) sample was not analyzed for this analysis; TR, total recoverable; SEM, simultaneously extracted metals]

Matrix, analytes	LCS (% recovery)	Interference check (% recovery)	Analysis spike (% recovery)	Analysis duplicates (RPD)	Dilution analyses (% difference)	CRMs (% recovery)	Method replicates (RSD or RPD)	Method spikes (% recovery)
Surface and pore water								
Fluoride	99.2	NA	95.0–106.5	0	NA	NA	NA	NA
Chloride	102.4	NA	90.0–122.0	0–8.2	NA	NA	NA	NA
Sulfate	99.8	NA	88.7–116.0	0–8.0	NA	NA	NA	NA
Dissolved organic carbon	NA	NA	85.8–106.2	[1]0–9.5	NA	NA	NA	NA
Zinc	93.7–110.9	83.0–119.0	90.6–107.3	0–5.9	0–7.3	NA	NA	NA
Cadmium	95.4–105.6	[2]86.6–128.2	91.8–106.4	0–5.2	0–5.0	NA	NA	NA
Lead	90.7–105.0	85.0–99.8	90.2–105.2	0–4.9	0–6.3	NA	NA	NA
Nickel	97.0–106.6	89.5–91.5	95.7–104.5	0–0.6	1.3–3.7	NA	NA	NA
Copper	91.0–107.5	88.8–89.3	94.7–97.6	2.7–2.8	0.3–0.7	NA	NA	NA
Sodium	91.7–101.7	96.0–106.0	96.0–104.0	0–4.5	0–8.2	NA	NA	NA
Magnesium	97.0–106.7	98.0–104.0	94.0–104.0	0–5.0	0–5.1	NA	NA	NA
Potassium	93.1–104.0	96.0–108.0	100.0–114.0	0–6.9	0–9.5	NA	NA	NA
Calcium	[3]91.4–120.0	94.0–106.0	96.0–120.0	1.9–6.9	0–3.1	NA	NA	NA
Iron	100.0	96.0–106.0	95.6–104.0	0–4.1	0–4.1	NA	NA	NA
Corbicula fluminea tissue								
Zinc	99.3–102.3	92.5–98.8	96.7–101.7	0–2.2	0.5–6.5	97.2–100.0	9.4–31.4	[4]96.8–101.3
Cadmium	100.8–106.6	116.4–119.0	93.6–100.8	1.2–2.2	1.0–5.4	96.2–100.0	9.7–22.7	[5]93.8–104.9
Lead	98.4–100	91.4	95.6–98.0	1.0–1.5	0.4–2.7	[6]86.8–100.0	9.3–11.6	95.8–108.6
Sediment								
TR-nickel	97.6–99.0	87.0	90.4–95.1	0–1.9	0.5–1.7	83.2–86.1	18.0	78.3–95.1
TR-copper	100.9–101.1	92.3	95.1–101.1	0.9–7.2	1.2–1.8	88.7–100.0	17.4	96.7–98.9
TR-zinc	100.8–103.3	86.9–89.3	91.4–97.2	0.8–4.9	1.4–4.3	84.0–91.8	12.2–19.3	94.8–106.4
TR-cadmium	97.9–105.3	104.0–114.2	96.3–98.4	0.6–1.4	1.2–1.9	91.2–100.0	14.8–17.5	96.6–103.9
TR-lead	94.7–101.1	90.8–93.4	96.6–98.7	0–0.9	0.9–1.5	69.0–100.0	2.9–35.7	97.8–113.6
Acid-volatile sulfide	NA	NA	103.0–134.4	0–2.2	NA	NA	0–18.2	103.4–136.5

Table 2.2. Summary of quality control results.—Continued

[Target recoveries for quality control samples are generally 90 to 110 percent for analytes in laboratory control standard (LCS), analysis spikes, and method spikes, with the exception of major ions and dissolved organic carbon, which have a target recovery range of 80 to 120 percent. The target recovery range for metals in the interference check solution is 80 to 120 percent, and recoveries of analytes from certified reference materials (CRMs) depend on the quantification method. For example, a total recoverable metal in a tissue will have a much more comparable recovery (80 to 120 percent) relative to a sediment material. Sediments are certified based on total extractions rather than total recoverable or simultaneous extractions with acid-volatile sulfide; therefore, it is reasonable to expect a much lower recovery of analytes from sediment CRMs. Target differences between analysis duplicates, method replicates, and dilution analysis samples (undiluted versus 5x diluted) are generally less than a 10-percent relative standard deviation or relative percentage difference. Few outliers from the target ranges were detected; outliers are not included in the ranges below (see footnotes). %, percent; RPD, relative percentage difference; RSD, relative standard deviation; NA, not applicable, this type of quality control (QC) sample was not analyzed for this analysis; TR, total recoverable; SEM, simultaneously extracted metals]

Matrix, analytes	LCS (% recovery)	Interference check (% recovery)	Analysis spike (% recovery)	Analysis duplicates (RPD)	Dilution analyses (% difference)	CRMs (% recovery)	Method replicates (RSD or RPD)	Method spikes (% recovery)
				Sediment—Continued				
SEM-nickel	95.2–103.5	88.5–103.0	99.3–100.7	0	1.5–2.4	22.6–44.1	5.8	88.7–97.7
SEM-copper	93.3–102.0	91.9–113.0	100.0–101.9	1.9–3.9	0–1.0	48.1–58.8	6.1–10.7	89.7–99.3
SEM-zinc	94.7–103.9	88.1–108.0	97.0–101.3	0–4.6	0–2.6	67.7–83.9	6.1–8.3	90.2–110.0
SEM-cadmium	94.4–102.4	111.0–120.2	95.2–102.0	0.7–2.2	0.7–2.7	66.7–89.8	0–8.8	89.6–105.0
SEM-lead	93.6–103.3	84.3–91.3	94.2–96.1	0–1.9	0.5–2.8	67.8–79.4	5.2–18.5	85.7–104.9

[1]One analysis duplicate pair for dissolved organic carbon had a 28.6-percent RPD; all other QC results indicated that the analyses were in control.

[2]Two cadmium results in the interference check solution had greater than expected recoveries (173.4 percent and 189.4 percent), which was likely due to an uncorrected interference in the presence of significantly elevated concentrations of molybdenum in the check solution. Results for cadmium in the other QC samples indicated that this was isolated to the interference check solution and that the results were ultimately in control.

[3]Calcium in one laboratory control solution had a recovery of 140.0 percent; all other QC results were within the acceptable range.

[4]One method spike for zinc had a recovery of 68.2 percent. This was likely due to underspiking of the sample.

[5]One method spike for cadmium had a recovery of 71.9 percent. This was likely due to underspiking of the sample.

[6]One certified reference material had a lead recovery of 135.1 percent; all other QC results indicated that the analyses were in control.

Appendix 3. Estimation of Effect Concentrations for Metals in Water and Sediment

The estimation of effect concentrations for toxic effects of metals in water and sediment on freshwater mussels (*Lampsilis siliquoidea*; Barnes, 1823) and amphipods (*Hyalella azteca*; Saussure, 1858) are shown in figures 3.1 to 3.8. In figures 3.1 and 3.2, the models were produced using Toxicity Relationship Analysis Program software (version 1.30a). When filtered zinc in the control was less than the detection limit, the background zinc concentration was estimated (for example, control=3 micrograms per liter). In figures 3.3 to 3.8, the models were derived using three-parameter logistic regression in SigmaPlot (version 14.5).

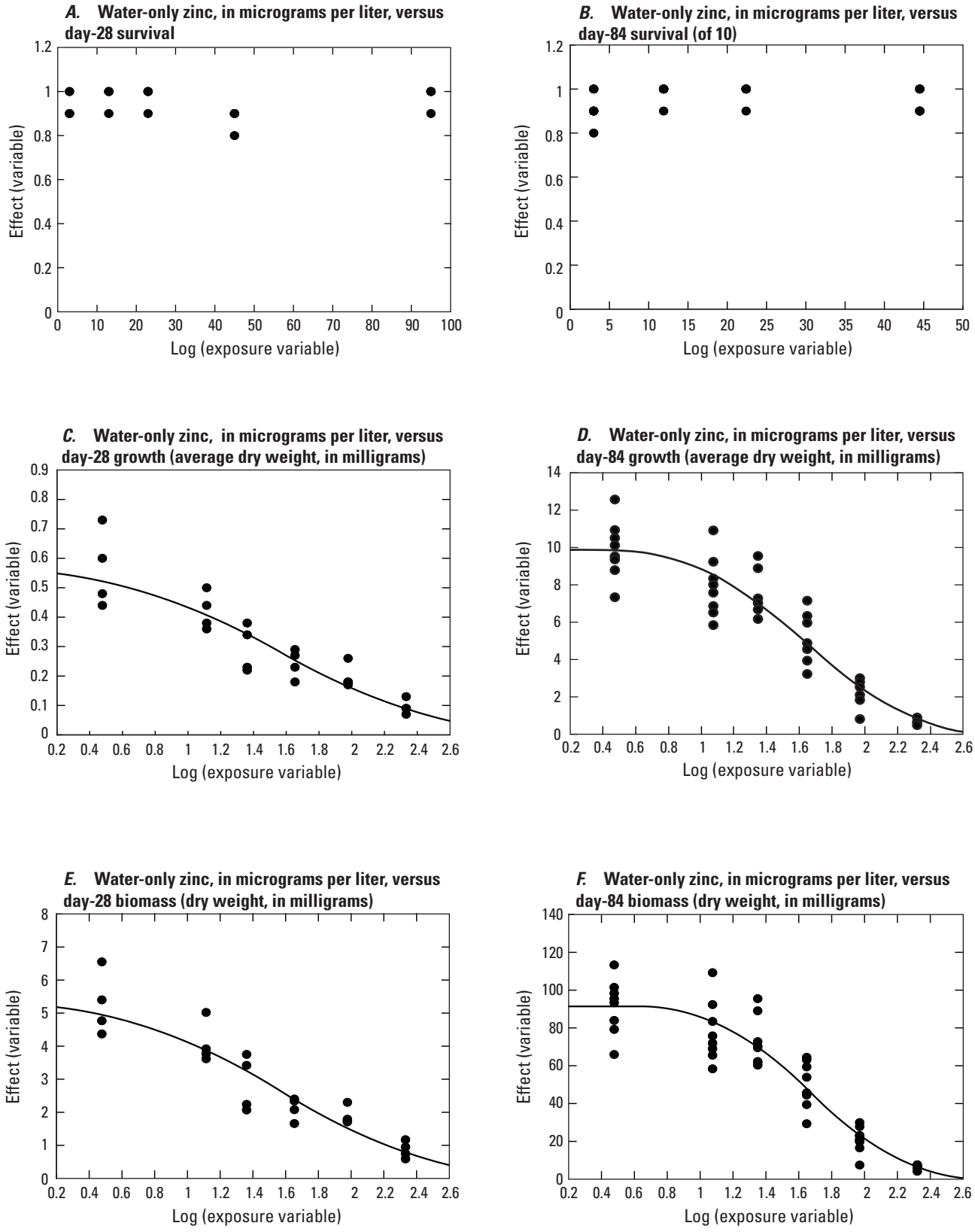

Figure 3.1. Toxicity Relationship Analysis Program estimates of mussel effect concentration values for filterable zinc (in micrograms per liter) in 12-week water-only tests with zinc, spring 2017. *A*, water-only zinc versus day-28 survival; *B*, water-only zinc versus day-84 survival; *C*, water-only zinc versus day-28 growth; *D*, water-only zinc versus day-84 growth; *E*, water-only zinc versus day-28 biomass; *F*, water-only zinc versus day-84 biomass. Circles indicate treatment means; lines indicate regressions.

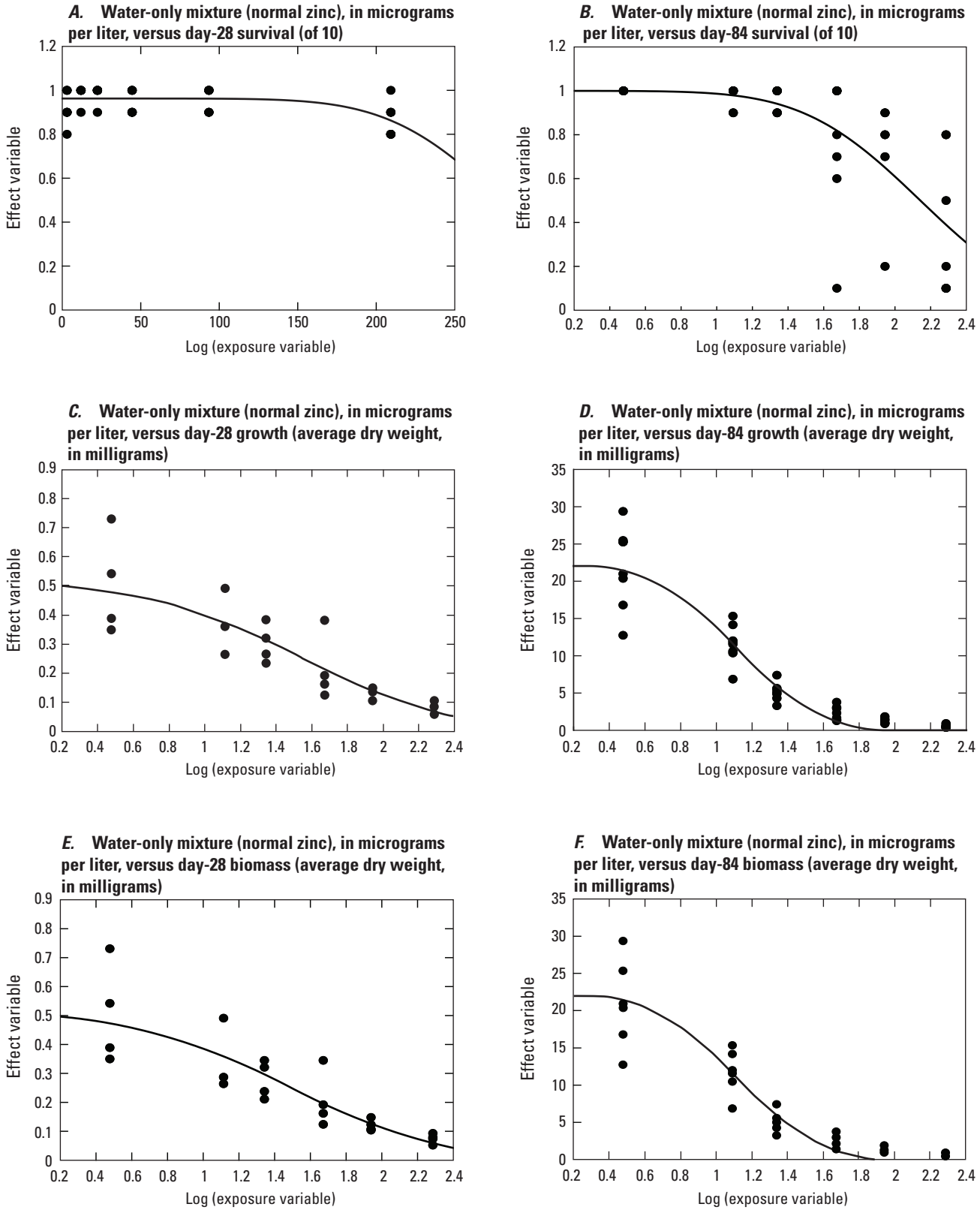

Figure 3.2. Toxicity Relationship Analysis Program estimates of mussel effect concentration values for filterable zinc (in micrograms per liter) in 12-week water-only tests with three-metal mixture, spring 2017. *A*, water-only mixture versus day-28 survival; *B*, water-only mixture versus day-84 survival; *C*, water-only mixture versus day-28 growth; *D*, water-only mixture versus day-84 growth; *E*, water-only mixture versus day-28 biomass; *F*, water-only mixture versus day-84 biomass. Circles indicate treatment means; lines indicate regressions.

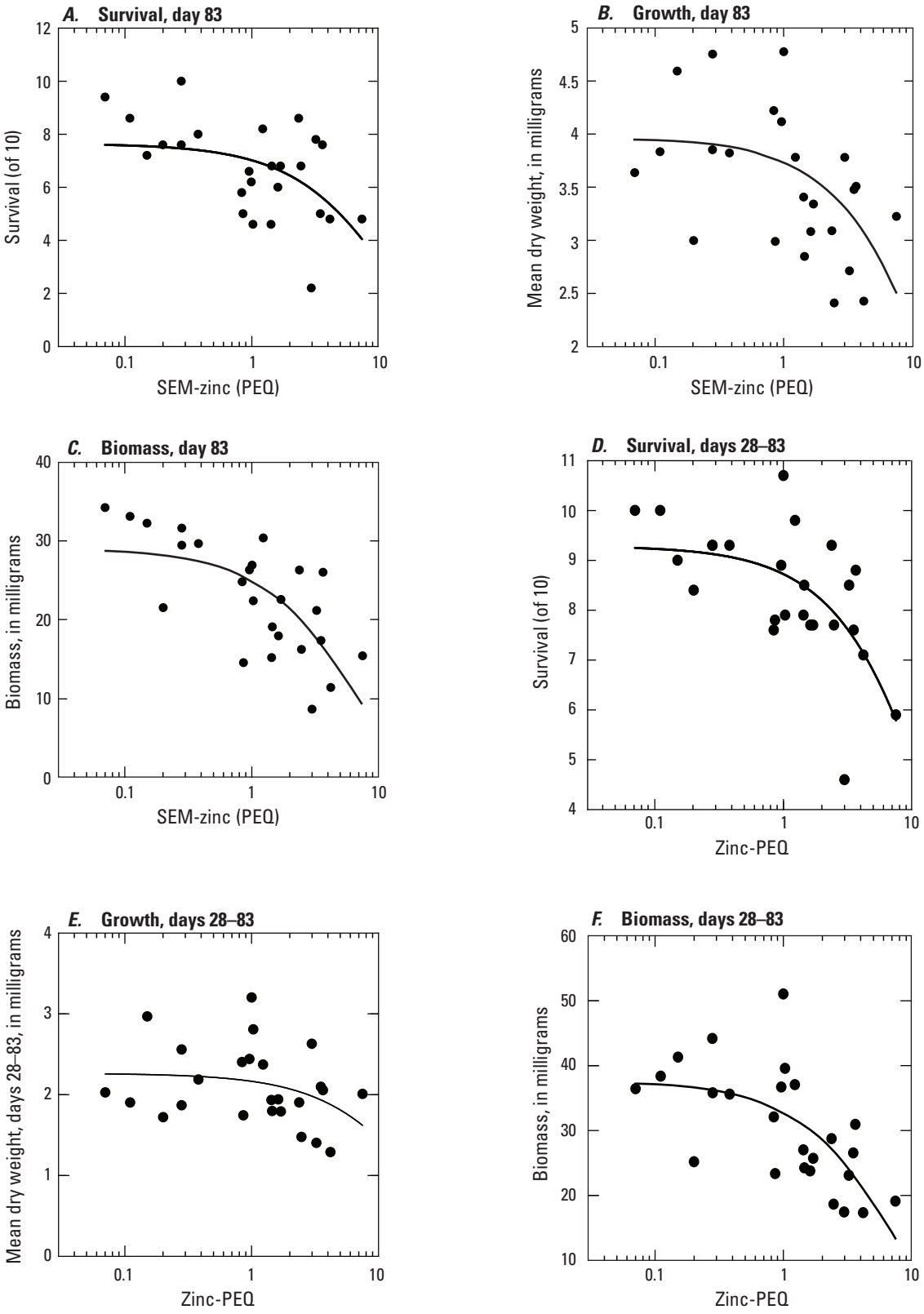

Figure 3.3. SigmaPlot estimates of mussel 20-percent effect concentrations for simultaneously extracted metals- (SEM-) zinc (as probable effect quotient [PEQ]) in 12-week sediment toxicity tests, spring 2017. *A*, survival, day 83; *B*, growth, day 83; *C*, biomass, day 83; *D*, survival, days 28–83; *E*, growth, days 28–83; *F*, biomass, days 28–83. Circles indicate treatment means; lines indicate regressions.

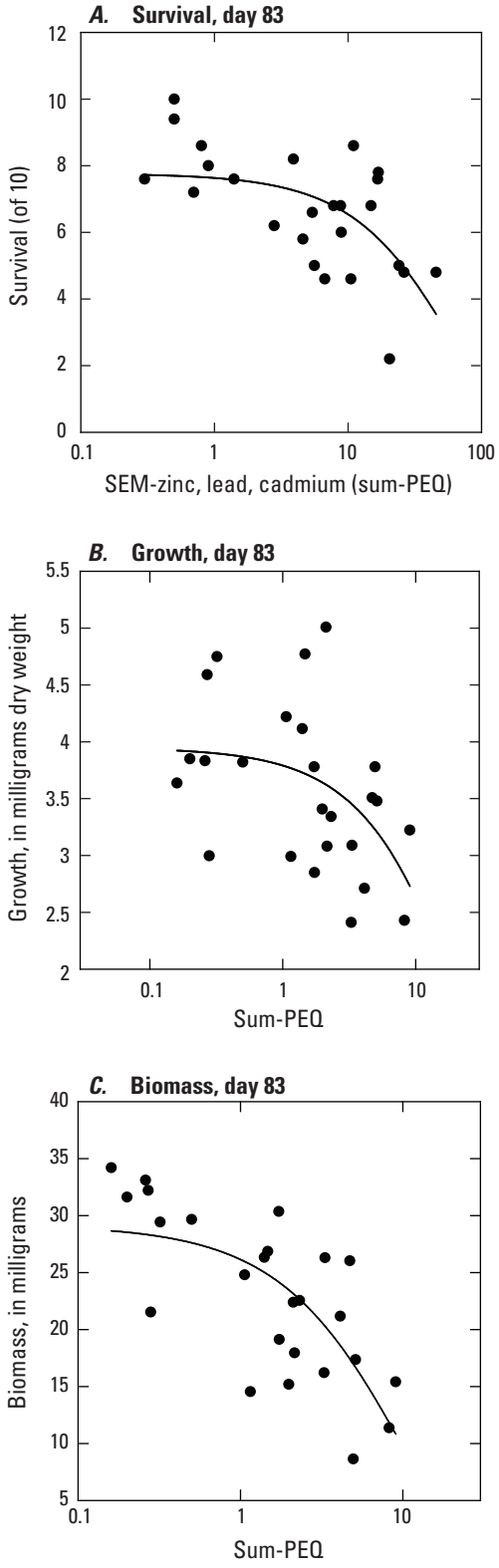

Figure 3.4. SigmaPlot estimates of mussel 20-percent effect concentrations for sum of zinc+lead+cadmium (as probable effect quotient [PEQ]) in 12-week sediment toxicity tests, spring 2017. *A*, survival, day 83; *B*, growth, day 83; *C*, biomass, day 83. Circles indicate treatment means; lines indicate regressions. [SEM, simultaneously extracted metals].

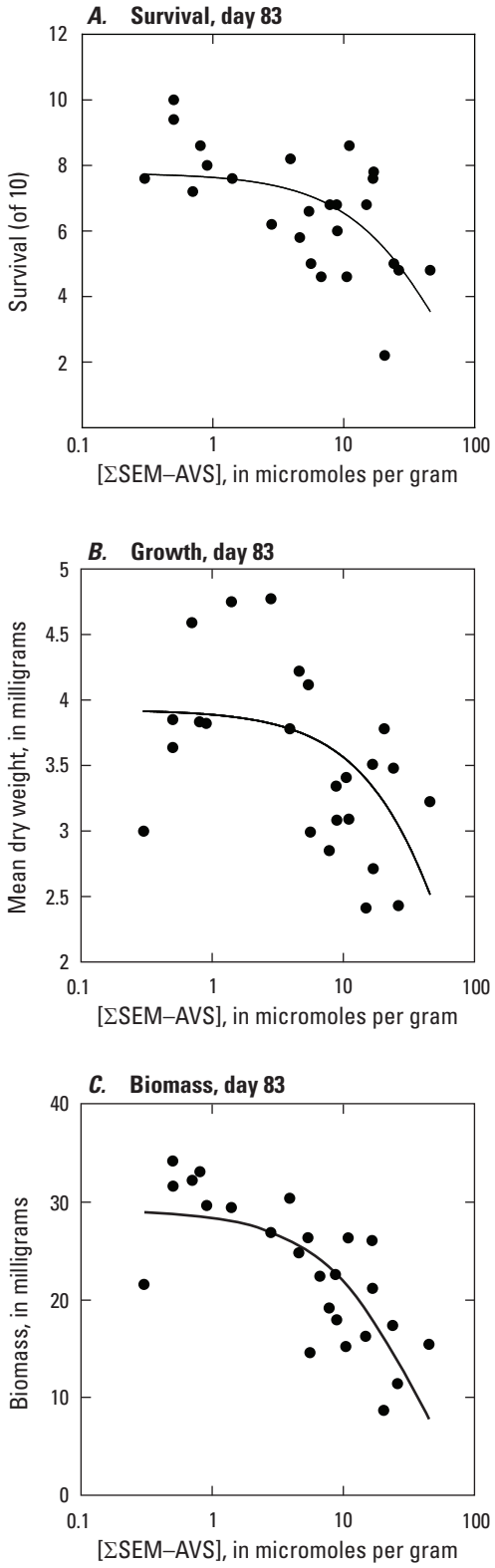

Figure 3.5. SigmaPlot estimates of mussel 20-percent effect concentrations for summed molar concentrations of simultaneously extracted metals relative to acid-volatile sulfide ([ΣSEM–AVS]; in micromoles per gram) in 12-week sediment toxicity tests, spring 2017. *A*, survival, day 83; *B*, growth, day 83; *C*, biomass, day 83. Circles indicate treatment means. Solid lines indicate regressions.

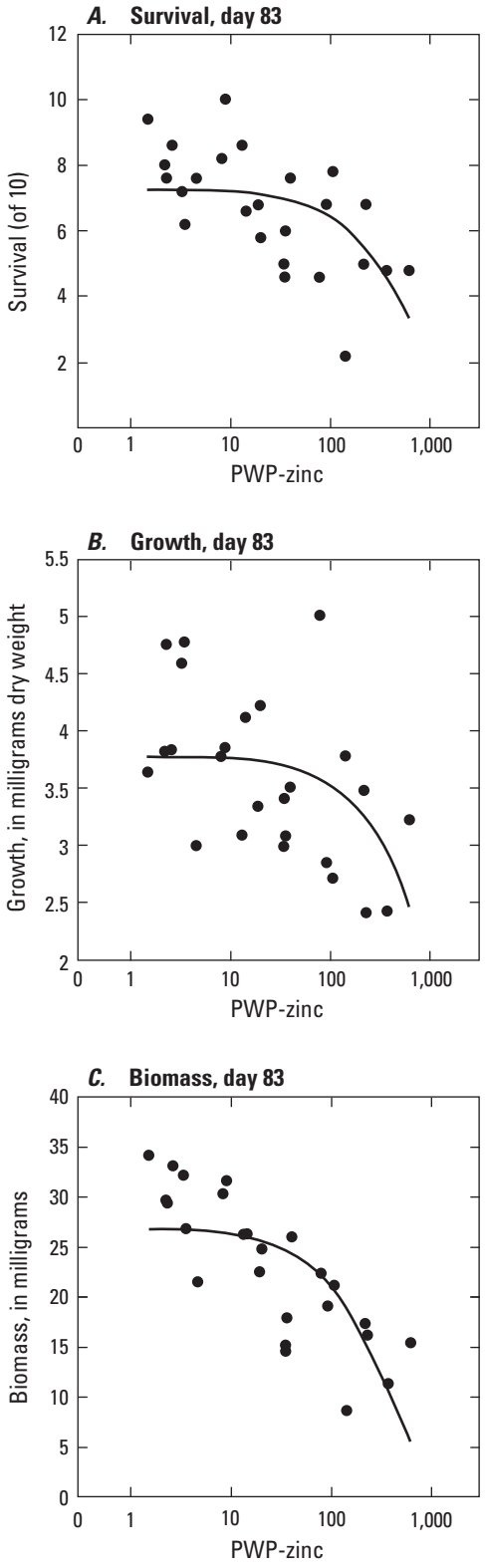

Figure 3.6. SigmaPlot estimates of mussel 20-percent effect concentrations for zinc in pore-water peepers (PWP-zinc; in micrograms per liter as zinc) in 12-week sediment toxicity tests, spring 2017. *A*, survival, day 83; *B*, growth, day 83; *C*, biomass, day 83. Circles indicate treatment means. Solid lines indicate regressions.

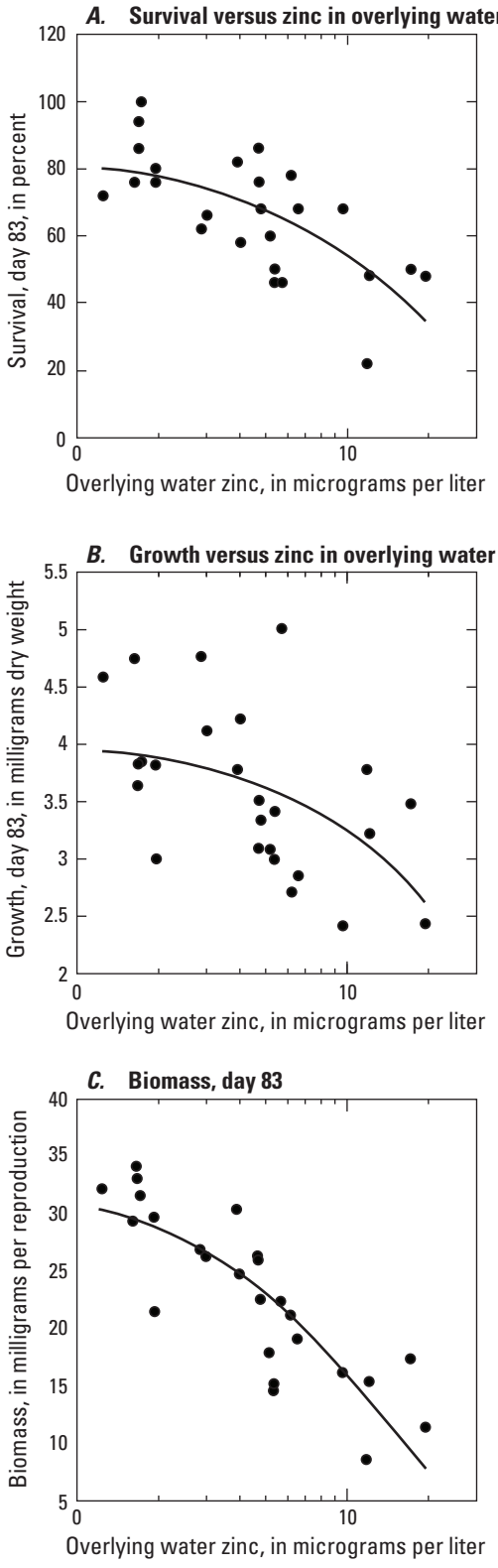

Figure 3.7. SigmaPlot estimates of mussel 20-percent effect concentrations for zinc in overlying water (in micrograms per liter as zinc) in 12-week sediment toxicity tests, spring 2017. *A*, survival versus zinc in overlying water; *B*, growth versus zinc in overlying water; *C*, biomass, day 83. Circles indicate treatment means. Solid lines indicate regressions.

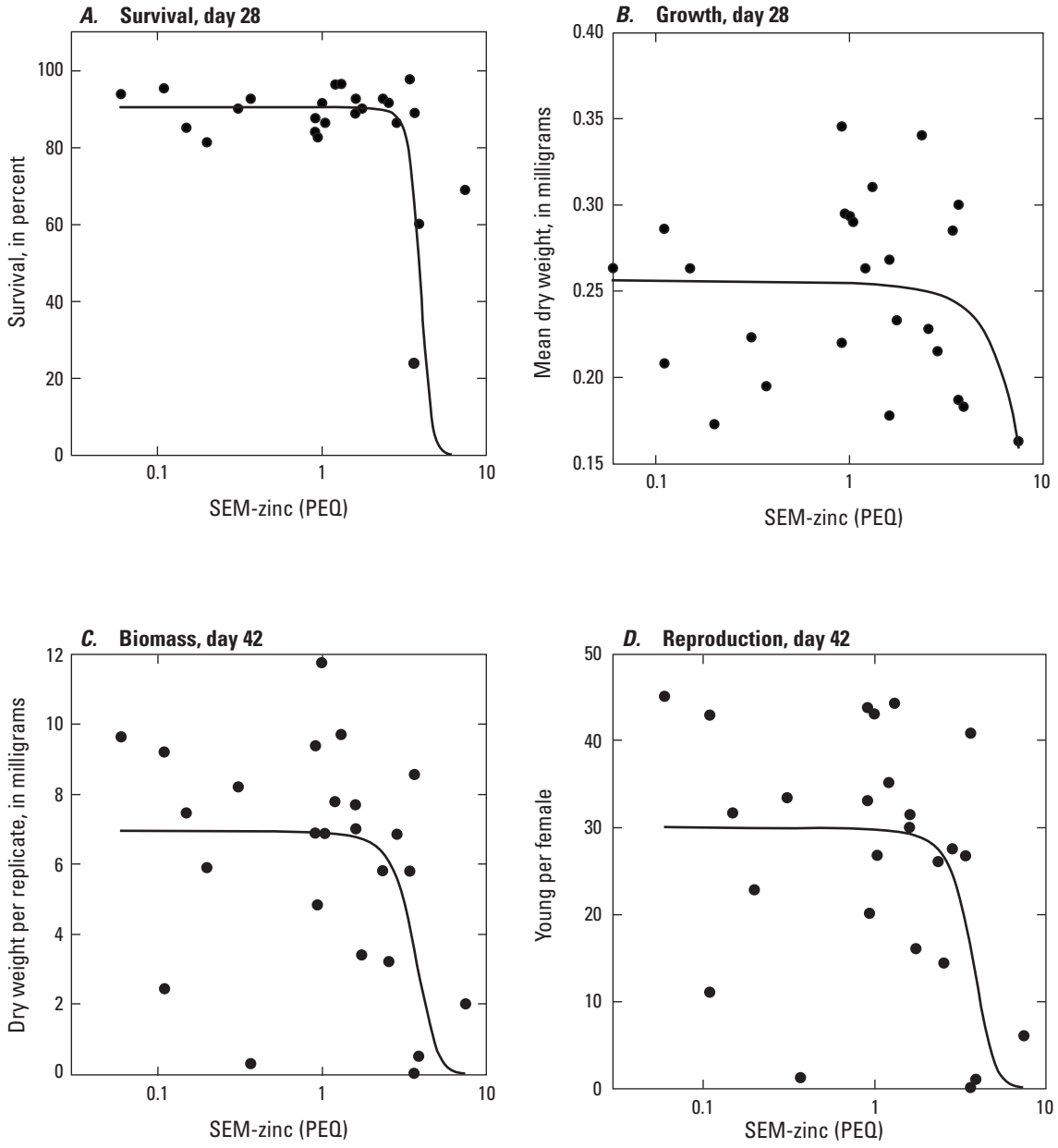

Figure 3.8. SigmaPlot estimates of amphipod 20-percent effect concentrations for simultaneously extracted metals- (SEM-) zinc (as probable effect quotient [PEQ]) in 12-week sediment toxicity tests, spring 2017. *A*, survival, day 28; *B*, growth, day 28; *C*, biomass, day 42; *D*, reproduction, day 42. Circles indicate treatment means. Solid lines indicate regressions.

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