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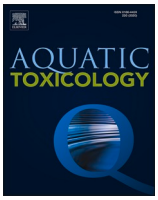

Combined effects of copper, nickel, and zinc on growth of a freshwater mussel (*Villosa iris*) in an environmentally relevant context

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ABSTRACT

Trace metals rarely contaminate freshwaters independently, hence regulatory limits based on single-metal toxicity may be underprotective of aquatic life. This could be especially the case for rare and sensitive fauna like freshwater mussels, such as those suppressed in the Clinch and Powell Rivers in eastern USA where trace metals are long-term contaminants but at concentrations below regulatory limits. We hypothesized metal mixtures may be exerting combined effects on mussels, resulting in greater toxicity than would be predicted based on single-metal exposures. To test that hypothesis, we conducted two experiments exposing juvenile rainbow mussels (*Villosa iris*) for 42 days to dissolved copper, nickel, and zinc, individually and in three-metal mixtures, in an environmentally-relevant context of water with chemistry (hardness 155 mg/L as $CaCO_3$, dissolved organic carbon 1.7–2.3 mg/L, pH 8.4) similar to that of the Clinch River, which receives alkaline mine drainage. We used a toxic unit approach, selecting test concentrations based on literature values for the lower of 28-day survival or growth (length) effect concentrations for *Villosa iris* or *Lampsilis siliquoidea* (fatmucket). Our first experiment confirmed survival and growth effects when acute and chronic water quality criteria, respectively, are approached and/or exceeded. Our second experiment, at lower concentrations, showed no effects on survival but combined effects on growth were evident: a mixture of Cu, Ni, and Zn (7.2 ± 1.2, 65.3 ± 6.1, 183 ± 32 μg/L, respectively) inhibited growth (dry weight) by 95% versus 73%, 74%, and 83% inhibition for single-metal exposures to Cu, Ni, and Zn of similar concentration (8.0 ± 1.1, 63.5 ± 4.8, 193 ± 31 μg/L, respectively). Furthermore, a mixture of Cu, Ni, and Zn with individual concentrations 21%, 29%, and 37% of their water quality criteria (3.4 ± 1.2, 21.8 ± 1.8, and 62.1 ± 8.4 μg/L, respectively) inhibited growth (dry weight) by 61% relative to controls. Our observation of combined effects suggests that regulatory limits based on single-metal toxicity may be underprotective of freshwater mussels when multiple metals are present.

1. Introduction

Anthropogenic activities can increase trace-metal concentrations in aquatic ecosystems to levels potentially toxic to aquatic life. Sources of trace-metal contaminants in freshwaters include metal and coal mining and processing, industrial discharges, atmospheric deposition from coal combustion and metal smelting, and leaching from contaminated terrestrial environments (Swaine et al., 2000; Clements et al., 2000; Deonarine et al., 2015; Vareda et al., 2019). Complicating management is the fact that environmental contamination by single trace metals is rare, as freshwaters affected by such sources are often characterized by elevated concentrations of multiple trace metals (Swaine, 2000; Vareda et al., 2019).

Elevated trace metal concentrations can cause ecotoxicological effects; hence, environmental regulators commonly establish limits on allowable concentrations. Although water contaminants commonly co-

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occur (e.g., Velasco et al., 2019) and contaminant mixtures can exert ecotoxicological effects at concentrations lower than where such effects would be evident for individual constituents (Norwood et al., 2003), regulatory limits are commonly established on a single-constituent basis. For example, in the USA in-stream water quality is regulated by water quality criteria which establish both chronic and acute limits (criteria continuous concentration, CCC and criteria maximum concentration, CMC, respectively) on concentrations for >100 individual constituents including 13 trace elements (USEPA, 2019), but each criterion is applied independently and without consideration of whether other potential toxicants are present. Hence, there are questions concerning the adequacy of such regulatory approaches for aquatic-life protection in real-world, multi-contaminant exposures (Spehar and Fiandt, 1986; Norwood et al., 2003).

Such questions are of particular concern for waters harboring rare species (e.g., Wright et al., 2017). One faunal group notable for its rare and endangered species is freshwater mussels, which are especially susceptible to trace-metal pollution owing to their unique ecology. They are benthic organisms with limited mobility, long lifespans, and complex life histories, all of which make them vulnerable to many aquatic pollutants. Adult mussels are filter feeders that process large water volumes to extract particulate organic matter for nutrition, a process that increases their potential for exposure to water contaminants. They also spend time burrowed into sediments, feeding on deposited organic materials and are therefore subject to influence by contaminants incorporated into the deposited organic matter or otherwise deposited into sediments (Cope et al., 2008). During early life stages, mussels reside within the interstitial waters of aquatic sediments, feeding on deposited organic materials and similarly exposed to water and sediment contaminants. Worldwide as well as in North America, these traits have caused freshwater mussels to be among the most imperiled faunal groups (Haag and Williams, 2014).

In the USA, the Clinch and Powell River systems of southwestern Virginia and northeastern Tennessee are examples of waters where aquatic biota are at risk from trace-metal pollution, as they harbor what are among the most biodiverse freshwater faunal assemblages in North America. In addition to >100 native species of fish (Jenkins and Burkhead, 1994; VaFWIS, 2021), rich freshwater mussel assemblages are major contributors to the high biodiversity status of these two river systems. Currently, 49 extant freshwater species occur in these systems, with 20 species classified as threatened or endangered in accord with the U.S. Endangered Species Act (Ahlstedt et al., 2016). Mussel assemblages have declined in richness and density, most severely in river sections directly impacted by the extensive coal mining that occurs in both watersheds (Johnson et al., 2014; Ahlstedt et al., 2016; Zipper et al., 2016). Multiple solutes of apparent mining origin are elevated above non-mining-influenced reference levels in both rivers, including several trace metals and major ions (Johnson et al., 2014; Zipper et al., 2016; Phipps, 2019), and polycyclic aromatic hydrocarbons (Phipps, 2019; Cope et al., 2021). Although no constituents are known to exceed CCC or CMC and pollutant concentrations have decreased relative to historical levels (Price et al., 2014), faunal declines persist and only minimal, if any, recovery is evident in affected reaches.

This study was motivated by our observation in the Clinch River of suppressed mussel populations in river reaches with multiple trace metals co-occurring, yet available water chemistry data do not suggest overt metal toxicity based on single-metal water quality criteria. Therefore, we hypothesized that exposure to a combination of elevated trace metals is a possible mechanism causing mussel population suppression (the "combined-effects" hypothesis). Thus, the objective of this study was to test the prediction that a multiple-metal mixture would increase toxicity relative to single-metal exposures. We tested this combined-effects hypothesis in an environmentally-relevant context by exposing juvenile rainbow mussels (*Villosa iris*) to three trace metals (Cu, Ni, and Zn) at elevated levels, both individually and in combination, in water with major-ion concentrations comparable to those observed in the Clinch River.

2. Methods

2.1. Test organisms

Juvenile rainbow mussels (*Villosa iris*) were cultured at the Freshwater Mollusk Conservation Center at Virginia Tech (FMCC). We used juvenile mussels rather than adults because research has shown the sensitivity of mussel juveniles to contaminants is similar or greater than that of adults (Cope et al., 2008). Wild gravid female *Villosa iris* brooding mature glochidia were collected and held in FMCC culture tanks while awaiting propagation. Glochidia were flushed from 5 females, combined, and allowed to infest 5 wild-caught host fish (*Ambloplites rupestris*) for 10 min. Infested fish were held in individual tanks until glochidia completed transformation, at which point juvenile mussels were transferred to 3 L aquaria and introduced into the FMCC culture apparatus (detailed in Carey et al., 2013). Mussels were raised in recirculating filtered pond water (approximately 25 °C, dissolved oxygen 7.8 mg/L, pH 8.5, hardness 200 mg/L as $CaCO_3$, alkalinity 175 mg/L as $CaCO_3$, specific conductance 400 µS/cm) and fed 3 mL of a concentrated algae mixture three times daily consisting of 500 mL conditioned water, 1.88 mL Nanno 3600, and 8.25 mL Shellfish Diet 1800 (Reed Mariculture, Campbell, California). where they grew until use in testing. One day prior to test initiation, juvenile mussels from the propagation cohort, aged approximately 90 days (Experiment 1) or 150 days (Experiment 2), were combined and sorted by size using a progressive sieve stack. Mussels passing through 1000 µm mesh and retained on 800 µm mesh (Experiment 1) or passing through a 1200 µm mesh and retained on 1000 µm mesh (Experiment 2) were collected into one tank and acclimated to test dilution water and temperature by partial water changes over the course of the day.

2.2. Dilution water

Our intent to test the hypothesis of combined metal effects was motivated by observations in the Clinch River where metal exposure occurs in combination with elevated major ions (i.e., freshwater salinization). Water hardness, in addition to dissolved organic carbon (DOC), are known to have a strong influence on the toxicity of metals (Paquin et al., 2002). Therefore, we endeavored to expose mussels to metals within an environmentally-relevant context with respect to background water chemistry. Toward that end, we prepared test dilution water to approximate the major-ion composition of Clinch River water during late summer, the period when salinized rivers in the region typically reach annual maximum solute concentration (Timpano et al., 2018). We selected a target sum of ions based on the highest-observed ion sum in the Clinch River dataset for August and October 2015 to 2017 (359 mg/L; VDEQ/TDEC, 2018) and used that value to calculate individual ion concentration targets (Table 1).

We prepared dilution water weekly by mixing filtered (5 µm) FMCC pond water (Ciparis et al., 2015) approximately 1:1 with reverse-osmosis processed tap water (Blacksburg, Virginia) then reconstituting to target levels with reagent-grade salts. We prioritized week-to-week consistency over absolute ion concentrations when preparing each batch. Dilution water contained DOC and low levels of trace metals present in FMCC pond water and were not manipulated, but in the case of DOC, was fortunately similar to the Clinch River target (Table 1). See Supplement for more details on preparation of dilution water.

2.3. Metal exposure concentrations

We standardized metal exposure concentrations using the toxic unit (TU) approach (Sprague and Ramsay, 1965). A TU is the concentration of a toxicant that causes a specific toxic effect to the test organisms.

Table 1
Water chemistry of dilution water approximating annual maxima in Clinch River.

Water Quality Parameter	Units	Actual[a] mean (and standard deviation)		
		Clinch Target	Experiment 1	Experiment 2
Specific Conductance	µS/cm	476	505 (12)	521 (7)
Sum of Major Ions	mg/L	359	357 (9)	337 (16)
Ca	mg/L	36.8	33.2 (1.1)	34.6 (1.1)
Mg	mg/L	17.3	17.4 (0.6)	16.7 (0.7)
Na	mg/L	22.7	36.5 (2)	37.2 (1.9)
K	mg/L	2.64	3.95 (0.1)	4.10 (0.1)
Cl	mg/L	16.3	17.2 (0.7)	17.2 (0.7)
SO_4	mg/L	60.6	59.9 (2.5)	60.5 (2.4)
HCO_3	mg/L	203	187 (7.9)	165 (17)
Alkalinity (as $CaCO_3$)	mg/L	166	154 (7)	141 (5)
Hardness (as $CaCO_3$)	mg/L	163	155 (4)	155 (5)
pH		8.4	8.2 (0.1)	8.4 (0.1)
Ca:Mg mass ratio		2.13	1.91 (0.09)	2.08 (0.06)
Cl:SO_4 mass ratio		0.27	0.29 (0.004)	0.28 (0.002)
Dissolved organic carbon	mg/L	2.1	2.3 (0.2)[b]	1.7 (0.1)[b]
Temperature	°C	24.8	25.3 (0.3)	24.8 (0.3)

[a] Mean values measured across all six treatments on Days 1 and 7 during each week of the 42-day exposure ($n = 12$ measurements per treatment, 72 total). Except for specific conductance and pH, all parameters were measured on filtered samples (0.45 µm).

[b] Concentration in dilution water prepared from diluted pond water, plus any dissolved organic carbon from algal feed mixture; not manipulated.

Although the numeric effect concentration for a given toxicological endpoint may vary among toxicants, each should cause the same response at a concentration equivalent to 1 TU. This standardized approach allowed evaluation of whether metal mixtures had a greater effect than would be predicted by single-metal concentrations.

Toxic units were selected based on hardness-adjusted 28-day EC_{20} (20% effect concentration) values for length or survival observed in the available literature on freshwater mussel water-column toxicity for *Villosa iris* (Cu: Wang et al., 2011) and *Lampsilis siliquoidea* (Ni and Zn: Kunz et al., 2016 and Wang et al., 2020; Zn: Wang et al., 2010). We used the USEPA aquatic life criteria calculator (USEPA, 2018) to adjust effect concentrations in the literature from the tested hardness (approximately 50 to 100 mg/L as $CaCO_3$) to our target hardness of 160 mg/L as $CaCO_3$. We then used the adjusted EC_{20} values (all of which were greater than those in the literature) as the basis for our target TU values (see Supplement and Table S2 for details). To incorporate consideration of DOC into TU selection for Cu, we used the mean of two EC_{20} values from two DOC levels (0.5 and 2.5 mg C/L) in Wang et al. (2011) that bracketed our expected dilution water DOC (1.5–2 mg C/L). Note that we did not convert our exposure concentrations to equivalents at hardness of 100 mg/L (as $CaCO_3$) as is often done for standard toxicity tests. We made that decision because our test conditions were far from those of a standard toxicity test, and for that reason our study was never designed to derive effect concentrations. Therefore, we have no value comparable to EC_{20} to use for comparison with standard studies. Furthermore, and perhaps most importantly, our dilution-water chemistry simulates waters receiving alkaline mine drainage with multiple elevated major ions (e.g., Ca, Mg, SO_4, HCO_3), complicating any comparisons of our findings with effect concentrations from standard studies. Instead, we chose to adjust water quality criteria based on our environmentally-relevant exposure-water chemistry (i.e., essentially site-specific criteria) and compare our exposure concentrations to those adjusted criteria. In this way we place our findings in an environmentally-relevant context, providing a standardized perspective on toxicity potential of our exposure concentrations in an environmentally-relevant context.

We initiated testing by conducting what would become Experiment 1, using 1 TU for single-metal exposures for Cu, Ni, and Zn, and 1 TU and 1/3 TU for exposures applied as mixtures of the three metals. Complete mortality by Day 42 in three of five treatments in Experiment 1 prompted us to conduct Experiment 2 because we needed to compare differences in effect among treatments, which was impossible with the data from Experiment 1. We present results of Experiment 1, as they provide confirmation of survival and growth effects when acute and chronic water quality criteria, respectively, are approached and/or exceeded. However, we focus our presentation and discussion – and base conclusions – on results of Experiment 2 because it provided data sufficient to address our objective.

For Experiment 2 we reduced our exposure concentrations to 1/3 that of Experiment 1 with the intention of avoiding survival effects. In Experiment 2 there were three single-metal treatments: 1/3 TU Cu, 1/3 TU Ni, 1/3 TU Zn, and 2 treatments containing all three metals: 1/3 TU Each metal (1/3 TU EA), and 1/9 TU Each metal (1/9 TU EA). The treatments with 1/3 TU (individual metal and EA) were intended to constrain metals to concentrations lower than individual CCCs (USEPA, 2018), and the 1/9 TU EA treatment contained metals at concentrations ranging from 21% to 37% of their respective CCCs.

We predicted mixture effects would be greater than single-metal effects, because we expected concentration addition (as TUs) given that Cu, Ni, and Zn share the same toxic mode of action: metallic iono/osmoregulatory impairment (Barron et al., 2015). That is, because the three metals exert toxicity in the same way, they could be considered toxicologically equivalent per TU. This means combining multiple metals should sum toxic effects, much like increasing the concentration of a single-metal treatment.

2.4. Test procedures

Test conditions (Table S1) were based on guidance for conducting water-only chronic toxicity tests to early life stages of freshwater mussels (ASTM, 2013). We adapted the approach to our apparatus, which has been used successfully in prior studies (Carey et al., 2013; Ciparis et al., 2015; Phipps, 2019). The exposures were static with weekly renewal, twice-daily feeding, 5 metal treatments and 1 control, 5 replicates per treatment, 60 (Experiment 1) or 20 (Experiment 2) organisms per replicate, and of 42-day duration (see Supplement for details). Test temperature was 25 ± 1 °C, as it approximates both the mean temperature observed in the Clinch River impacted zone during August (24.8 °C; VDEQ/TDEC, 2018), as well as the optimal growth temperature (26 °C) for two species of juvenile mussel in our apparatus as determined by Carey et al. (2013).

Our experimental apparatus was based on that of Ciparis et al. (2015), with slight modifications for temperature control and to accommodate the greater number of treatments used here. Exposure chambers (replicate experimental units) were 18 L downweller-bucket systems (Barnhart, 2006) distributed by stratified random assignment to one of 6 water baths maintained at 25 ± 1 °C. Each exposure chamber held (6) 2-inch diameter sieve chambers enclosed on top and bottom with 500 µm nylon mesh, with the bottom screen supporting mussels during the experiments (no sediment substrate was used). A 600 L/hr submersible pump circulated water continuously downward through the sieve chambers (Supplement). Exposure chambers were pre-conditioned with nitrifying biofilms (Supplement), which controlled ammonia to < 0.01 mg/L NH_3-N during the experiment.

One day prior to test initiation, treatment waters were prepared by adding appropriate volumes of metal stock solution to dilution water to achieve target concentrations based on dosing rates determined in a prior trial run (Supplement). Treatment waters were heated to 25 ± 1 °C and aerated until distribution to exposure chambers. The same procedure was used to prepare treatment waters prior to weekly renewal, at which point we exchanged 100% of water in each replicate.

At test initiation, 60 (Experiment 1) or 20 (Experiment 2) juvenile mussels from the acclimation tank were selected randomly, measured for maximum shell length on the long axis, and transferred at random to

one of six sieve chambers within an exposure chamber filled with fresh treatment water. This process was repeated until all 30 exposure chambers were populated. Thus initial length and subsequent lengths were measured at the replicate level. We measured dry weight for Experiment 2 but not for Experiment 1 because of substantial mortality in the latter. At initiation of Experiment 2 we obtained a single mean initial dry weight from a subsample of 60 mussels selected from the cohort used in the test.

Each exposure chamber was fed 1 mL of a concentrated algae mixture twice daily, yielding approximately 94,000 algal cells added per mL of exposure water at each feeding. A batch of algal mixture was prepared weekly, consisting of 400 mL test dilution water, 10 mL Nanno 3600, and 50 mL Shellfish Diet 1800 (Reed Mariculture, Campbell, California). Algae mixture and feeding rates were comparable to those used for mussel culture at FMCC as well as those used in a prior experiment in our apparatus (Ciparis et al., 2015). In addition, mussels had uncontrolled access to food in the form of any seston (e.g., phytoplankton, fine particulate organic matter) present in the dilution water as sourced from the FMCC pond.

We measured physicochemical parameters and water chemistry each week using composited equal subsamples from each replicate of a treatment/control. On Day 7 of each week (i.e., prior to renewal) we measured dissolved oxygen, temperature, pH, and specific conductance by multi-parameter sonde. We measured total ammonia (spectrophotometry) as well as dissolved values for alkalinity (potentiometric titration), chloride and sulfate (ion chromatography), major cations and trace metals (including Cu, Ni, and Zn; inductively-coupled plasma mass spectrometry), and dissolved organic carbon (carbon analyzer). We also measured DOC and trace metals on Day 1 of each week (i.e., 24 h after renewal). Dilution water was sampled on Day 0 of each week (i.e., at renewal) for alkalinity, DOC, chloride, sulfate, major cations, and trace metals (see Supplement for more details).

2.5. Observations

We measured survival ($\geq$ 80% required in control) and length every 14 days in both experiments. At test initiation and termination (Days 0 and 42) we examined all mussels and on Days 14 and 28 we examined the same subset of 10 individuals in each replicate by measuring mussels in the same sieve chamber within the same replicate each time.

Mussels were recorded as alive if foot movement was detected during a 5-minute period of observation. Maximum shell length parallel to the hinge (long axis) was measured to the nearest micrometer graduation (25–50 μm depending on magnification) using a stereo dissecting microscope with calibrated ocular micrometer. Length was calculated as the mean length among surviving mussels in each replicate and compared to that replicate's initial length to determine change in length for each replicate. At test termination for both experiments, we measured mussel length and for Experiment 2 we also preserved mussels in 70% ethanol for subsequent measurement of dry weight. Mussels were aggregated by replicate and dried in pre-weighed aluminum weigh boats at 60 °C for 24 h then weighed. Mean individual dry weight was calculated per replicate for subsequent comparison with the single mean initial dry weight to determine change in dry weight for each replicate.

In the course of measuring mussels upon termination of Experiment 2, we noticed that shells of some individuals were malformed, so deformity frequency was included as a sublethal endpoint for Experiment 2 (deformities were not noted, present or absent, for Experiment 1). Shell deformity was a binary endpoint and a mussel was considered to have shell deformity if the edges of shell halves did not meet over the entirety of the seam when valves were closed fully. For each surviving mussel we recorded deformity as present or absent based on those criteria.

2.6. Data analysis

We calculated several test endpoints derived from raw observations of survival, growth, and shell deformities. *Percent survival* was 100 times the number of live mussels per replicate divided by the total number of mussels in that replicate at the start of the test. We use the term growth to mean size (length or mass) increase over time. Therefore, growth endpoints were represented by relative changes in length and dry weight of surviving mussels (i.e., the proportion of initial size by which mussels increased between test initiation and a given time point). We chose to use relative measures of growth because we believe it enhances interpretability (e.g., 95% growth inhibition clearly conveys that almost no growth occurred), and it standardizes the growth endpoint when initial sizes of organisms may be non-standard and variable, such as in the case of our two experiments using juvenile mussels of different age and much older than the typical 1–10 days recommended in ASTM methods (ASTM, 2013). Percent length *change* was 100 times the difference between final and initial length, divided by initial length, calculated using replicate-level mean values for both initial and final lengths. *Percent dry weight change* was 100 times the difference between final and initial dry weight, divided by initial dry weight, calculated using replicate-level mean final weights and a single mean initial weight. *Percent deformed* was 100 times the number of live mussels with a shell deformity divided by all live mussels in that replicate. For growth measures we also calculated *percent inhibition* relative to control performance, which is the percentage of the control relative size increase by which relative size increase was reduced in metal treatments, calculated as: 100(1 – treatment mean relative size increase/control mean relative size increase). Percent inhibition values represent the degree to which each metal treatment limited potential growth compared to mussels growing in the absence of elevated metal concentrations. For each endpoint, all statistical analyses were conducted using one mean value per replicate and five replicates per treatment.

To provide environmentally-relevant perspective on toxicity potential of our exposure concentrations, we compared metal concentrations to their respective chronic water quality criteria using a chronic criterion quotient (CCQ) for each metal concentration. We calculated a CCQ as the measured concentration of dissolved metal divided by its adjusted chronic water quality criterion. Higher CCQ values indicate greater toxicity potential and a CCQ $>$ 1 indicates criterion exceedance. To account for our exposure-water chemistry, we adjusted Ni and Zn criteria to our experimental water hardness using USEPA equations and parameters in that agency's aquatic life criteria calculator (USEPA, 2018). We adjusted criteria for Cu following the Biotic Ligand Model (BLM) per USEPA criteria guidance for copper (USEPA, 2007) using BLM software (Hydroqual, 2007). We input into the BLM software our experimental data on temperature, pH, DOC, Ca, Mg, Na, K, SO_4, Cl, and alkalinity (Table 1); in the absence of data we used the suggested default parameters for humic acid (10%) and sulfide (10^{-6} mg/L).

Statistical analyses were conducted with R 3.5.3 (R Core Team, 2019). Treatment effects were assessed using package *lme4* (v1.1-21) to construct generalized linear mixed models (GLMM) with treatment as a fixed effect and water bath as a random effect. Tukey post-hoc pairwise contrasts were conducted using the glht() and mcp() functions of the *multcomp* package (v.1.4.10). For survival data, at least one treatment in each test at every time point was "completely separated" (i.e., 0 or 100% survival), as detected using package *brglm2* (v0.5.1). Complete separation of a treatment prevents proper fitting of a GLMM because that treatment can explain fully the binomial response (Heinze and Schemper 2002). Therefore, survival data were modeled using a modified GLMM with Bayesian weak priors using package *blme* (v1.0-4). Survival percent and deformity frequency data were modeled using a binomial error distribution, whereas length and weight data were modeled using a Gaussian error distribution. All statistical tests were conducted at a significance level of $\alpha = 0.05$.

3. Results

3.1. Experiment 1 water chemistry and metal treatments

Treatment water physicochemistry for Experiment 1 was considered acceptable and consistent among treatments and over time. Experiment-wide mean ± standard deviation ($n = 72$) for each parameter were as follows: specific conductance (505 ± 12 µS/cm); pH (8.2 ± 0.1); dissolved oxygen (7.5 ± 0.3 mg/L); temperature (25.3 ± 0.3 °C); NH_3–N (0.04 ± 0.4 mg/L); dissolved organic carbon (2.3 ± 0.2 mg/L). Major ion concentrations were also consistent and generally within 10% of targets meant to simulate environmentally-relevant conditions as observed in the salinized Clinch River (Table 1).

Mean exposure concentrations in Experiment 1 for Cu and Ni approximated target values, but mean Zn concentrations were higher than nominal target values for all treatments to which Zn was added (Table 2). All of the 1 TU treatment concentrations exceeded chronic criteria (CCC) and all treatments containing 1 TU Cu or 1 TU Zn had those metals at concentrations near or exceeding acute criteria (CMC), with Zn doing so by approximately 3-fold (Table 2). This could explain why we observed such mortality in those treatments in Experiment 1. The lowest dose treatment, 1/3 TU EA, contained metals approximately equal to target concentrations and near or below CCC, with Zn exceeding CCC in some weeks (Table 2).

3.2. Experiment 1 survival and growth

Mortality (≤ ~ 20%) was observed for all but the 1 TU Ni treatment at the first examination (Day 14), with complete mortality by Day 28 for the 1 TU Cu and 1 TU Zn treatments and complete mortality for the 1 TU EA treatment by Day 42 (Table 2, Fig. S1). Survival for the 1 TU Ni (76 ± 4.9%) and 1/3 TU EA (78 ± 9.0%) treatments on Day 42 were significantly lower than control survival (95 ± 3.1%). Among surviving mussels, growth (length increase) for the 1 TU Ni (36 ± 8.1%) and 1/3 TU EA (12 ± 3.0%) treatments were also significantly lower than the control (111 ± 33%) (Table 2).

3.3. Experiment 2 water chemistry and metal treatments

Treatment water physicochemistry for Experiment 2 was considered acceptable and consistent among treatments and over time. Experiment-wide mean ± standard deviation ($n = 72$) for each parameter were as follows: specific conductance (521 ± 7 µS/cm); pH (8.4 ± 0.1); dissolved oxygen (7.6 ± 0.4 mg/L); temperature (24.8 ± 0.3 °C); NH_3–N (< 0.01 mg/L); dissolved organic carbon (1.7 ± 0.1 mg/L). Major ion concentrations were also consistent and generally within 10% of targets meant to simulate environmentally-relevant conditions as observed in the salinized Clinch River (Table 1).

Mean exposure concentrations for Cu and Ni approximated target values, but Zn concentrations exhibited greater week-to-week variation and were generally higher than nominal target values (Table 3). The treatments containing 1/3 TU concentrations were on average near or below chronic criteria (CCQ ~ 1 or < 1), with 1/3 TU Cu and 1/3 TU Ni treatments consistently so over the 42-day exposure duration (Table 3). The 1/3 TU Zn treatment at times exceeded chronic and acute criteria (Table 3). The lowest dose treatment, 1/9 TU EA, contained metals approximately equal to target concentrations, with Zn exhibiting the greatest variation (Table 3). All metal concentrations in the 1/9 TU EA treatment were less than their chronic criteria with CCQs of 0.21, 0.29, and 0.37 for Cu, Ni, and Zn, respectively (Table 4).

3.4. Experiment 2 survival and growth

Survival in the control was 100% on Day 42, and ≥ 93% in all metal exposure treatments, none of which were significantly different from control survival (Table 3).

Growth was reduced significantly in all metal treatments relative to the control (Table 3, Fig. S2). In the single-metal treatments of 1/3 TU Cu (Cu 8.0 ± 1.1 µg/L), 1/3 TU Ni (Ni 63.5 ± 4.8 µg/L), and 1/3 TU Zn (Zn 193 ± 31 µg/L), length increase was inhibited by 61%, 66%, and

Table 2
Survival and growth at the end of 42-day Experiment 1.

Treatment (with target concentrations; µg/L)[a]	Mean metal concentration (µg/L)[b] Cu	Ni	Zn	Survival (%)[c,d]	Initial Length (mm)[c]	Length Increase (%)[c,d,e]	Length Increase % Inhibition[e]
Control	0.9 (1.0) 0.3 - 3.7	1.7 (2.0) 0.5 - 7.7	5.1 (4.5) 0.1 - 11.7	95 (3.1)a	1.101 (0.004)	111 (33)a	
1 TU Cu (24 Cu)	23.3 (1.2)[f] 21.5 – 25.0	0.9 (0.9) 0.4 - 3.6	7.4 (9.7) 0.1 - 34.8	0c	1.112 (0.005)	–	–
1 TU Ni (180 Ni)	0.6 (0.2) 0.3 - 0.9	180 (16)[f] 154 - 202	3.7 (3.6) 0.1 – 11.0	76 (4.9)b	1.112 (0.016)	36 (8.1)b	67
1 TU Zn (450 Zn)	0.8 (0.5) 0.3 - 1.9	1.0 (0.8) 0.4 - 2.8	518 (64)[f] 380 - 607	0c	1.124 (0.022)	–	–
1 TU EA (24 Cu, 180 Ni, 450 Zn)	20.7 (3.0)[f] 16.3 - 25.6	191 (8.3)[f] 180 - 201	493 (75)[f] 335 - 587	0c	1.121 (0.012)	–	–
1/3 TU EA (8 Cu, 60 Ni, 150 Zn)	7.6 (0.5)[f] 6.7 - 8.4	63.0 (3.3)[f] 57.9 - 68.1	172 (21)[f] 140 - 206	78 (9.0)b	1.121 (0.025)	12 (3.0)b	89
Water Quality Criteria[g]							
USEPA CCC	17.0	75.4	170				
USEPA CMC	27.3	678	170				

[a] Treatment abbr. – TU: toxic unit; EA: a mixture containing each metal (Cu, Ni, Zn) at the individual TU concentrations indicated.
[b] Mean with standard deviation in parentheses (n = 8 [1 TU Cu, 1 TU Zn] or 12) and range; dissolved concentration (0.45 µm filter) measured in a 5-replicate composite sample on Days 1 and 7 during each week with surviving mussels.
[c] Mean with standard deviation in parentheses (n = 5) and range.
[d] For each endpoint, treatments with the same letter are not significantly different from one another (Tukey's HSD, $p < 0.05$).
[e] Sublethal endpoints not applicable with complete mortality.
[f] Values were manipulated; unmanipulated values are as they occurred in dilution water prepared from diluted pond water.
[g] U.S. Environmental Protection Agency water quality criteria; CCC = Criteria Continuous Concentration, CMC = Criteria Maximum Concentration. Adjusted for mean water chemistry in Experiment 1 using USEPA hardness calculator (Ni, Zn; mean hardness 155 mg/L as $CaCO_3$) and Biotic Ligand Model 2007 revision (Cu).

Table 3
Survival and growth at the end of 42-day Experiment 2.

Treatment (with target concentrations; µg/L)[a]	Metal Concentration (µg/L)[b] Cu	Ni	Zn	Survival (%)[c,d]	Initial Length (mm)[c]	Length Increase (%)[c,d]	Length Increase % Inhibition	Dry Weight Increase (%)[c,d,e]	Dry Weight Increase % Inhibition
Control	0.6 (1.2) 0.1 - 4.4	0.5 (0.2) 0.3 - 0.9	6.7 (7.8) 0.1 - 29.3	100 (0)a	1.628 (0.038)	117 (5.8)a		834 (80)a	
1/3 TU Cu (8 Cu)	8.0 (1.1)[f] 6.4 - 10.3	0.7 (0.4) 0.4 - 1.6	5.6 (4.7) 0.1 - 13.9	93 (10)a	1.597 (0.026)	46 (7.8)bc	61	225 (55)c	73
1/3 TU Ni (60 Ni)	0.5 (0.4) 0.2 - 1.5	63.5 (4.8)[f] 52.4 - 69.6	5.5 (3.5) 0.1 - 11.1	98 (2.7)a	1.617 (0.034)	40 (7.6)cd	66	216 (40)c	74
1/3 TU Zn (150 Zn)	0.5 (0.5) 0.1 - 1.7	0.6 (0.2) 0.4 – 1.0	193 (31)[f] 139 - 246	100 (0)a	1.608 (0.027)	31 (5.3)d	74	144 (11)c	83
1/3 TU EA (8 Cu, 60 Ni, 150 Zn)	7.2 (1.2)[f] 6.1 - 10.8	65.3 (6.1)[f] 50.8 - 71.3	183 (32)[f] 116 - 223	98 (2.7)a	1.620 (0.041)	6.0 (4.3)e	95	42 (15)d	95
1/9 TU EA (2.7 Cu, 20 Ni, 50 Zn)	3.4 (1.2)[f] 2.3 - 6.3	21.8 (1.8)[f] 17.6 - 24.1	62.1 (8.4)[f] 47.8 - 78.2	98 (4.4)a	1.617 (0.059)	56 (5.2)b	52	326 (77)b	61
Water Quality Criteria[g]									
USEPA CCC	15.7	75.4	170						
USEPA CMC	25.3	678	170						

[a] Treatment abbr. – TU: toxic unit; EA: a mixture containing each metal (Cu, Ni, Zn) at the individual TU concentrations indicated..
[b] Mean with standard deviation in parentheses (n = 12); dissolved concentration (0.45 µm filter) measured in a 5-replicate composite sample on Days 1 and 7 during each week with surviving mussels. Shaded values were manipulated; non-shaded values are as they occurred in dilution water prepared from diluted pond water.
[c] Mean with standard deviation in parentheses (n = 5).
[d] For each endpoint, treatments with the same letter are not significantly different from one another (Tukey's HSD, $p < 0.05$).
[e] Initial weight of random subsample of 60 mussels from test cohort on Day 0 (mean and standard deviation): 0.250 (0.036) mg.
[f] Values were manipulated; unmanipulated values are as they occurred in dilution water prepared from diluted pond water.
[g] U.S. Environmental Protection Agency water quality criteria; CCC: Criteria Continuous Concentration, CMC: Criteria Maximum Concentration. Adjusted for mean water chemistry in Experiment 2 using USEPA hardness calculator (Ni, Zn; mean hardness 155 mg/L as $CaCO_3$) and Biotic Ligand Model 2007 revision (Cu).

Table 4
Metal chronic criterion quotients in Experiment 2.

Treatment[a]	Mean metal CCQ (with CCC, µg/L)[b]		
	Cu (15.7)	Ni (75.4)	Zn (170)
Control	0.04	0.01	0.04
1/3 TU Cu	0.51[c]	0.01	0.03
1/3 TU Ni	0.03	0.84[c]	0.03
1/3 TU Zn	0.03	0.01	1.14[c]
1/3 TU EA	0.46[c]	0.87[c]	1.08[c]
1/9 TU EA	0.21[c]	0.29[c]	0.37[c]

[a] Treatment abbr.: TU = toxic unit; EA = a mixture containing each metal (Cu, Ni, Zn) at the individual TU concentrations indicated.
[b] CCQ: chronic criterion quotient is the mean measured concentration of dissolved metal divided by its chronic water quality criterion; CCQ > 1 indicates criterion exceedance. CCC: U.S. Environmental Protection Agency criteria continuous concentration. Adjusted for mean water chemistry in Experiment 2 using USEPA hardness calculator (Ni, Zn; mean hardness 155 mg/L as $CaCO_3$) and Biotic Ligand Model 2007 revision (Cu).
[c] Values were manipulated; unmanipulated values are as they occurred in dilution water prepared from diluted pond water.

74%, and dry weight increase was inhibited by 73%, 74%, and 83%, respectively. The least toxic treatment was 1/9 TU EA (Cu: 3.4 ± 1.2, Ni: 21.8 ± 1.8, and Zn: 62.1 ± 8.4 µg/L), which inhibited length increase and dry weight increase by 52% and 61%, respectively. The most-toxic treatment was 1/3 TU EA (Cu: 7.2 ± 1.2, Ni: 65.3 ± 6.1, Zn: 183 ± 32 µg/L), in which mussels grew very little, with both length increase and dry weight increase inhibited by 95%. Dry weight increase was slightly more inhibited for most treatments relative to control, with 1/3 TU Cu, 1/3 TU Ni, and 1/3 TU Zn inhibited by 73%, 74%, and 83%, respectively. The 1/9 TU EA treatment increase in dry weight was inhibited by 61% relative to control, and the 1/3 TU EA treatment gained little weight, inhibited by 95% compared with control. In addition, we observed mussel tissues in the most-toxic treatment (1/3 TU EA) appeared diminished and retracted from the shell edge relative to less-toxic treatments and the control (Fig. 1).

3.5. Experiment 2 shell deformities

We observed three types of shell deformity: (1) *splayed ventral margin*; outward curvature of shell halves, often folded back onto exterior surface, with visceral mass exposed (Fig. 2a), (2) *aperture hole*; oval gap or shell formed in the shape of a pronounced tubular structure on the posterior margin near apertures (Fig. 2b), and (3) *shell gap*; shell halves do not meet along the shell edge other than on the posterior margin near the apertures; often on the ventral margin, but occasionally anterior or posterior corners; shell edges lacking splayed outward curvature (Fig. 2c). Some individuals had more than one deformity – mussels with shell gaps tended to also have aperture holes but the opposite was not necessarily the case.

Frequencies of shell deformities were generally higher in metal treatments than in the control, but did not co-vary with toxicity (Fig. 3). Rather, deformity frequency exhibited a curvilinear relationship with toxicity. Deformity frequency was lowest when growth inhibition was low and high (i.e., control and 1/3 EA treatments) whereas deformity frequency was highest when growth inhibition was moderate (i.e., 1/3

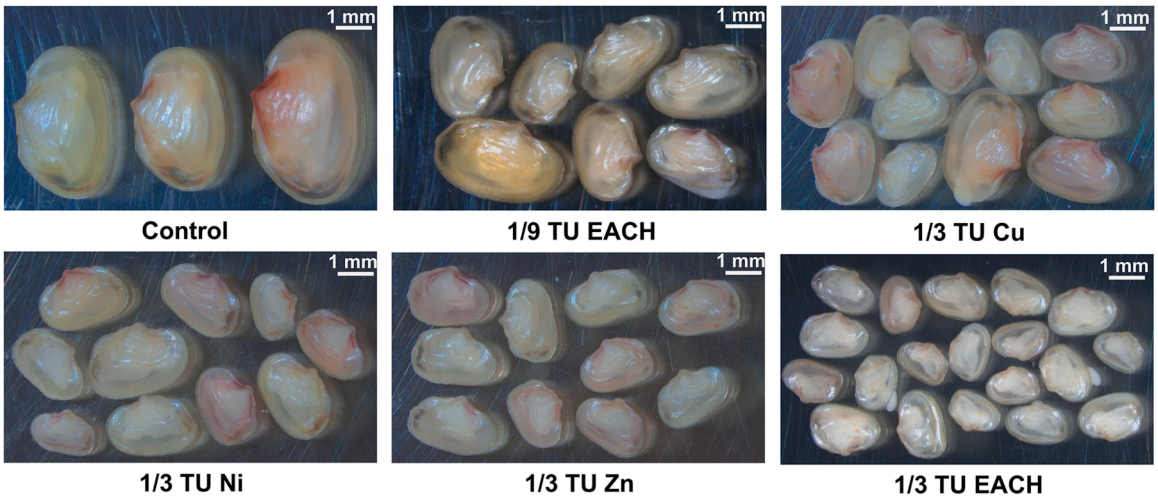

Fig. 1. Photographs of mussels from each treatment after 42 days in Experiment 2. Photos are to identical scale and arranged in order of increasing toxicity (increased growth inhibition) left to right and down. Treatment abbr. – 1/9 TU EACH: mixture of 1/9 TU (toxic unit) each of Cu, Ni, and Zn; 1/3 TU Cu, 1/3 TU Ni, 1/3 TU Zn: 1/3 TU of an individual metal as indicated; 1/3 TU EACH: mixture of 1/3 TU each of Cu, Ni, and Zn.

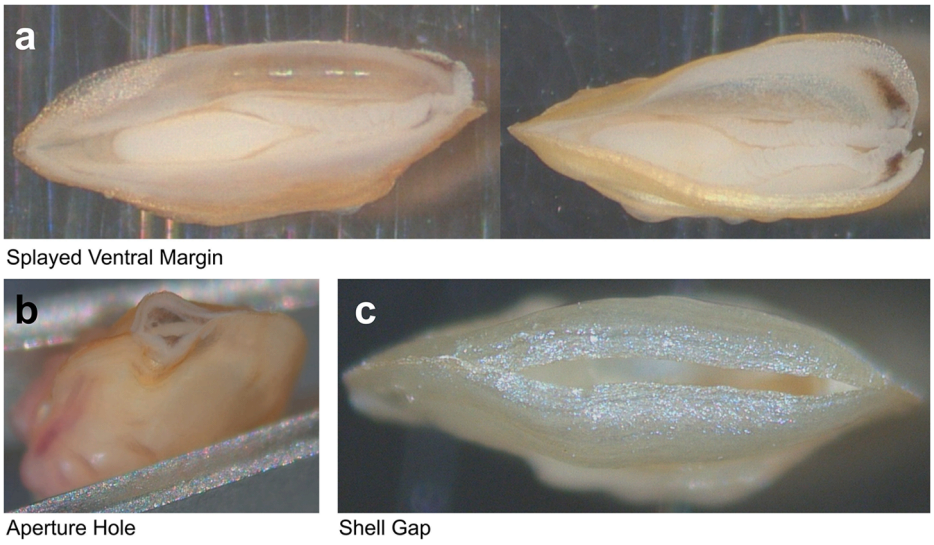

Fig. 2. Representative photos of the three types of shell deformity observed: (a) splayed ventral margin, (b) aperture hole, (c) shell gap. Photos not to scale. Taken from Experiment 2 1/9 TU (toxic unit) EACH treatment after 42 days (a mixture containing each metal – Cu, Ni, Zn – at the individual TU concentration indicated).

Ni and 1/9 EA treatments). No deformities were observed for organisms taken directly from FMCC culture at initiation of the experiment.

4. Discussion

We tested three trace metals, Cu, Ni, and Zn individually and in combination, for toxicity to juvenile mussels of a species native to the Clinch and Powell Rivers, *Villosa iris*, in two experiments. These three metals were selected for testing because of their occurrences at elevated concentrations in mining-influenced sections of Virginia's mussel-bearing Clinch and Powell Rivers, both currently and historically (Johnson et al., 2014; Price et al., 2014; Zipper et al., 2016; VDEQ/T-DEC, 2018). The three metals are also released from weathering of coal-mine spoils and have been found elevated in waters, biofilms, sediments, leaf detritus, and other environmental media of mining-influenced headwater streams of the Appalachian coalfield (Clark et al., 2018).

In Experiment 1, we expected some mortality because we selected test concentrations based on mussel studies that documented survival and/or growth effects at similar metal concentrations (Wang et al., 2010, 2011, 2020; Kunz et al., 2016), we did not anticipate complete mortality in any treatment. The 1 TU Cu and 1 TU Zn treatments resulted in greater effects (mortality) in Experiment 1 than was observed at similar concentrations in studies that informed selection of TU (Wang et al., 2010, 2011, 2020; Kunz et al., 2016). We suspect these differences were likely the result of one or more differences in experimental conditions, which include: duration, species tested, organism age, culture conditions, water source/type, major ion matrix, test chamber substrate, experimental apparatus, and feeding regime.

Therefore, with complete mortality in the 1 TU Cu, 1 TU Zn, and 1 TU EA treatments, and growth endpoints only available for the 1 TU Ni and 1/3 TU EA treatments, we could not test our hypothesis of combined effects for Experiment 1. That experiment informed our selection of lower, sublethal metal concentrations for Experiment 2. We also note that Experiment 1 results suggest survival and growth effects to juvenile *Villosa iris* are probable when acute and chronic water quality criteria, respectively, are approached and/or exceeded amid a background of elevated major ions. However, given that only results from Experiment 2

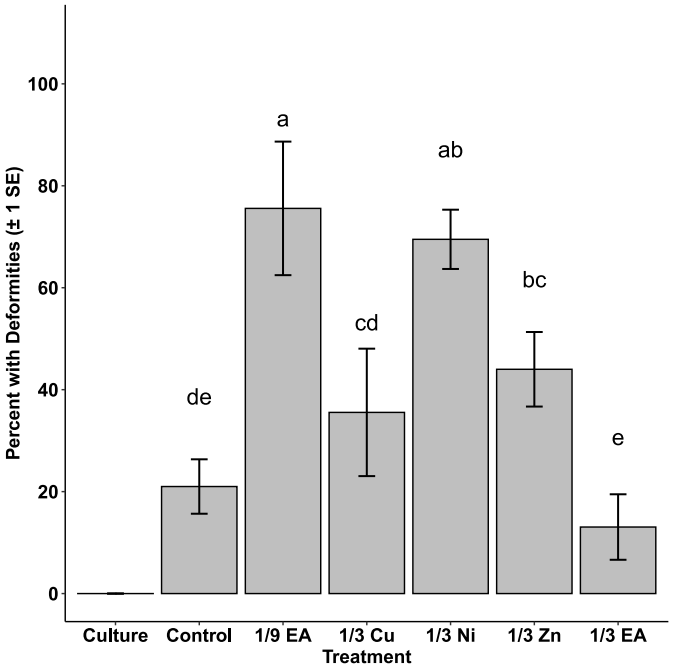

Fig. 3. Shell deformity frequency, Day 42 of Experiment 2 (mean ± 1 SE, $n \leq$ 20 surviving mussels per treatment). Treatment toxicity (growth inhibition) increases left to right. Treatment abbr. – Culture: 20 mussels examined on Day 0 from the culture cohort used for Experiment 2 (no deformities observed). 1/9 EA: mixture of 1/9 Toxic Unit (TU) each of Cu, Ni, and Zn; 1/3 Cu, 1/3 Ni, 1/3 Zn: 1/3 TU of an individual metal as indicated; 1/3 EA: mixture of 1/3 TU each of Cu, Ni, and Zn; Treatments with different letters are significantly different from one another (Tukey's HSD, $p < 0.05$).

were sufficient to address our objective, we focus discussion on findings of that experiment.

Combined toxic effects were evident in Experiment 2 as predicted, given that the 1/3 TU EA mixture treatment inhibited growth to a greater extent than did any of the single-metal 1/3 TU treatments. Those combined effects appear at least somewhat additive, which we expected given the common mode of toxic action among Cu, Ni, and Zn. This demonstrates that trace metals when applied in combinations can induce toxic effects at lower individual concentrations than when they are applied alone. We also observed substantial growth inhibition (> 50%) in treatments with individual metal concentrations below regulatory limits (1/3 TU Cu CCQ = 0.51; 1/3 TU Ni CCQ = 0.84), with one treatment being well below those limits (1/9 TU EA mean CCQ = 0.29; Table 4). This suggests that mussel toxicity may occur in environmentally-relevant contexts even when regulatory limits for single metals are not exceeded. Together, these two findings suggest that regulatory limits based on single-metal toxicity may be underprotective of freshwater mussels when multiple metals are present.

Thus, our findings suggest that regulatory limits based on single-metal toxicity may be underprotective of freshwater mussels when multiple metals are present. Although our results justify considering mixture effects when setting regulatory limits, we caution that whether developing single- or multiple-contaminant limits, inclusion of data on toxicity to sensitive fauna such as freshwater mussels is a critical step toward ensuring limits are fully protective of aquatic life. As an example, the chronic ammonia criterion was reduced by more than 50% after being updated to include substantial data on juvenile mussel toxicity (USEPA, 2013).

We observed growth impairments as early as Day 14 and throughout the 42-day exposures. Those mussels surviving the growth-impairing treatments appeared to us as unhealthy and as unlikely to survive if the experimental exposures had been continued for longer durations. For example, the mussels exposed to the 1/3 TU EACH treatment exhibit reduced growth and their soft-bodies are emaciated, indicating they are unlikely to survive for much longer (Fig. 1). Impaired growth indicates that affected animals were either not feeding or were not metabolizing food as effectively as control-group mussels. The term "ecological death" has been used to describe a condition induced by sublethal concentrations of environmental contaminants that impair organisms' fitness and behaviors essential to survival in the wild (Scott and Sloman, 2004). Although the mussels exposed to metals in Experiment 2 survived for 42 days, their smaller size and apparent reduced fitness relative to control mussels suggest ecological death would be likely with metal exposure of longer duration.

Upon microscopic examination to measure shell length we were surprised to observe a high frequency of shell deformity (e.g., Fig. 1a), although no shell deformity was evident in mussels taken directly from FMCC culture. Standard culture conditions have juvenile mussels living in a thin layer of fine sediment, not subsisting on a nylon mesh as in the test exposure. Although it is possible that the rough texture of the mesh contributed to shell deformities as mussels moved across it, we observed significantly greater deformity frequency in most metal treatments relative to controls, suggesting that metal toxicity does contribute to deformities. Notably, deformity frequency followed a non-monotonic pattern along the continuum of toxicity (Fig. 3). At lowest toxicity (controls), deformity frequency was low, presumably because metal toxicity has not altered physiology or behavior. At highest toxicity (1/3 EA), deformity frequency is also low as organisms are barely growing at all, perhaps indicating their nutritive status and physical motion has been impaired. At moderate toxicity (1/3 Ni, 1/9 EA) however, shell deformity frequency is highest, suggesting that metal toxicity may cause physiological and/or behavioral changes sufficient to cause deformation while still permitting moderate growth. The pattern of deformity response along the toxicity continuum suggests this phenomenon is worthy of further study, as shell malformation and resultant loss of shell seal may be an indirect mode of metal toxicity in juvenile mussels.

The finding that mixtures of toxicants can induce effects in excess of those induced by individual toxicants acting alone is well established in scientific literature. Bliss (1939) proposed that types of "joint action" by toxicants could be classified based on the nature of the combined effects, while citing still-older studies that demonstrated such. Norwood et al. (2003) reviewed 210 tests of metal-mixture effects on aquatic organisms and found the majority demonstrated combined effects exceeding those predicted based on the single-metal exposures. It is also known that combined effects by multiple potential toxicants, with each applied at concentrations or dosages below the minimum known to induce a measurable effect when acting independently, can induce toxicity, though this is not always necessarily the case. For example, Walter et al. (2002) found that mixtures of common pesticides, all applied at No Observed Effect Concentrations (NOECs), caused toxicity due to their combined effects. Working with the freshwater mussel *Anodonta imbecilis,* Keller and Zam (1991) found that two-metal mixtures including Cu, Ni, and/or Zn were generally more toxic, as determined by 24- and 48 h median lethal concentrations (LC50s), than single-metal exposures; but two-metal mixtures including Cd tended to be less toxic by that measure. Gillis et al. (2021) observed greater sensitivity of freshwater mussel *Lampsilis fasciola* glochidia (larvae) to chloride when a complex mixture of metals was included in the exposure. Both Appalachian coal-mine spoil leachates and mining-influenced sections of the Clinch and Powell Rivers are known to be elevated in multiple constituents, including Al, As, Cd, Mn, and/or Se as well as the three metals studied here (Zipper et al., 2016; Clark et al., 2018; VDEQ/TDEC, 2018). Such findings suggest a need for additional research concerning effects by mixtures including still-larger suites of elements but at lower-concentration exposures, such as occurs in the wild.

Mussels in rivers influenced by humans can be and often are exposed to multiple water contaminants simultaneously. Anthropogenic elevated salinity in freshwaters, for example, often occurs in association with

elevated trace metals (Velasco et al., 2019); that is also the case in the Clinch and Powell Rivers (Johnson et al., 2014; Zipper et al., 2016). Here, we applied our test concentrations in association with elevated salinity characteristic of the Clinch River in an effort to simulate environmental conditions, but the role played by that elevated salinity as a potential stressor in combination with elevated trace metals is not clear. Elevated salinity characteristic of the Powell River (and approximately double the level of salinity in our experiments) was found to induce physiological stress to the freshwater mussel *Lampsilis fasciola*, albeit with no detectable survival or growth impairments (Ciparis et al., 2019), suggesting the possibility that elevated salinity may be inducing stress in addition to that induced by the elevated trace metals. The predominant cations in both rivers and in the test waters were Ca and Mg, which provide hardness ameliorative of toxicity from metals such as Cu, Ni, and Zn both generally (Paquin et al., 2002; Borgmann et al., 2005) and specifically for freshwater mussels (Gillis et al., 2008). The presence of DOC can also mitigate toxicity of certain trace metals, as has been demonstrated clearly for Cu (Gillis et al., 2008; Wang et al., 2011). Although DOC and major ions could influence toxicity of the metals studied here, those factors do not influence our conclusions because our conclusions are based on comparisons among treatments with the same DOC and major ion matrix, both dictated by dilution water used for all treatments. Discussion of DOC effects on metal toxicity generally are beyond the scope of our study, but we would expect any influence DOC might have on effect concentrations of a single metal should apply equally when comparing those effects to effects of a mixture of contaminants containing that metal, thus removing DOC from the calculus of single vs. combined effects, all else equal.

Exposure concentrations of dissolved Cu, Ni, and Zn exceeded those known to occur in the two rivers' water columns, but were comparable to concentrations observed in substratum interstitial water. Water-column dissolved concentrations were predominantly < 1 μg/L for all three metals in the Clinch River (VDEQ/TDEC, 2018), and predominantly < 2 μg/L, < 2 μg/L, and < 12 μg/L for the three metals, respectively in the Powell River (Phipps, 2019; Timpano and Jones, 2021). In the Powell River interstitial-water dissolved concentrations can be considerably higher, as upper-percentile (but not outlier) values ranged up to 10 μg/L, ~2.5 μg/L, and 10–100 μg/L for Cu, Ni, and Zn, respectively; total (unfiltered) concentrations can be higher still (Phipps, 2019; Timpano and Jones, 2020). Hence, the dissolved Cu test concentrations for 1/3 and 1/9 TU were realistic relative to what mussels are exposed to in interstitial waters of the Powell River, while the Ni and Zn test concentrations exceeded those levels somewhat. Water-column dissolved concentrations comparable to our 1/9 EA test concentrations for Cu and Ni have been documented in another of Appalachia's coal-mining-influenced rivers (Lindberg et al., 2011).

Our experiments did not simulate the full complement of trace-element exposure modes and pathways that mussels may experience in the wild. As we sought only to test the combined-effects hypothesis as a possible toxicity mechanism for dissolved water-column metal-mixture exposures, we necessarily have not tested for effects from metalloids such as selenium known to occur in rivers draining surface coal mines, nor for effects of trophic-particulate exposures. Given that water-column dissolved trace elements (metals and metalloids such as selenium) are taken up by phytoplankton, bacteria, and benthic algae, it is reasonable to expect that trace-element concentrations are likely elevated in the food particles mussels ingest relative to the commercial algae mixture fed to mussels in our experiments. As a direct result of such processes, a primary mode of trace-element exposure to upper trophic-level organisms in natural environments is commonly dietary (Mason et al., 2013). Biological uptake of dissolved trace elements from water can in some cases expose consumer organisms to dietary ecotoxicity even when the water concentrations themselves are not overtly harmful (Deforest and Meyer, 2015). Similarly, filter-feeding mussels are exposed to solid-phase trace elements in particulate form when such are carried by the water column, while mussels occupying interstitial spaces and deposit-feeding are exposed to such within the sediments. While the extent to which such exposures result in uptake by freshwater mussels is not known, a freshwater bivalve with similar physiology and trophic mechanism, *Corbicula fluminea*, has been shown to metabolize particle-bound metals when ingested (Cd, Cr, and Zn; Lee and Lee, 2005). Furthermore, ingestion of metal-oxide nanoparticles by aquatic organisms including mussels can result in uptake and incorporation of those metals into tissue and body fluids through mechanisms that include dissolution within the animals (Gagné et al., 2013; Made et al., 2017). However, it is not clear the extents to which mussels ingest non-organic particles bearing trace-elements, nor is it evident how physiological processes that affect nanoparticles might also affect larger particles if ingested by freshwater mussels.

Another aspect of environmental realism not replicated by our experiment was that additional water contaminants can co-occur with salinity and multiple trace elements. For example, polycyclic aromatic hydrocarbons (PAHs) have been detected in the water column of the Clinch River (Cope et al., 2021) and in the water column and interstitial waters of the Powell River (Phipps, 2019). Research demonstrates that the combined exposure of aquatic animals to metals and PAHs increases ecological risk above levels caused by individual exposure to such contaminants (Gauthier et al., 2014). Nonetheless, our finding of combined toxic effects of metal mixtures suggests that the presence of additional contaminants such as PAHs would be expected to further increase toxicity of the resultant mixture, underscoring the importance of considering multiple stressors when evaluating toxicity potential.

In the USA and elsewhere, current water quality criteria are developed and applied on a single-constituent basis, yet trace-element pollution sources to freshwaters commonly release multiple trace elements (Vareda et al., 2019). Similarly, organisms residing in trace-element-impacted freshwaters are often exposed simultaneously to other co-occurring pollutants (Gauthier et al., 2014; Velasco et al., 2019). The limitations of single-constituent regulatory approaches for the protection of aquatic life in multi-stressor aquatic environments has been known for many years (Spehar and Fiandt, 1986), yet such approaches persist. Thus, adoption of multi-stressor regulatory frameworks would be especially beneficial for high-biodiversity aquatic resources that are at risk of decline.

CRediT authorship contribution statement

Anthony J. Timpano: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Visualization. **Jess W. Jones:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition. **Braven Beaty:** Conceptualization, Writing – review & editing, Funding acquisition. **Matthew Hull:** Conceptualization, Writing – review & editing. **David J. Soucek:** Conceptualization, Methodology, Writing – review & editing. **Carl E. Zipper:** Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2021.106038.

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