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Effects of environmentally relevant mixtures of major ions on a freshwater mussel

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ABSTRACT

The Clinch and Powell Rivers (Virginia, USA) support diverse mussel assemblages. Extensive coal mining occurs in both watersheds. In large reaches of both rivers, major ion concentrations are elevated and mussels have been extirpated or are declining. We conducted a laboratory study to assess major ion effects on growth and survival of juvenile *Villosa iris*. Mussels were exposed to pond water and diluted pond water with environmentally relevant major ion mixtures for 55 days. Two treatments were tested to mimic low-flow concentrations of Ca^{2+}, Mg^{2+}, SO_4^{2-}, HCO_3^{+}, K^{+} and Cl^{-} in the Clinch and Powell Rivers, total ion concentrations of 419 mg/L and 942 mg/L, respectively. Mussel survival (>90%) and growth in the two treatments showed little variation, and were not significantly different than in diluted pond water (control). Results suggest that major ion chronic toxicity is not the primary cause for mussel declines in the Clinch and Powell Rivers.

1. Introduction

Major ions of geologic origin are fundamental components of freshwater ecosystems. However, anthropogenic activities are causing major ion concentrations to increase in freshwaters worldwide (Williams, 2001). Toxic effects of elevated major ion concentrations to freshwater species have been observed in laboratory studies, and elevated major ions in natural waters have been correlated with aquatic species losses (Canedo-Arguelles et al., 2013; Kefford et al., 2004). Elevated major ion concentrations in freshwaters can induce osmotic stress in freshwater organisms (McCulloch et al., 1993). The toxicity of major ions to freshwater organisms depends upon both the concentration and the ionic composition (Goodfellow et al., 2000; Mount et al., 1997; Soucek and Kennedy, 2005).

Mining is a common cause for freshwater salinization (Canedo-Arguelles et al., 2013; Hancock et al., 2005; Pond et al., 2008; Schreck, 1995), defined as an increase in the total concentration of dissolved inorganic ions in water and estimated by measuring total dissolved solids (TDS) as an evaporative residue (Hem, 1985). Mining operations move unweathered geologic materials into the ambient environment, where exposure to O_2 and H_2O enables accelerated weathering and major ion release (Orndorff et al., 2015). Mining-origin elevated stream salinity and associated alterations of aquatic communities have been documented throughout Appalachian USA (Bernhardt et al., 2012; Cormier et al., 2013; Evans et al., 2014; Griffith et al., 2012; Hitt and Chambers, 2014; Pond et al., 2008; Timpano et al., 2015). The dominant major ions in mining-influenced Appalachian waters are generally SO_4^{2-}, HCO_3^{-}, Ca^{2+}, and Mg^{2+}; but K^{+}, Na^{+}, and Cl^{-} can also become elevated (Cormier et al., 2013; Pond et al., 2008; Timpano et al., 2015).

Increasing salinization of Appalachian streams and rivers is of concern because they often support exceptionally high biodiversity. The Clinch and Powell Rivers occur within the Upper Tennessee River system in eastern USA (Fig. 1); both originate in Virginia and flow into northeastern Tennessee. Both river mainstems are established refugia for diverse biotic assemblages, as they remain free-flowing throughout Virginia and their watersheds have low human population densities relative to most other eastern USA areas. The Clinch and Powell Rivers support 49 extant freshwater mussel species, 21 of which are federally endangered (Johnson

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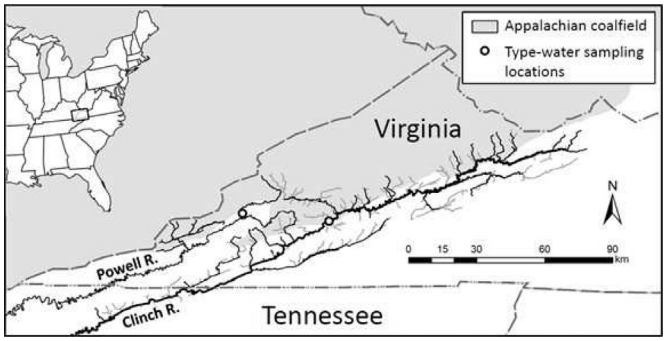

Fig. 1. Locations of the Clinch and Powell Rivers in Virginia relative to the Appalachian coalfield (gray shading). Samples collected from the type-water sampling locations (open circles), Big Stone Gap on the Powell River and Dungannon on the Clinch River, were used to prepare treatments simulating ion concentrations in each river.

et al., 2012; Jones et al., 2014). However, surface coal mining occurs within upper portions of the Powell River watershed and in watersheds of the Clinch River's northwestern tributaries (Fig. 1). Long-term water quality monitoring of the Clinch and Powell Rivers has documented increasing temporal trends of TDS concentrations since the 1960s (Price et al., 2011, 2014). Independent biological monitoring, initiated in 1979, has documented significant declines of freshwater mussel richness and density in sections of Clinch River and throughout the Powell River (Ahlstedt et al., 2005; Johnson et al., 2012; Jones et al., 2014). Recent research has found a spatial association between elevated TDS concentrations and declining freshwater mussel populations in the Clinch River; reaches of the river experiencing the greatest declines in mussel richness and density have elevated concentrations of major ions relative to other reaches of the river where mussel populations are stable (Johnson et al., 2014). In upper segments of the Powell River, TDS concentrations are greater than at any location in the Clinch River (Price et al., 2011) and severe declines of mussel richness and density have been observed in surveyed reaches of the Powell River located downstream of the high TDS reaches (Ahlstedt et al., 2005; Johnson et al., 2012).

This study addresses a fundamental question concerning potential influence by anthropogenic activities on Clinch and Powell River biota: Are major ions acting as toxicants to native mussels? We conducted a laboratory study to assess effects of major ion combinations and concentrations characteristic of the Clinch and Powell Rivers on survival and growth of juveniles of a native mussel species.

2. Materials and methods

2.1. Juvenile mussel production

Juvenile mussels were produced at the Freshwater Mollusk Conservation Center (FMCC), Virginia Tech, Blacksburg, VA following standard propagation procedures (Carey et al., 2013; Zale and Neves, 1982). Gravid female rainbow mussels (*Villosa iris*) were collected from Copper Creek, Scott County, VA on 14 May 2014. Host fish (*Ambloplites rupestris*) were collected from Sinking and Toms Creeks, Montgomery County, VA in early May 2014. Fish were infested with mussel glochidia on 17 May and held in recirculating aquaculture systems at 22 °C. Juveniles excysted from fish hosts 2–3 weeks post-infestation and were cultured in 18 L glass aquariums at 24 °C for approximately 3.5 months prior to study initiation. Juvenile culture methods are detailed in Carey et al. (2013). The water used to hold infested fish and culture juvenile mussels was obtained from a man-made pond at the FMCC which has an on-site well as its source water. Chemical characterization of the pond water was conducted upon initiation of this study (Supplemental Information, Table S-1).

2.2. Test concentrations

The Clinch and Powell treatments were based on data obtained from the Virginia Department of Environmental Quality (DEQ). The highest recorded TDS concentrations (Storet 70300) for impacted sections of the two rivers were 854 mg/L (23 August 2008, at station 6BPOW179.20, Big Stone Gap) for the Powell, and 338 mg/L (5 December 2000, at station 6BCLN237.09, Dungannon) for the Clinch (Fig. 1). The measured TDS values were scaled up to 942 and 419 mg/L (major ion concentration sums), respectively, considering measured alkalinity values as indicators of bicarbonate concentrations, and assuming a 40% volatilization loss of bicarbonate during the evaporative TDS measurement (Howard, 1933). Analyses of other Clinch and Powell River data collected by DEQ, where all major ions and TDS were measured, confirms the ~40% adjustment as appropriate. These data were used to create regressions between TDS and concentrations of individual ions for preparation of treatment waters.

2.3. Treatment preparation

Pond water from the FMCC pond was filtered through a 5 μm polypropylene microfiber filter (Vortex Filter, Filter Specialists, Inc., Michigan City, IN). Undiluted filtered pond water (Pond) and a 50:50 mixture of filtered pond water: deionized water (1/2 Pond) were used as controls and as base waters to prepare treatments. Two different base waters were used to make the two TDS treatments because background ion concentrations in Pond water were too high for synthesis of simulated Clinch River water. The Clinch treatment was 1/2 Pond water with major ions added, and the Powell treatment was Pond water with major ions added. Nominal ion concentrations (Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, SO_4^{2-}, and HCO_3^-) for the Clinch and Powell treatments (Table 1) were based on the regressions of measured TDS concentrations and individual ion concentrations obtained from DEQ data. Recipes for the Clinch and Powell treatments included ion concentrations measured in their respective base (control) waters. Controls and treatments were prepared weekly. Treatments were prepared from base waters using certified American Chemical Society (ACS) reagent grade salts. Potassium chloride (KCl), potassium bicarbonate ($KHCO_3$), sodium bicarbonate ($NaHCO_3$), magnesium sulfate heptahydrate ($MgSO_4{}^*7H_20$), calcium chloride dihydrate ($CaCl_2{}^*2H_2O$), and sodium sulfate (Na_2SO_4) were purchased from Fisher Chemical (Fair Lawn, NJ). Calcium sulfate ($CaSO_4$) was purchased from Sigma–Aldrich (St. Louis, MO). Ion concentrations in the base waters were verified prior to preparation of treatment waters. A rain event during week 2 of the exposure required adjustment of the recipes to elevate HCO_3^-. Otherwise, ion concentrations in the base waters (and resulting recipes) remained constant. Salts were mixed into 90 L of base water in a 150 L vat with a conical bottom and held for 24 h prior to water exchanges. Each control water (90 L) also was held for 24 h. All waters in the vats were aerated and heated to the target exposure temperature (25 °C).

2.4. Mussel exposure system

Mussels were held in 18 L downweller-bucket systems (Barnhart, 2006); photographs are available in Carey et al. (2013). Each bucket was an experimental unit and held six chambers containing juvenile mussels. Chambers were constructed from

Table 1
Ions as nominal concentrations, mean measured concentrations (standard deviation in parentheses), and % deviation from nominal concentrations (minimum and maximum deviation in parentheses). Total dissolved solids (TDS) is the sum of all ion concentrations.

Ions (mg/L)	1/2 Pond control			Clinch treatment			Pond control			Powell treatment		
	Nominal	Mean[a]	% Dev.	Nominal	Mean[a]	% Dev.	Nominal	Mean[a]	% Dev.	Nominal	Mean[a]	% Dev.
Ca^{2+}	15.6	16.7 (1.5)	106 (87–120)	50.0	49.6 (2.1)	99 (94–106)	31.3	33.4 (3.1)	106 (88–122)	86.0	78.0 (6.7)	90 (73–99)
K^{+}	1.15	1.35 (0.21)	116 (78–155)	3.00	3.17 (0.24)	105 (92–122)	2.30	2.40 (0.30)	104 (76–127)	6.00	6.10 (0.27)	102 (92–108)
Mg^{+}	15.7	14.8 (1.3)	94 (77–104)	21.0	19.7 (1.3)	94 (80–105)	31.3	29.4 (2.4)	94 (77–102)	49.0	45.2 (2.4)	92 (80–98)
Na^{+}	2.70	3.03 (0.78)	109 (74–177)	33.0	40.0 (2.3)	121 (114–38)	5.40	5.22 (0.91)	95 (72–131)	114	122 (6)	107 (98–119)
SO_4^{2-}	7.75	8.19 (0.79)	105 (86–124)	118	118 (5)	100 (94–106)	15.5	16.0 (1.3)	103 (88–112)	452	434 (32)	96 (79–104)
Cl^{-}	5.10	6.02 (1.9)	113 (68–212)	15.0	16.5 (3.3)	109 (91–177)	10.2	11.5 (2.2)	111 (81–162)	19.0	16.1 (2.1)	84 (73–109)
HCO_3^{-}	110	122 (15)	110 (84–129)	184	194 (10)	105 (98–113)	220	239 (29)	108 (86–132)	229	242 (16)	106 (96–119)
TDS	**158**	**172**	**108**	**424**	**441**	**104**	**316**	**337**	**106**	**955**	**944**	**99**

% deviation calculated as [geometric mean concentration/nominal]*100 with a range in parentheses of [minimum concentration/nominal]*100 – [maximum concentration/nominal]*100.
[a] Mean is the arithmetic mean.

3.81 cm diameter PVC pipe; 10.16 cm long and capped with 500 micron mesh to allow for water and food circulation. Four buckets were placed into a 757 L container filled with water to serve as a temperature control bath (water bath). Temperature (target = 25 °C) was maintained in the water baths using aquarium heaters. This exposure temperature was based on optimal survival and growth conditions determined by Carey et al. (2013). Five water baths were used, each contained a replicate (bucket) of ½ Pond control, Pond control, Clinch treatment, and Powell treatment randomly arranged (n = 5 for all controls and treatments). A blocked design was used to account for any temperature differences between water baths. A total of 1200 juvenile mussels were randomly selected from the original cohort. Sixty mussels were allocated to each bucket (10 individuals per chamber), and the length of each mussel was recorded using a dissecting microscope and ocular micrometer (accuracy 0.1 mm). The average length of mussels was 3.3 mm on day 0. Mussels were fed daily with a 1:1 algal cell ratio from two premixed commercial micro-algae diets (Nanno 3600 and Shellfish Diet 1800, Reed Mariculture, Campbell, California). Initial algal concentrations were based on Carey et al. (2013), but algal concentrations were reduced to a concentration of 95,000 cells per bucket after the first week of exposure due to elevated ammonia levels. Algal solutions were distributed to each bucket over 24 h using a drip bottle.

For each bucket, a 100% water exchange occurred weekly. Temperature (°C), specific conductance (μS/cm), dissolved oxygen (DO; mg/L and % saturation) and pH were measured just prior to and 24 h after water exchanges using a YSI 556 Multi-Probe Sensor (YSI Inc., Yellow Springs, OH). Concentrations of NH_3–N, alkalinity, Cl^{-}, and elements (Na, K, Mg, Ca, and S) were measured by filtering (0.45 μm) 25 ml from each replicate bucket and combining aliquots into a pooled sample for each control/treatment. Ammonia (NH_3–N) was measured weekly just prior to water exchanges using a HACH DR/2400 spectrophotometer following the manufacturer's methods. Chloride, alkalinity, and element concentrations were measured just prior to and 24 h after water exchanges. Chloride was measured using an Orion Star Plus Meter (Thermo Scientific, Beverly, MA) with a Cl^{-} Ion Selective Electrode (Cole-Parmer, Vernon Hills, IL) following the manufacturer's instructions. Total alkalinity (mg/L $CaCO_3$) was measured using a standard titration method and converted to HCO_3^{-} using the equation mg/L HCO_3^{-} = mg/L $CaCO_3$*1.22. Measurement of elements in solution was performed by the Virginia Tech Soil Testing Laboratory using Inductively Coupled Plasma Atomic Emission Spectrometry (Spectro ARCOS ICP, Spectro Analytical Instrumentation, Kleve, Germany) following standard methods (USEPA Method 200.7 and APHA Method 3120). The laboratory's standard operating procedure and quality assurance/quality control (QA/QC) procedures include single element calibration standards and an internal standard (5 ppm Yttrium) for quantification and multi-element QC check solutions. The QC check solutions were run at initiation of analysis and at 10-sample intervals thereafter (acceptance criteria 90–110% recovery). Calibration standards and QC check solutions were purchased from SPEX CertiPreps (Metuchen, NJ) and Environmental Express (Mt. Pleasant, SC). Sulfate concentration was calculated from measured total S; all S was assumed to be present as SO_4^{2-} because of the buckets' oxygenated environment.

Juvenile mussels were exposed for 55 days. Mussels in half of the chambers in each bucket ($n = 3$) were measured every two weeks. Remaining chambers ($n = 3$) were not sampled throughout the duration of the study to assess any handling effects on mussel survival and growth. Mussels in all chambers were measured at the final sampling event. Mussels were not marked; the average mussel length per chamber was used to calculate growth between sampling events. Means for each replicate (bucket) were used in statistical analyses.

2.5. Data analysis

Statistical tests were conducted using SAS software (SAS 9.3, SAS Institute, Inc., Cary, NC) with a significance level of $\alpha = 0.05$. The probability of survival was compared between treatments and controls using interval-censored survival analysis (Proc LIFEREG). Overall survival was compared between treatments and controls and between chambers sampled at 2-week intervals and those sampled on day 55 only using a mixed model with number of surviving mussels out of total mussels in the chamber as a binomial response (Proc GLIMMIX). The model had two predictors, treatment and treatment*sampling with one random factor, water bath (block). Mussel lengths and growth rates were compared between treatments and controls at each sampling interval using a mixed model with a normal distribution (Proc GLIMMIX). The model had two predictors, treatment and treatment*day, and two random variables, water bath (block) and day (repeated). Total mussel growth during the 55-day exposure was compared between treatments and controls and between chambers sampled at 2-week intervals and those sampled on day 55 only using a linear mixed model with a normal distribution (Proc GLIMMIX). The model had two predictors, treatment and treatment*sampling, with one random factor, water bath (block). For all GLIMMIX models, significant terms were further investigated using a Tukey post-hoc test, with p-values adjusted for multiple comparisons. Prior to inclusion in GLIMMIX models, mussel lengths, growth rates, and growth totals were tested for fit to the normal distribution using the Shapiro–Wilk W test ($p > 0.05$ for all).

3. Results

Mean measured ion concentrations and TDS concentrations, as the sum of measured ions, were similar to nominal concentrations (Table 1). Geometric mean concentrations of measured ions were generally within 10% of nominal concentrations and geometric mean TDS concentrations were within 10% of nominal values (Table 1). The greatest variation in measured concentrations occurred for Cl^- across all treatments. Some variation was expected due to the use of natural water as the base water for each treatment.

Water quality measurements were generally within acceptable ranges for toxicity tests with freshwater mussels. Mean calculated hardness (mg/L $CaCO_3$) was 103 (range 84–114) in the ½ Pond control, 204 (range 167–226) in the Pond control, 205 (range 187–223) in the Clinch treatment and 381 (range 318–410) in the Powell treatment. Mean minimum DO saturation was 88% during the first week of exposure and increased to 91% during remaining weeks after the feeding rate was adjusted. The lowest recorded DO saturation during week 1 was 78% in a Pond control bucket and the lowest recorded saturation during the remaining weeks was 88% in a Powell treatment bucket (week 3). Mean measured temperatures were similar between treatments (Table 2). Mean water bath temperatures ranged from 22.5 °C to 25.0 °C, but the blocked design (one replicate of each treatment/control within each water bath) accounted for this variation. Specific conductance was similar within treatments over the course of the study (Table 2). Measured pH was also generally stable over the course of the study, but pH was elevated in the Pond control and Powell treatment relative to the ½ Pond control and Clinch treatment (Table 2). Ammonia levels were elevated during the first week of exposure in all controls and treatments (Table 2). However, once the feeding rate was adjusted, ammonia levels decreased and remained low for the duration of the study (Table 2).

Survival in all treatments and controls was ≥97% during the first two weeks of the study (Fig. 2). However, survival in the Pond control decreased dramatically between days 14–28 (Fig. 2). The overall probability of survival was significantly lower in the Pond control compared to all other treatments (LIFEREG, Chi-Square, $p < 0.0001$). The probability of survival was similar (LIFEREG, Chi-Square, $p \geq 0.748$) in the ½ Pond control, Clinch treatment, and Powell treatment (Fig. 2). At the end of the study, mean survival was >90% and was not significantly different (GLIMMIX, $p > 0.887$) between the ½ Pond control, Clinch treatment, and Powell treatment (Figs. 2 and 3). Mean 55-day survival in the Pond control was <20% (Figs. 2 and 3), significantly lower than the ½ Pond control and the two treatments (GLIMMIX, $p < 0.0001$). Within all controls and treatments, mean 55-day survival was similar between chambers that were sampled at 2-week intervals and chambers that were only sampled on day 55 (week 8; GLIMMIX, $p = 0.599$; Fig. 3).

Mussel lengths and growth rates were compared between the ½ Pond control, Clinch treatment, and Powell treatment for chambers sampled at 2-week intervals. The Pond control was excluded from these analyses due to the low survival after week 2. Mussel lengths were not significantly different between the ½ Pond control, Clinch treatment, and Powell treatment at any sampling point (GLIMMIX, $p \geq 0.301$; Fig. 4).

On day 14 (week 2), the growth of mussels in several chambers from each of these treatments and control was lower than measurement error, as evidenced by many negative values for growth across all controls and treatments. Therefore, growth rate was only compared for the subsequent three sampling intervals; days 14–28 (weeks 2–4), days 28–42 (weeks 4–6), and days 42–55 (weeks 6–8), when values were all positive. Within each sampling interval, there was no significant difference in growth rates between the ½ Pond control, Clinch treatment, and Powell treatment (GLIMMIX, $p \geq 0.133$; Fig. 5). Growth rates during each interval were compared within treatments/control. In the ½ Pond control, growth rates were significantly lower (GLIMMIX, $p < 0.006$) in the second and third intervals compared to the first (Fig. 5). In the Clinch treatment, growth rates in the third sampling interval were significantly lower than in the first (GLIMMIX, $p = 0.004$; Fig. 5). In the Powell treatment, there were no significant differences in growth rates between sampling intervals (GLIMMIX, $p \geq 0.295$; Fig. 5).

Total mussel growth during the 55-day exposure was compared between the ½ Pond control, Clinch treatment, and Powell treatment for chambers sampled at 2-week intervals and chambers sampled only on day 55. Within each treatment, there was no difference in mussel growth between chambers sampled at 2-week intervals and chambers sampled only on day 55 (GLIMMIX, $p = 0.502$; Fig. 6). There was no statistically significant difference in overall mussel growth between treatments ($p = 0.076$).

4. Discussion

Prior research has found spatial and temporal associations of elevated TDS concentrations with freshwater mussel declines in the Clinch River in Virginia (Johnson et al., 2014; Price et al., 2014), suggesting that elevated major ions may be causing toxicity in the Clinch River. This idea also applies to the Powell River, where TDS concentrations are substantially higher in the upper reaches of the river and mussel populations have experienced long-term declines at monitoring locations downstream of these high TDS reaches (Ahlstedt et al., 2005; Johnson et al., 2012). However, our findings do not support the suggestion that chronic toxicity of major ions is the primary cause of declining mussel populations in these rivers.

Freshwater mussel sensitivity to major ions has been most studied in laboratory settings with NaCl in reconstituted waters. The total ion concentrations in the treatment waters of the current study (441 and 944 mg/L) were within the range of NaCl concentrations causing acute toxicity to mussel glochidia (24 h NaCl LC_{50}s for eight species: 216–3310 mg/L; Bringolf et al., 2007; Gillis, 2011; Valenti, 2004), but were lower than concentrations causing acute toxicity to juvenile mussels (96 h NaCl LC_{50}s for four species: 1660–5230 mg/L; Bringolf et al., 2007; Pandolfo et al., 2012). However, our results are not directly comparable with the acute

Table 2
Mean concentrations of water quality parameters, with standard deviation in parentheses, for each treatment/control (n = 5 replicates) measured one day after addition and prior to each water change, with the exception of ammonia which was only measured prior to each water change.

Parameters	1/2 Pond control	Clinch treatment	Pond control	Powell treatment
Temperature (°C)	23.9 (1.7)	23.8 (1.7)	24.0 (1.6)	23.9 (1.6)
Specific conductivity (μS/cm)	220 (20)	570 (10)	400 (30)	1190 (60)
pH	8.42 (0.25)	8.74 (0.19)	8.82 (0.25)	8.84 (0.19)
NH_3–N (mg/L; Day 7)[a]	0.64 (0.39)	0.71 (0.29)	0.50 (0.29)	0.77 (0.25)
NH_3–N (mg/L; Day 14–49)	0.06 (0.04)	0.04 (0.02)	0.06 (0.04)	0.08 (0.05)

[a] The feeding rate was adjusted after Day 7 due to elevated ammonia concentrations.

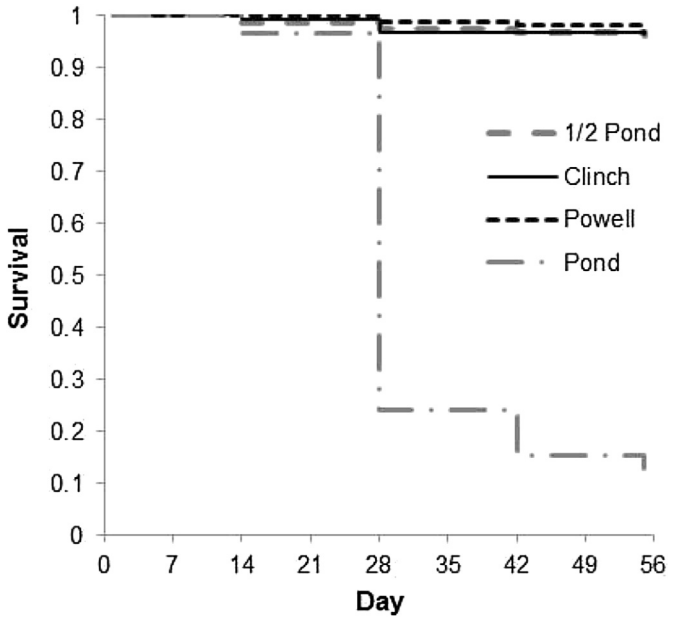

Fig. 2. Probability of mussel survival in each treatment (n = 5 replicates each) at each 14-day sampling interval. Survival in the pond water control was significantly lower than in all other treatments ($p < 0.0001$). Survival was not significantly different between the ½ Pond control, Clinch treatment, and Powell treatment ($p \geq 0.748$).

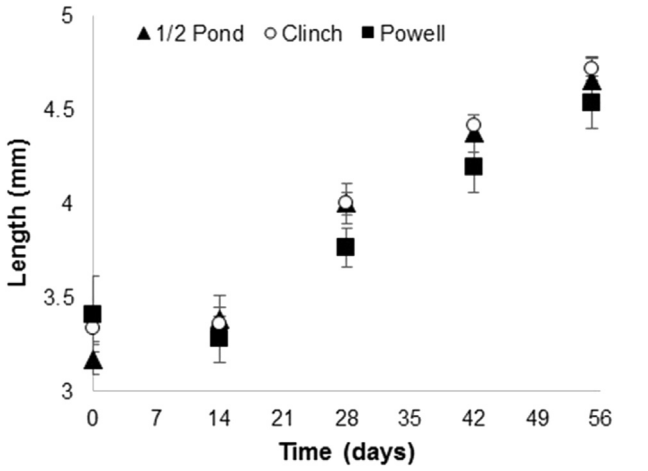

Fig. 4. Mean length of mussels sampled at 2-week intervals in the three treatments (n = 5 replicates each) with high survival. Error bars represent standard error of the mean. Mussel lengths were not significantly different between treatments at any sampling point ($p \geq 0.301$).

toxicity of NaCl, as previous studies have demonstrated that major ion toxicity to freshwater organisms is dependent on ionic composition as well as concentration (e.g. Mount et al., 1997). Treatments in the current study were designed to reflect ion mixtures in the Clinch and Powell Rivers, where predominant ions are SO_4^{2-}, HCO_3^-, Ca^{2+}, and Mg^{2+}, not Na^+ and Cl^-.

Another recent study examined effects of reconstituted waters representative of Appalachian streams affected by coal mining on juvenile *Lampsilis siliquoidea* (Kunz et al., 2013). After 28-days, Kunz et al. (2013) observed significant effects on survival and growth of *L. siliquoidea* in treatments representing 10% and 33% dilutions of stream water. In the Kunz et al. (2013) study, concentrations of SO_4^{2-}, Ca^{2+}, Mg^{2+}, K^+, Na^+, and Cl^- in the 10% stream water dilutions were similar to Clinch and Powell treatment concentrations in the current study, and effects were observed at 504 and 565 μS cm^{-1}, conductivities similar to our Clinch treatment (570 μS cm^{-1}) and lower than our Powell treatment (1190 μS cm^{-1}). Thus, there were similar ion concentrations in the two studies but vastly different responses observed between the two studied species of freshwater mussels. Hardness has been demonstrated to have an ameliorating effect on ion toxicity to freshwater mussels (Gillis, 2011). However, hardness was similar in the current study and Kunz et al. (2013); both studies were conducted in hard water. Hardness is a measure of multiple polyvalent cations, including Ca^{2+} and Mg^{2+}. Incrementally increasing the molar ratios of Ca:Mg from 0.7 to 7.0 reduced the toxicity of Na_2SO_4 to *Daphnia magna* and *Hyalella azteca* (Davies and Hall, 2007). In the current study, mean molar Ca:Mg was 1.5 in the Clinch treatment and 1.1 in the Powell treatment. In comparison, in the 10% dilutions used in Kunz et al. (2013), molar Ca:Mg was lower (0.7–0.8) in two treatments, but similar in the third (1.5). Thus, molar Ca:Mg alone is likely not the primary cause of differences between the two studies.

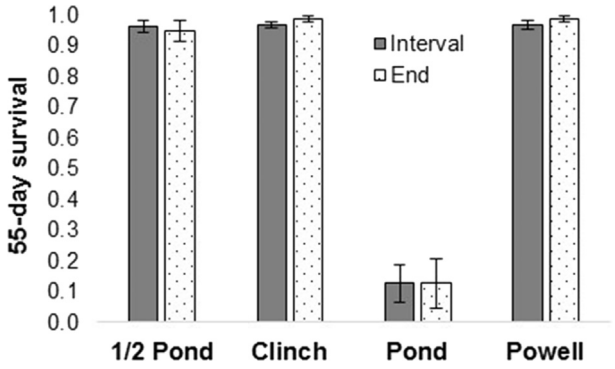

Fig. 3. Mean proportion of surviving mussels in each treatment (n = 5 replicates each) at 55 days. Error bars represent standard error of the mean. There was no significant difference in survival between chambers sampled at 2-week intervals and chambers sampled only at the end of the study within each treatment ($p = 0.599$). Survival in the Pond control was significantly lower ($p < 0.0001$) than in all other treatments/control, which were statistically similar to each other ($p \geq 0.887$).

Major differences in water chemistries between the current study and the 10% stream water treatments in Kunz et al. (2013) are pH (8.4–8.8 in the current study and 8.1–8.3 in Kunz et al. (2013)), and HCO_3^- (110–229 mg/L in the current study and 102–135 mg/L in Kunz et al. (2013), calculated from reported alkalinity). Recent studies have documented the toxicity of HCO_3^- to aquatic organisms, with toxic effects to freshwater mussels occurring at $NaHCO_3$ concentrations >900 mg/L (Farag and Harper, 2014; Harper et al., 2014). The mechanism for HCO_3^- toxicity is thought to involve interference with ion exchange via Na^+/K^+-ATPase and the HCO_3^-/Cl^- co-transporter (Farag and Harper, 2014; Harper et al., 2014). However, sublethal concentrations of HCO_3^- can lessen the toxicity of Cu to freshwater organisms (Daly et al., 1990; Hyne et al., 2005; Wurts and Perschbacher, 1994). In bivalves, Cu affects the activities of several enzymes and co-transporters involved in ionoregulation (Jorge et al., 2013; Lopes et al., 2011). It seems plausible that HCO_3^- could mitigate toxic effects of other major ions presumed to negatively affect freshwater organisms through ionoregulatory pathways, but this idea requires further study.

Source water in the current study was pond water (natural water) compared to a mixture of diluted well water and deionized water (reconstituted water) in Kunz et al. (2013). Gillis (2011) found that natural water reduced sensitivity of mussel glochidia to NaCl compared to exposures in reconstituted waters, suggesting that natural waters may reduce ion toxicity. Dissolved organic carbon reduced the toxicity of Cu to both *V. iris* and *L. siliquoidea*, but the

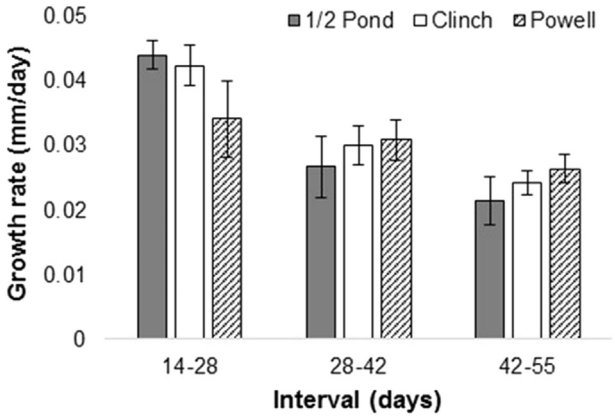

Fig. 5. Mean growth rate of mussels sampled at 2-week intervals in the three treatments (n = 5 replicates each) with high survival. Error bars represent standard error of the mean. There was no significant difference in growth rate between treatments for any sampling interval ($p \geq 0.133$). Growth rates in the second and third sampling intervals were significantly lower than the first in the ½ Pond control ($p < 0.006$). Growth rates in the third sampling interval were significantly lower than the first in the Clinch treatment ($p = 0.004$). There were no significant differences in growth rate between intervals for the Powell treatment ($p \geq 0.295$).

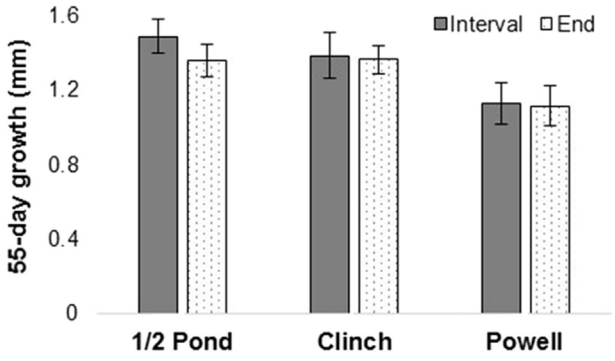

Fig. 6. Mean growth of mussels in the three treatments (n = 5 replicates each) with high survival at 55 days. Error bars represent standard error of the mean. There was no significant difference in growth between chambers sampled at 2-week intervals and chambers sampled only at the end of the study within each treatment ($p = 0.502$). Growth differences between treatments at study end were not statistically significant ($p = 0.076$).

mechanism of toxicity reduction is thought to be complexation rather than competition for receptors and binding sites (Wang et al., 2009). In contrast, synthetic and natural waters produced similar EC_{50}s for *Ceriodaphnia dubia* exposed to $NaHCO_3$ (Vera et al., 2014). The effect of natural water compared to reconstituted water on the toxicity of ion mixtures also requires further study.

It is possible that sensitivity differences between tested mussels contributed to the difference in findings between this study and Kunz et al. (2013). *L. siliquoidea* may be more sensitive to ionic stress than *V. iris*, but the two species have not been directly compared; comparisons of values across different studies suggest similar sensitivity to NaCl. Measured 24 h NaCl LC_{50}s for *L. siliquoidea* glochidia range from 550 mg/L (Bringolf et al., 2007) to 2340 mg/L (Gillis, 2011). Measured 24 h NaCl LC_{50}s for *V. iris* include a range of 310–1150 mg/L (glochidia from 8 individuals; Valenti, 2004) and 1700 mg/L (Pandolfo et al., 2012). Measured 96 h NaCl LC_{50}s for juveniles of the two species include 4560 mg/L for *L. siliquoidea* (Bringolf et al., 2007) and 1660 for *V. iris* (Pandolfo et al., 2012). The differences in age of the juveniles may have also contributed to observed differences between this study, which utilized 3.5 month old mussels, and Kunz et al. (2013), which utilized 2 month old mussels. Generally, sensitivity of freshwater mussels to contaminants is expected to decrease with age, however duration of exposure may equalize these effects (Cope et al., 2008).

The broodstock (gravid females) of *V. iris* used in this study were collected from a reference stream in the Clinch River watershed with measured summer specific conductance 385 μS/cm, intermediate between that of the ½ Pond control and the Clinch treatment, and their progeny may be better able to acclimate to changes in major ion concentrations compared to progeny of mussels collected from low conductivity streams. Gillis (2011) found that *L. siliquoidea* glochidia collected from different streams had significantly different sensitivities to NaCl. The juveniles used in this study were reared in Pond water with mean specific conductivity 400 μS/cm. It is possible that acclimation of individuals occurred during the 3.5 month rearing process, resulting in a reduced response to elevated major ion concentrations. While not statistically significant, growth rates in the Powell treatment were slightly lower than in the ½ Pond control and Clinch treatment during the day 14–28 interval, but were almost identical between the three treatments during the remaining sampling intervals. This suggests that some acclimation to the Powell treatment, requiring energy expenditure, may have been occurring early in the exposure. The overall growth in the Powell treatment was also slightly lower (but not significantly different) than in the ½ Pond control and Clinch treatment, which may have been a result of early acclimation.

An obvious confounding factor in the current study was the mortality in the Pond control. Given the high survival in the ½ Pond control (and in the Clinch and Powell treatments), we are confident that there was not an overall flaw in the exposure system, but rather an effect specific to the pond water. The Pond control had a higher mean pH compared to the ½ Pond control, including individual readings up to pH 9.2. This is likely due to the productivity of the pond, as removal of CO_2 by primary producers can increase pH. The majority of mussel mortality in the Pond control occurred during the interval between week 2 and 4. Ammonia was low in all treatments and controls after week 1. The lowest pH values in the Pond Control were recorded during weeks 1–2 (pH 8.1–8.8). Measurements of pH > 9 began to occur between week 2 and 3, and intermittently occurred during the remainder of the study. At pH 9 a greater proportion of ammonia exists in the unionized form, which is most toxic to aquatic organisms. Thus, even though measured NH_3–N concentrations were well below the USEPA's acute criterion (0.41 mg/L at pH 9, 25 °C; USEPA, 2013) and a measured 10-day EC_{50} for survival of 2 month old *V. iris* (1.2 mg/L at pH 8.4, 20 °C; Wang et al., 2007), it is possible that there was enough in unionized form to cause toxicity. The Powell treatment had a similar pH to the Pond control, but much higher salinity. Increasing salinity has been demonstrated to reduce toxicity of unionized ammonia to aquatic organisms due to a reduced proportion of ammonia existing in the unionized form, facilitated ammonia excretion, and reduced uptake (Barbieri, 2010; Costa et al., 2008; Kir and Oz, 2015; Lin and Chen, 2001; Sink, 2010). We did not monitor nitrite (NO_2–N) concentrations, but freshwater mussels do not appear to be highly sensitive to NO_2–N (96 h LC_{50} = 177 mg/L; Soucek and Dickinson, 2012). In addition to potential interactive effects of pH and ammonia, it is also possible that reduced survival in the Pond control was due to microbial activity, reduced by dilution (1/2 Pond control and Clinch treatment) and elevated salinity (Powell treatment). Juvenile mussels are reared in pond water (identical to Pond control) at the FMCC and do not demonstrate adverse effects. However, rearing is conducted in flow-through systems and the toxicity in the Pond control appears to be due to holding the pond water in a static-renewal system.

Overall, results of this study suggest that direct effects of

elevated major ion concentrations in the Clinch and Powell Rivers of Virginia may not be the primary cause of mussel declines, as salt solutions representative of the rivers' ion compositions did not cause mortality or reduced growth in juvenile *V. iris* over a 55-day period. We selected juvenile mussels for this study because this is the earliest lifestage when chronic effects become important. However, we recognize that the life histories of freshwater mussels are complex, and elevated major ions in the Clinch and Powell Rivers may have direct toxic effects on glochidia or reproductive success of adults, which could ultimately lead to observed declines in populations. In addition, major ions are not the only contaminants present in the Clinch and Powell Rivers. Elevated concentrations of trace elements and polycyclic aromatic hydrocarbons are present in bed sediment of both rivers (Johnson et al., 2014, and S. Ciparis, unpublished data). The potential contribution of persistent contaminants to mussel declines in the Clinch River is currently under investigation. However, the combined effects of elevated major ion concentrations and persistent contaminants on freshwater mussels requires further study, as the potential for interactive effects has received little attention, particularly under laboratory conditions.

4.1. Directions for future research

Results of this study illustrate that predicting effects of increasing major ion concentrations on freshwater mussels in natural waters is complex and likely cannot be accomplished by testing single lifestages of single species for short time periods. The ion composition of the test waters appears to be critically important; the mechanism of action of major ion toxicity to mussels requires further study, as does the effect of natural water on ion mixture toxicity. Future studies should consider potential differences in species sensitivity, sources of gravid females/glochidia, and the age and rearing conditions of juvenile mussels. Although the duration of our study was twice as long as standard chronic toxicity tests, it is a very small portion of freshwater mussel lifespan (decades). While logistically difficult, longer duration tests should also be considered.

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Appendix A. Supplementary information

Supplementary information related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.09.023.

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