

**Evaluation of the Effects of Mining Related Contaminants on Freshwater Mussels  
(Bivalvia: Unionidae) in the Powell River of Virginia and Tennessee**

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**Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial  
fulfillment of the requirements for the degree of**

**Master of Science**

**In**

**Fisheries and Wildlife**

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**April 5, 2019**

**Blacksburg, Virginia**

**Keywords:** Specific Conductance, Coal Mining, Appalachia, Freshwater Mussels, Bivalve, Unionidae

## Evaluation of the Effects of Mining Related Contaminants on Freshwater Mussels (*Bivalvia: Unionidae*) in the Powell River of Virginia and Tennessee

### ABSTRACT

The Powell River is located in southwestern Virginia and northeastern Tennessee, USA and supports a diverse freshwater mussel assemblage of 29 extant species. Throughout the river major ion and trace element concentrations have increased over the last several decades due to extensive surface coal-mining in the headwaters in Virginia. As watershed area affected by mining has increased, mussel populations have declined, especially in Virginia where populations have been severely reduced or extirpated. The upper watershed now has been extensively mined for coal, causing widespread effects on water and sediment quality. To investigate how mining may be affecting mussel populations, I first conducted a laboratory bio-assay to assess the effects of elevated major ions and the trace element nickel (Ni) on growth and survival of juvenile mussels, including one common species (*Villosa iris*) and one endangered species (*Epioblasma capsaeformis*). No significant differences in overall survival between treatments and control were observed for either species over a 70 day test period. Total growth was not significantly different between treatments and control for either species. However, overall growth varied significantly ( $p=0.009$ ) between species, with *V. iris* (2.49 mm) exhibiting greater growth compared to *E. capsaeformis* (1.97 mm). Results suggest that major ion chronic toxicity alone or in combination with Ni at or below my test concentration is not a likely source of toxicity to juvenile mussels in the Powell River. Secondly, I conducted a field study in the Powell River using two cohorts of juveniles of *Villosa iris* to assess the effects of trace elements and PAH contamination related to mining on mussel survival and growth. Specific conductance was elevated throughout the Powell River, where site means ranged from 450 to 900  $\mu\text{S}/\text{cm}$ . While mortality was high at all eight sites it was not significantly different among these sites ( $p>0.28$ ); however, growth of juvenile mussels was significantly higher ( $p<0.001$ ) in the lower river in Tennessee. Regression analysis showed significant relationships ( $p<0.001$ ) of river kilometer with temperature, specific conductance, and aqueous major ion concentrations. A principal component analysis (PC) was conducted on all trace element data. Growth of Cohort 1 on Day 106 was best explained by the PC dominated by aqueous major ion concentrations ( $p<0.0001$ ,  $R^2= 0.65$ ) and growth of Cohort 2 on Day 106 was best explained by specific conductance ( $p<0.0001$ ,  $R^2= 0.68$ ). Growth of Cohort 2 at Day 423 was best explained by tissue trace element concentration PC1 and PC2 ( $p<0.0001$ ,  $R^2= 0.73$ ). This study suggests major ions and select trace elements (Ba, Ni, Fe, Se, and Sr) in the Powell River are negatively affecting the growth of freshwater mussels and that the source of these contaminants is primarily from mining in the headwaters.

## **ABSTRACT (General Audience)**

The Powell River is located in southwestern Virginia and northeastern Tennessee, USA and supports a diverse freshwater mussel assemblage of 29 extant species. Throughout the river major ion and trace element concentrations have increased over the last several decades. As watershed area affected by coal mining has increased mussel populations have declined, especially in Virginia where populations have been severely reduced or extirpated. The upper Powell River watershed has been extensively mined for coal, causing widespread decline in the river's water and sediment quality. My study consisted of a laboratory and field exposure to assess the toxicity of the mining related contaminants, such as major ions, trace elements, and polycyclic aromatic hydrocarbons (PAHs) to freshwater mussels. Further, the study investigated the concentrations of these contaminants in the river and their effects on the survival and growth of exposed juvenile mussels. In my laboratory study, mussels of a common species (*Villosa iris*) and an endangered species (*Epioblasma capsaeformis*) showed no effect when exposed to a suite of major ions and the trace element Ni similar to levels measured in the Powell River. When juvenile *Villosa iris* were exposed in the Powell River at eight sites in Virginia and Tennessee, high rates of mortality were observed at all eight sites and growth of juveniles showed a significant spatial trend, with higher growth observed downstream in Tennessee. Water quality analysis revealed increased concentrations of major ions at all sites but concentrations of trace elements were generally below EPA water quality criteria. Further, many of the major ions and trace elements trended spatially with higher concentrations measured in the headwaters in Virginia and lower concentrations observed downstream in Tennessee. Statistical analysis revealed that major ions and trace elements (Ba, Ni, Fe, Se, and Sr) may have negatively affected growth of exposed mussels. This study revealed that laboratory conditions may not adequately be representing river conditions and that in the river major ions and trace elements likely are negatively effecting growth and survival of freshwater mussels. This study revealed that conditions in the Powell River likely are not suitable for mussel reintroduction and that mining is the main source of the contaminants in the river.

## **ACKNOWLEDGEMENTS**

I offer special thanks to the staff at Virginia Tech's Freshwater Mollusk Conservation Center, especially Anna Dellapenta, Katie Ortiz, Garrett Rhyne, Murray Hyde, and Lee Stephens who assisted with field work and propagating and culturing the mussels used in this study. I would like to thank my committee members Dr. Jess Jones, Dr. Serena Ciparis, and Dr. Carl Zipper for all of their work in helping me to plan and execute this project. I want to especially thank Dr. Madeline Schreiber at Virginia Tech Department of GeoSciences for allowing me to use some of her equipment and laboratory space to conduct analyses for my project. I also thank Brian Watson at Virginia Department of Game and Inland Fisheries, Don Hubbs at Tennessee Wildlife Resources Agency, Sarah Sweeten and Caitlin Carey at Conservation Management Institute at Virginia Tech for their assistance with field deployment and sample collection. I extend special thanks to Dr. Braven Beaty and The Nature Conservancy for providing funding to my project and for the use of their specific conductance loggers. I would also like to thank Athena Tilley and Jeff Parks at the Soils Testing Laboratory at Virginia Tech for all of their assistance in the processing of samples. Finally, I would like to thank U.S. Fish and Wildlife for the funding they have provided for this project. Without the efforts and assistance of those listed above this project would not have been possible.

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## **CHAPTER 1**

# **TOXICITY OF AN ENVIRONMENTALLY RELEVANT MIXTURE OF MAJOR IONS AND THE TRACE ELEMENT NICKEL TO JUVENILE RAINBOW MUSSEL (*VILLOSA IRIS*) AND OYSTER MUSSEL (*EPIOBLASMA CAPSAEFORMIS*).**

## ABSTRACT

The Powell River is located in southwestern Virginia and northeastern Tennessee, USA and supports a diverse freshwater mussel assemblage of 29 extant species. Throughout the river major ion and trace element concentrations have increased over the last several decades. As watershed area affected by coal mining has increased mussel populations have declined. I conducted a laboratory bio-assay to assess the effects of elevated major ions and the trace element nickel (Ni) on growth and survival of juvenile mussels, including one common species (*Villosa iris*) and one endangered species (*Epioblasma capsaeformis*). Mussels (3.5 months old) were exposed to environmentally relevant concentrations of major ions and nickel, to assess individual and combined toxicity. A control water (TDS concentration 153 mg/L) was compared to three treatment conditions: (1) a high TDS concentration of 936 mg/L, with  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ ,  $\text{HCO}^-$  and  $\text{SO}_4^{2-}$  at concentrations which mimicked low-flow conditions in the Powell River, (2) the high TDS treatment with the addition of Ni (14  $\mu\text{g/L}$ ), and (3) Ni at 14  $\mu\text{g/L}$  in water with a TDS concentration of 153 mg/L, similar to the control. The control water had a TDS of 153 mg/L and no added ions or trace elements. Mussel mean survival was significantly different between species ( $p=0.0029$ ): 84.2% for *E. capsaeformis* and 92.7% for *V. iris*. Overall growth varied significantly ( $p=0.009$ ) between species, with *V. iris* (2.49 mm) exhibiting greater growth compared to *E. capsaeformis* (1.97 mm). For both species, survival and growth were not significantly lower in any of the three treatment conditions compared to the control. Results suggest that under laboratory conditions major ion chronic toxicity alone or in combination with Ni at or below my test concentration is not a likely source of toxicity to juvenile mussels in the Powell River.

## INTRODUCTION

Numerous anthropogenic activities such as mining, urbanization, and farming are causing major ion concentrations to increase in freshwaters worldwide (Williams 2001). These anthropogenic activities are shifting the composition of major ions in freshwater and increasing salinization and alkalization in streams throughout North America (Kaushal et al. 2018). Coal was first mined in Southwest Virginia in the late 1800's (Hibbard 1987). Mining in the headwaters of Appalachian streams has resulted in the release of a suite of major ions of geologic origin, including  $\text{SO}_4^{2-}$ ,  $\text{HCO}_3^-$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$ , and  $\text{Cl}^-$  (Cormier et al. 2013; Pond et al. 2008; Timpano et al. 2015). Major ions have been increasing in concentration in the Powell River, Virginia over a four-decade period since the 1960's (Zipper et al. 2016).

Toxic effects of elevated major ion concentrations to freshwater species such as *Daphnia*, benthic macroinvertebrates, and freshwater mussels have been observed in laboratory studies (Kefford et al. 2004; Canedo-Arguelles et al. 2013; Kunz et al. 2013) and associations of elevated major ions with mussel declines have been observed in natural waters (Zipper et al. 2016). A significant decline in mussel diversity and density has been documented in the Powell River since 1979 (Johnson et al. 2012; Ahlstedt et al. 2016). A quantitative survey of 22 sites in the Powell River, Tennessee and Virginia, USA over a two-year period (2008-2009) found 29 extant mussel species and reported that six species are likely extirpated from the river (Johnson et al. 2012). Declines in populations of endangered mussel species in the Powell River, including the last reproducing population of Appalachian monkeyface (*Theiloderma sparsa*), is of great concern to natural resource managers (Johnson et al. 2012), and *Epioblasma capsaeformis* which was last recorded live in the Powell in 1988 (Ahlstedt et al. 2016). Much of the Powell River is designated critical habitat for *Epioblasma capsaeformis* and several other mussel species listed as endangered.

Even common non-listed species such as *Villosa iris* have been impacted in the Powell (Phipps et al. 2018). However, many of the non-listed species appear to be more tolerant of current river conditions. For example, live individuals of *V. iris* have been found in small numbers (18 individuals in 2016-2017), including young individuals <6 years old upstream of RKM 250 but are more abundant at sites downstream of river kilometer (RKM) 171 (Phipps et al. 2018).

Kunz et al. (2013) assessed survival and growth of juvenile *Lampsilis siliquoidea* exposed to a suite of ions representative of mining influenced waters and found significant impacts on their survival and growth. In contrast, Ciparis et al. (2015) found no significant impacts on survival and growth of *Villosa iris* exposed to a suite of ions representative of mining influenced waters. One major difference between the two studies was the use of natural pond water by Ciparis et al. (2015), whereas Kunz et al. (2013) used reconstituted water. A second potentially important difference between the two studies was a higher  $\text{HCO}_3^-$  concentration in Ciparis et al. (2015) compared to Kunz et al. (2013). Kunz et al. (2013) also had a higher Mg:Ca ratio in their waters than Ciparis et al. (2015). Recent studies have documented the toxicity of  $\text{HCO}_3^-$  to aquatic organisms, with toxic effects to freshwater mussels occurring at  $\text{NaHCO}_3^-$  concentrations >900 mg/L (Farag and Harper 2014; Harper et al. 2014). However, sub-lethal concentrations of  $\text{HCO}_3^-$  can lessen the toxicity of Cu to freshwater organisms (Daly et al. 1990; Hyne et al. 2005; Wurts and Perschbacher 1994).

Mining also releases a suite of trace elements, and Ni has been observed at elevated levels in mining influenced surface waters (Baruah et al. 2010; Griffith et al. 2012) including the Powell River (Zipper et al. 2016). Olem (1980) documented mine seeps in the upper Powell River watershed where Ni and Cu values exceeded by orders of magnitude the hardness-adjusted acute (~300 and ~22  $\mu\text{g/L}$ ) and chronic (~30 and ~15  $\mu\text{g/L}$ ) toxicity thresholds established by the U.S. Environmental Protection Agency ([www.epa.gov/wqc/national-recommended-water-quality-](http://www.epa.gov/wqc/national-recommended-water-quality)

[criteria-aquatic-life-criteria-table](#)). The Virginia Department of Environmental Quality (VADEQ) measured Ni levels as high as 2.9 µg/L in surface water and as high as 49 mg/kg in the sediment at a long-term water quality monitoring station in the Powell River (Station 6BPOW184.19, Big Stone Gap, VA) (MapTech Inc. and New River Highlands RC&D, 2011). The sediment concentration is above the threshold effect concentration (TEC) of 22.7 mg/kg and the probable effect concentration of 48.6 mg/kg (MacDonald et al. 2000). Even with available VADEQ data, information about Ni concentrations in the Powell River is sparse. Nickel has been found to be toxic to freshwater mussels. For example, Wang et al. (2017) observed a Final Acute Value of 520 µg/L for Ni for three species of freshwater mussels. Mussel sensitivity to trace elements has been shown to vary little among mussel species (Wang et al. 2017). Understanding the influence of Ni and other trace elements is important because juvenile mussels burrow into stream bottom sediments where concentrations are often higher than in the water column (Yeager et al. 1994; Cope et al. 2008). Although experiments have been conducted to determine the acute effects of Ni (Wang et al. 2013), few experiments have been conducted to assess its chronic effects or the combined effects elevated major ions and trace elements on freshwater mussels.

The purpose of this study was to investigate the toxicity of environmentally relevant concentrations of major ions and Ni on the survival and growth of two mussel species native to the Powell River, where concentrations of these contaminants are elevated relative to natural conditions. A 70-day chronic exposure using juveniles of the federally listed endangered oyster mussel (*Epioblasma capsaeformis*) and the common rainbow mussel (*Villosa iris*) was conducted to examine the effects of both Ni and a suite of major ions on their survival and growth.

## METHODS

*Juvenile mussel production* – Juvenile mussels were produced in May and June of 2015 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 *Villosa iris* were collected from Indian Creek, Tazewell County, VA and host fish (*Ambloplites rupestris*) were collected from Sinking Creek, Giles County, VA in May. Fish were infested with mussel glochidia on May 21, 2015 and held in recirculating aquaculture systems at 25 °C. Gravid female *Epioblasma capsaeformis* were collected from the Cinch River at Wallen Bend, Hancock County, TN on June 3, 2015 and host fish (*Cottus baileyi*) were collected from the South Fork of the Holston River near Sugar Grove, Smyth County, VA on June 12, 2015. Host fish were infested on June 15, 2015 and held at 20°C in a re-circulating aquaculture system. Juveniles of both species 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 initiating the study.

*Test concentrations* – Preparation of treatment waters and ion concentrations were based on methods used in Ciparis et al. (2015). The Powell River treatment water (Powell) was based on data obtained from VADEQ at station 6BPOW179.20, Big Stone Gap, Virginia. The target TDS value was 936 mg/L (major ion concentration sums). The target sum-of-ion concentration estimate was distributed among the major ions based on plots of measured TDS concentrations and concentrations of component ions: ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ , and  $\text{HCO}_3^-$ ) obtained from VADEQ data (Table 1).

The Ni treatment concentration was based on environmentally relevant values measured in the Clinch and Powell Rivers. Values of 39 mg/kg were recorded in the Clinch River in sediments samples collected by Johnson et al. (2014). Generally, water quality data from the Clinch River

has shown higher concentrations of Ni in pore water than in surface water. Unfiltered pore water data collected from the Clinch River in 2012–2013 in the vicinity of sediment collected by Johnson et al. (2014), measured Ni values as high as 8 µg/L (Cope and Jones 2016). The VADEQ data collected from the Powell River documented sediment concentrations of Ni as high as 49.00 mg/kg; however, pore water data have not been collected in the river. Given the higher sediment Ni concentration in the Powell River compared to the Clinch River, a higher pore water concentration was considered likely and justified. Thus, a concentration of 15 µg/L was set for this study.

*Treatment preparation* – A 50:50 mixture of filtered (5 µm) pond water from the FMCC pond and deionized water were used to create a base water for the study, referred to as ½ Pond Control. A suite of major ions was added to ½ Pond Control water to create the simulated Powell River treatment waters, referred to as the Powell and the Powell Ni treatments. Recipes for the Powell and Powell Ni treatments were based on ion concentrations measured in the ½ Pond Control water, and then adjusted accordingly by addition of major ions to reach target constituent concentrations. Ion concentrations in the base waters were measured and verified prior to preparation of treatment waters. Control and treatment waters were prepared weekly. Potassium chloride (KCl), potassium bicarbonate (KHCO<sub>3</sub>), sodium bicarbonate (NaHCO<sub>3</sub>), magnesium sulfate heptahydrate (MgSO<sub>4</sub>\* 7H<sub>2</sub>O), calcium sulfate (CaSO<sub>4</sub>), calcium chloride dihydrate (CaCl<sub>2</sub>\*2H<sub>2</sub>O), calcium bicarbonate (CaCO<sub>3</sub>) and sodium sulfate (Na<sub>2</sub>SO<sub>4</sub>) were used to prepare the Powell and Powell Ni treatments from the ½ Pond Control. All salts were certified American Chemical Society (ACS) reagent grade. 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. Following the addition of ion mixtures to vats, the Powell Ni and ½ Pond Ni treatments were prepared from a concentrated stock



solution (262.2 µg/ml Ni) created using NiSO<sub>4</sub>\*6H<sub>2</sub>O and ultrapure water. Concentrated stock (1 ml) was added to treatment waters to achieve a concentration of 15 µg/L. All waters in the vats were aerated and heated to the target exposure temperature (24°C).

*Mussel exposure system* – Mussels were held in 18 L downweller-bucket systems (Barnhart 2006; Carey et al. 2013). Each bucket served as an experimental unit with 6 chambers per bucket containing juvenile mussels. Chambers were constructed from 3.81 cm diameter PVC pipe; each chamber was 10.16 cm long and capped with 500 micron mesh to hold juveniles and 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). The target temperature 24°C was maintained in the water baths using aquarium heaters. Buckets were placed in baths and filled with pond water and 3 grams of Proline ammonium chloride (Aquatic Eco-Systems, Inc., Apopka, FL) were added three weeks prior to the study, and held at 26°C for 21 days and then flushed thoroughly with pond water two days prior to the study. This conditioning step was necessary to establish a nitrifying bacterial community. Five water baths were used, each containing a replicate (bucket) of ½ Pond Control, ½ Pond Ni, Powell, and Powell Ni treatments, randomly arranged (n=5 for the control and treatments). A blocked design, where each bath represents a block, was used to account for any temperature differences between water baths. A total of 1200 juvenile mussels (600 *Villosa iris*, 600 *Epioblasma capsaeformis*) were randomly selected from the original cohorts upon initiation of the study. Sixty mussels were allocated to each bucket (10 individuals per chamber and 3 chambers per species). 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). Nominal algal concentration for food was 95,000 cells per ml per bucket. Algal food solution was added to each bucket twice daily at 0.5 ml per bucket per event.

For each bucket, a 100% water exchange was conducted 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 YSI 556 Multi-Probe Sensor (YSI Inc., Yellow Springs, OH). Ammonia (NH<sub>3</sub>-N) was measured weekly just prior to water exchanges using a HACH DR/2400 meter following the manufacturer's methods. Total alkalinity (mg/L as CaCO<sub>3</sub>) was measured weekly using a standard titration method. Total alkalinity was converted to HCO<sub>3</sub><sup>-</sup> using the equation HCO<sub>3</sub><sup>-</sup> (mg/L) = Alkalinity (mg/L as CaCO<sub>3</sub>)\*1.22. Dissolved (<0.45 microns) element concentrations (Na, K, Mg, Ca, Ni, and S) were measured 24 h after water exchanges. Measurement of elements in solution was performed by the Virginia Tech Soil Testing Laboratory using an Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES) (Spectro Analytical Instrumentation, Kleve, Germany) following the laboratory's standard operating procedure and quality assurance/quality control methods, as detailed in Ciparis et al. (2015). Sulfate concentration was calculated from measured total S; all S is assumed to be present as SO<sub>4</sub><sup>2-</sup> because of the buckets' oxygenated environment. Concentrations of NH<sub>3</sub>-N, alkalinity, and elements were measured by filtering (45 µm) 25 ml from each replicate bucket and combining aliquots to form a pooled sample for each control/treatment.

Juvenile mussels were exposed to control and treatment conditions for 70 days. On day 0, shell length of each mussel along the longest anterior-posterior axis was measured to the nearest 0.1 mm using a dissecting microscope and ocular micrometer. Mean length of *Villosa iris* and *Epioblasma capsaeformis* juveniles on Day 0 was 2.3 mm and 1.6 mm, respectively. Juvenile mussel survival was recorded every 7 days and juvenile mussels were measured every 14 days during the study. Individual mussels were not marked because of their small size (<2.6 mm);

therefore, the average mussel length per chamber was used to calculate growth between sampling events. Mean length for each replicate (bucket) was used in statistical analyses.

*Data analysis* – An interval censored survival analysis model was developed in SAS using PROC LIFEREG to determine the effects of species and water treatment on survival throughout the course of the experiment. A generalized linear mixed model was fit using PROC GLIMMIX in SAS to assess the effects of species and treatment on overall mussel growth (normal distribution) and overall survival (binomial distribution) for the entire study. A generalized linear mixed model was also used for growth rate over each sampling interval but the data required square root transformation in order to maximize fit to the normal distribution. For all mixed models, a random effect for bucket was used to accommodate the structure of the experiment and bath was included in the model as a blocking factor. When the effect of a factor was statistically significant, least-squares means (model-adjusted) were compared among different levels of the factor using a Holm-Tukey post-hoc test with a correction for multiple comparisons.

## RESULTS

*Water Quality and Ion Concentrations* – Mean measured temperature for all treatments was 23.9° C (SD 0.9) (range 21.7 to 26.3). Mean temperatures by bath ranged from 23.3° C to 24.0° C, minimal variation in temperature was observed among water baths. Throughout the experiment, pH was stable and similar among treatments, ranging from 8.4 to 8.7 (Table 2). Over the course of the study ammonia and nitrite were stable and maintained at non-toxic levels (<0.05 mg/L) in all treatments, and specific conductance was stable for all treatments (Table 2). Mean calculated

hardness (mg/L CaCO<sub>3</sub>) was 103 mg/L in the ½ Pond Control, 103 mg/L in the Ni treatment, 360 mg/L in the Powell treatment, and 367 mg/L in the Powell Ni treatment. Ion and TDS mean concentrations were maintained near target concentrations, with geometric mean concentrations of measured ions within 20% of their target concentrations (Table 1). However, sulfate concentrations in the ½ Pond Control and ½ Pond Ni treatment waters exceeded target levels by 1.9 and 3.4 mg/L (124% and 143% of target concentrations), respectively (Table 1). Calcium concentrations were lower than expected in Powell and Powell Ni treatments, 76.7% and 80.3% of target concentrations, respectively. Bicarbonate was lower than expected in these two treatments with 84.2% of target concentration in the Powell treatment and 79.5% of target concentration in the Powell Ni treatment.

*Survival* – Across all treatments, mean proportional survival of juvenile *Epioblasma capsaeformis* was 0.85 (SD 0.07), with maximum survival of 0.92 in the ½ Pond Ni treatment and minimum survival of 0.77 in the Powell treatment (Figure 2). Across all treatments, mean survival of juvenile *Villosa iris* was 0.92 (SD 0.009), with maximum survival of 0.93 in the Powell treatment and minimum survival of 0.91 in the ½ Pond Ni treatment (Figure 2). Treatment did not have a significant effect on overall survival ( $p=0.6193$ ). Species had a significant effect on overall survival ( $p=0.0004$ ) with more *V. iris* surviving than *E. capsaeformis*. There was a significant overall treatment species interaction ( $p=0.0093$ ). However, this interaction became insignificant when examined with Holm-Tukey Least Squared Means comparison. Both species and treatment had a significant effect on survival over time. Specifically, the overall probability of survival was lower for *Epioblasma capsaeformis* than it was for *Villosa iris* ( $p=0.0029$ ) (Figure 3). Survival was significantly higher for *E. capsaeformis* exposed to ½ Pond Ni ( $p=0.02$ ) and Powell Ni ( $p=0.035$ ) treatments than it was for the ½ Pond Control (Figure 3). Additionally, survival was

significantly higher for *E. capsaeformis* exposed to ½ Pond Ni ( $p=0.015$ ) and Powell Ni ( $p=0.027$ ) treatments than it was for the Powell (Figure 3).

*Growth* – Mean growth of *Epioblasma capsaeformis* across all treatments was 1.97 mm (SD 0.19), minimum mean growth was 1.75 mm in the Powell treatment, and maximum mean growth was 2.18 mm in the ½ Pond Control (Figure 4). Mean growth of *Villosa iris* across all treatments was 2.49 mm (SD 0.45), minimum mean growth was 1.92 mm in the Powell treatment, and maximum mean growth was 3.03 mm in the ½ Pond Ni treatment (Figure 4). The generalized linear mixed model for overall growth showed that: (1) species had a significant effect on overall growth ( $p=0.009$ ), with higher growth in *V. iris* (2) treatment had no significant effect on growth ( $p=0.1553$ ), and (3) there were no significant treatment species interactions ( $p=0.26$ ). The linear mixed model for growth rate at each sampling interval revealed no significant effect of treatment ( $p=0.27$ ) or significant interactions between treatment and other variables ( $p=0.11$ ) (Figure 5). However, there was a significant species effect ( $p<0.0001$ ) and a significant interaction of day\*species ( $p<0.0001$ ). The Holm-Tukey post-hoc comparison showed that growth rate of *V. iris* and *E. capsaeformis* was similar during the 0-14 and 28-42 day intervals, but was higher for *V. iris* during the 42-56 and 56-70 day intervals ( $p\leq 0.032$ ) (Figure 5).

## DISCUSSION

*Maintenance of Water Quality and Ion Concentrations* – All monitored water quality parameters and major ion concentrations were kept within 20% of target concentrations set for the study, except for sulfate and calcium. Sulfate concentrations were affected by changes in the base pond water over the course of the study. The water levels in the pond fluctuated during the study causing variations in its water chemistry; therefore, the simulated Powell River treatment mixtures

were adjusted to accommodate ion fluctuations in the pond water. However, the sulfate concentrations that varied more than 20% over the target concentrations were those that consisted of base water and no salt mixture. The only solutions to this problem would have been to further dilute the base water or remove sulfate, which were not feasible due to having to control the other major ion concentrations during the study. However, it is unlikely that the observed variation in sulfate concentrations affected survival and growth of mussels during the study (Timpano et al. 2015). Low calcium concentrations during the study were most likely the result of not being able to dissolve all of the calcium bicarbonate into solution. Maintaining steady sulfate concentrations and dissolving calcium bicarbonate into make-up waters are issues that will need to be resolved in future studies.

In a previous study conducted in our laboratory using the same exposure setup, Ammonia-N concentrations spiked during the first two weeks (Ciparis et al. 2015), likely caused by inadequate colonization of nitrifying bacteria within the buckets prior to conducting the study. Substantial improvement in reducing ammonia-N to negligible concentrations was achieved in the current study, due to seasoning the buckets and holding chambers 3 weeks prior to the start of the study with live pond water and ammonia salt.

*Major Ion Toxicity* – Mussel populations in the Powell River have been severely reduced or extirpated in the zone from Appalachia (RKM 293.7), VA downstream to Dryden (RKM 269.1), VA (Phipps et al. 2018) where recent surface water specific conductance measurements averaged ~703  $\mu\text{S}/\text{cm}$  from July of 2016 to September of 2017 (See Chapter 2). Mussel populations have declined in the zone from Dryden (RKM 269.1), VA to McDowell Shoal (RKM 171.9), TN (Johnson et al. 2012; Ahlstedt et al. 2016) where recent surface water specific conductance measurements averaged ~553  $\mu\text{S}/\text{cm}$  from July of 2016 to September of 2017 (Chapter 2). Specific

conductance for the Powell and Powell Ni treatments was 1200 (SD 200)  $\mu\text{S}/\text{cm}$ , which is comparable to high monthly average surface water specific conductance at Big Stone Gap (1050  $\mu\text{S}/\text{cm}$ ) (Chapter 2). Kunz et al. (2013) observed significant effects on survival and growth of *L. siliquoidea* during laboratory trials at conductivities of 504 and 565  $\mu\text{S}/\text{cm}$ , while Ciparis et al. (2015) observed no significant effects on survival and growth of *V. iris* during laboratory trials at conductivities of 570 and 1190  $\mu\text{S}/\text{cm}$ . Similarly, results of the current study support those of Ciparis et al. (2015) in that no significant effect on survival and growth of *V. iris* was observed at 1200  $\mu\text{S}/\text{cm}$ .

One reason survival may not have been significantly affected by the treatments is the length of the exposure. It appears that growth rate of mussels in the Powell treatment decreased during the exposure, especially toward the end of the study. It may simply be that mussels are not being exposed for a sufficient amount of time to see significant effects on survival. For example, survival of *Villosa iris* exposed to in stream conditions in the Powell River had significantly lower survival at day 423 versus day 106 (Chapter 2).

The effects of elevated concentrations and the composition of major ions on survival, growth, and reproduction of mussels is not well studied and mainly limited to laboratory studies. Mount et al. (1997) demonstrated that major ion toxicity to freshwater organisms is dependent on both ionic composition as well as concentration. Similar to Kunz et al. (2013) and Ciparis et al. (2015), my study tested a suite of major ions ( $\text{SO}_4^{2-}$ ,  $\text{HCO}_3^-$ ,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$ , and  $\text{Cl}^-$ ) associated with mining in the headwaters of Appalachian streams (Cormier et al. 2013; Pond et al. 2008; Timpano et al. 2015). My study had similar hardness levels to those in the Ciparis et al. (2015) and Kunz et al. (2013), ranging from 360 mg/L to 472 mg/L ( $\text{CaCO}_3$ ). Gillis (2011) found that *L. siliquoidea* glochidia collected from different streams had significantly different

sensitivities to NaCl ranging from 113 mg/L to 1430 mg/L. The juveniles used in my study were reared in Pond water with a mean specific conductance of 400  $\mu$ S/cm.

Diatoms and other seston are important sources of food for freshwater mussels Potapova and Charles (2003) found that major ion concentrations explained a statistically significant proportion of variation in assemblage composition of benthic diatoms. Patrick (1978) documented changes in diatom communities that were exposed to changes in trace element concentrations. These studies show that major ion and trace element concentrations can significantly affect the food-webs that freshwater mussels depend on. Drastic shifts in algal assemblage caused by changes in major ion compositions could help to explain mussel declines. The shifts in food assemblage could also explain why there was no difference in growth among our treatments yet populations in the wild are in decline. Each day mussels were fed a proven mixture of algae and diatoms (Nanno 3600 and Shellfish Diet 1800, Reed Mariculture, Campbell, California). Algae were pre-frozen and provided daily, therefore food availability could not be affected by ion compositions or concentrations. This could explain why mussels are showing growth and survival effects in the wild but not in the laboratory when held in the experimental buckets. This could also explain the difference in results between the current study and Kunz et al. (2013). Kunz used reconstituted water whereas this study and Ciparis et al. (2015) used live pond water. Live pond water contains diatoms and algae that would not have been available in the reconstituted water. The algae and diatoms may have been killed by the high concentrations of ions added during the current study but they would have still been available for consumption. These added energy inputs could have offset much of the energetic stress caused by increased osmo-regulatory response caused by the ions.



*Nickel Toxicity* – Nickel toxicity to juvenile mussels was not observed in this study at a concentration of 15µg/L. Contrary to expectations, the Powell Ni and ½ Pond Ni treatments had nominally higher survival for *E. capsaeformis* than treatments without Ni. Further, maximum (nominally) growth was observed for *V. iris* in the ½ Pond Ni treatment. However, it is unclear what role if any the Ni may have played as a contributor to higher survival and growth. These results were non-significant but hint at the possibility that low concentrations of Ni may have enhanced survival and growth of the study species. Ni is crucial to biological functions and critical enzymatic and metabolic reactions of both terrestrial and aquatic organisms (Muysen et al. 2004).

The dissolved Ni pore water concentrations recently measured in the Powell River at eight sites on three occasions over two years averaged 1.51 µg/L, with the maximum average concentration of Ni (2.04 µg/L) at Big Stone Gap, VA (see Chapter 2). These concentrations are well below established EPA water criteria for acute and chronic values of 470 and 52 µg/L, respectively of dissolved Ni. Wang et al. (2017) found dissolved Ni<sup>2+</sup> to be acutely toxic to juvenile *Lampsilis siliquoidea*, resulting in a 96 h LC50 of 350 µg/L at a hardness of 50 mg/L, and Ni<sup>1+</sup> had a 96 h LC50 ranging from 173 µg/L to 676 µg/L for 5 mussel species, with the most sensitive being *Megaloniaias nervosa* and the least sensitive *Utterbackia imbecillis*. Five water borne mechanisms of Ni toxicity to aquatic organisms have been identified, including disruption of Ca<sup>2+</sup>, Mg<sup>2+</sup>, and Fe<sup>2+/3+</sup> homeostasis, an allergic reaction at respiratory epithelia, and generation of reactive oxygen species (ROS) (Brix et al. 2017).

The behavior of Ni<sup>2+</sup> in natural waters is poorly understood. Xue et al. (2001) found that 99% of dissolved Ni in natural waters from Swedish lakes was bound by organic ligands at low concentrations of natural organic matter, neutral pH, and with few mg/L DOC. They also found that a higher fraction of Ni<sup>2+</sup> was bound to DOC in eutrophic and human impacted waters, which

may explain why a negative effect on survival and growth was not observed in Ni treatment waters in the current study. Water from the eutrophic FMCC pond was used in the buckets and likely led to sorption of the Ni<sup>2+</sup> ions to DOC in the test waters. Bourgeault et al. (2012) found that Ni uptake was inhibited in zebra mussels (*Dreissena polymorpha*) by increased calcium concentrations (range 43 to 133 mg/L) and natural organic matter concentrations. Calcium concentrations in the Powell Ni test water contained Ca<sup>2+</sup> concentrations averaging 69.1 mg/L, which may help further explain the lack of negative effects of Ni on mussels in this study.

*Summary and Conclusions* – This study examined survival and growth of juvenile freshwater mussels of two species, *V. iris* a relatively tolerant species and *E. capsaeformis*, a relatively sensitive species. The findings of this study were that survival and growth of juvenile mussels of each species showed no significant effects of treatment waters when exposed to an environmentally relevant suite of major ions. Further, I observed no obvious negative effects on mussel survival and growth in the Ni treatment waters. Future research is needed to examine the possible effects of major ions on sensitive species such as *E. capsaeformis* and to increase control survival to an acceptable test standard (e.g. >80%). Additional research also is needed to examine the trophic effects of major ions and trace elements on aquatic food-webs and the food mussels consume. The addition of consistent food concentrations to the laboratory exposure systems could have mask the effects of the major ions and Ni on the survival and growth of the exposed mussels.

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## TABLES

Table 1. Target concentrations of major ions and the trace element Nickel maintained in juvenile mussel culture buckets per control and treatment conditions during the study. Actual concentrations measured during the study are presented as means (SD) and as % of the target concentrations, with minimum and maximum % of target concentration values given in parentheses.

Ions and Trace Element	½ Pond Control			½ Pond Ni Treatment		
	Target (mg/l)	Mean (mg/l)	% of Target Concentration	Target (mg/l)	Mean (mg/l)	% of Target Concentration
SO <sub>4</sub> <sup>2-</sup>	7.75	9.6 (0.5)	124.1 (95–149)	7.75	11.1 (0.9)	143.3 (95–204)
Na <sup>+</sup>	2.7	3.2 (0.6)	117.4 (91–162)	2.7	3.5 (0.6)	126.3 (101–165)
Ca <sup>2+</sup>	15.6	17.8 (1.5)	113.9 (94–127)	15.6	17.9 (1.4)	114.5 (100–126)
K <sup>+</sup>	1.15	1.2 (0.1)	107.9 (85–126)	1.15	1.3 (0.1)	109.6 (89–122)
Mg <sup>+</sup>	15.7	14.3 (1.7)	90.6 (67–106)	15.7	14.3 (1.5)	90.9 (72–102)
HCO <sub>3</sub> <sup>-</sup>	110	110.8 (9.6)	100.4 (89–120)	110	119.1 (25.8)	106.5 (89–173)
Ni	0.0	0.0(0.0)	NA	0.015	0.013 (0.002)	84.6 (67–120)
TDS	153	157	110	153	168	118
Ions and Trace Element	Powell Treatment			Powell Ni Treatment		
	Target (mg/l)	Mean (mg/l)	% of Target Concentration	Target (mg/l)	Mean (mg/l)	% of Target Concentration
SO <sub>4</sub> <sup>2-</sup>	452.0	452.6 (10.1)	100.1 (88–114)	452.0	451.1 (7.5)	99.8 (94–109)
Na <sup>+</sup>	114.0	134.3 (5.9)	117.7 (110–126)	114.0	130.6 (5.4)	114.5 (109–120)
Ca <sup>2+</sup>	86.0	66.2 (6.0)	76.7 (69–91)	86.0	69.1 (3.2)	80.3 (73–86)
K <sup>+</sup>	6.0	7.1 (0.5)	117.3 (92–114)	6.0	6.9 (0.5)	115.4 (90–115)
Mg <sup>+</sup>	49.0	47.5 (2.8)	96.8 (91–106)	49.0	47.3 (3.0)	96.5 (89–106)
HCO <sub>3</sub> <sup>-</sup>	229.0	193.8 (21.6)	84.2 (72–106)	229	185 (30.1)	79.5 (47–96)
Ni	0.0	0.0 (0.0)	NA	0.0015	0.014 (0.003)	93.7 (73–127)
TDS	936	901	98.8	936	890	98

Table 2. Mean (SD) concentrations of water quality parameters measured in juvenile mussel culture buckets. Each treatment (n=5 replicates) was measured one day before and after each water change, with the exception of ammonia and nitrite which were measured once per week prior to water changes.

<b>Parameters</b>	<b>1/2 Pond Control</b>	<b>1/2 Pond Ni</b>	<b>Powell</b>	<b>Powell Ni</b>
Temperature (°C)	23.9 (0.9)	23.9 (0.9)	23.9 (0.9)	23.9 (0.9)
Specific Conductance (µS/cm)	200 (8.0)	200 (6.0)	1200 (200.0)	1200 (200.0)
pH	8.4 (0.2)	8.4 (0.1)	8.7 (0.1)	8.7 (0.1)
Ammonia (mg/L)	0.009 (0.02)	0.006 (0.009)	0.01 (0.03)	0.01 (0.02)
Nitrite (mg/L)	0.05 (0.09)	0.05 (0.06)	0.06 (0.1)	0.07 (0.1)

## FIGURES

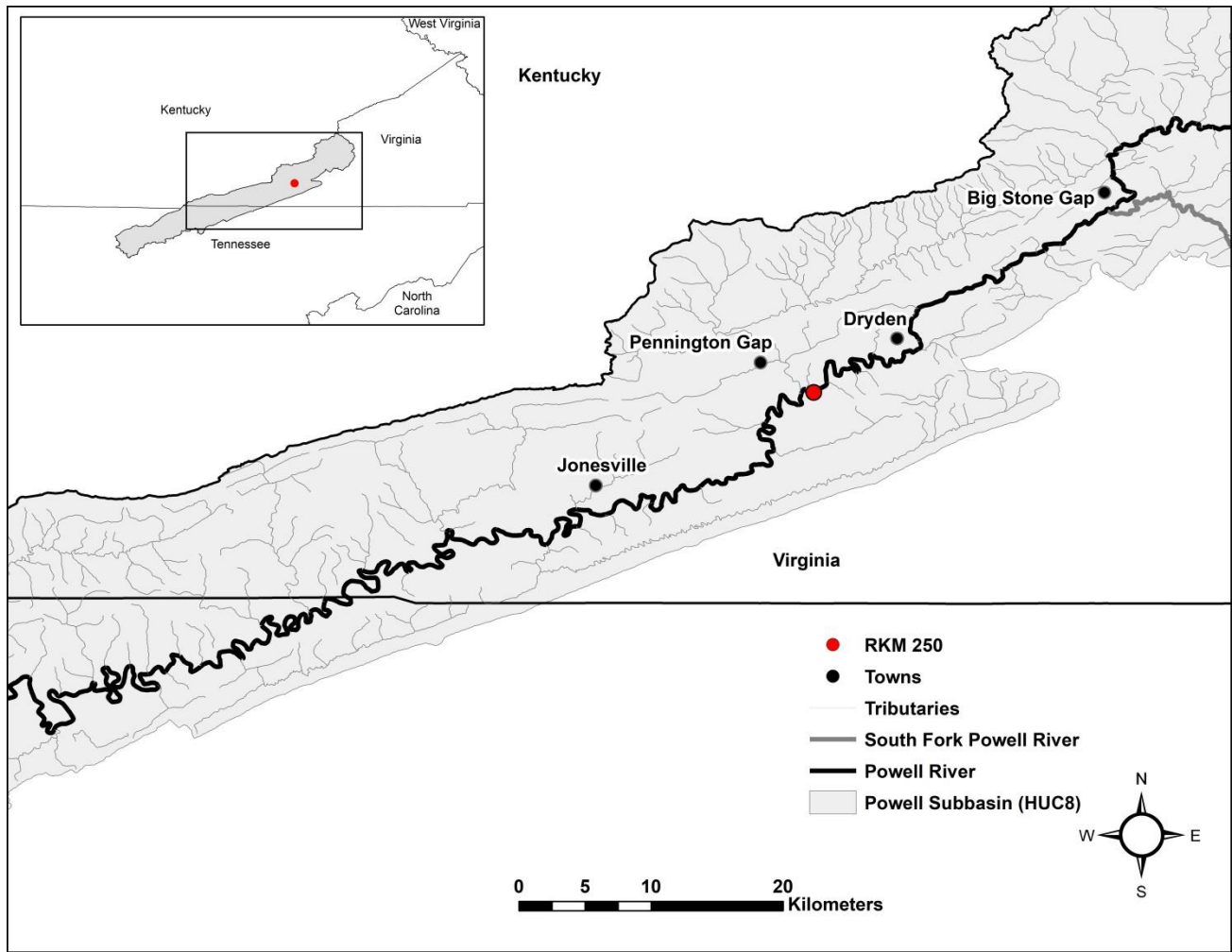


Figure 1. Location of the Powell River in Virginia and Tennessee. Water samples collected from Big Stone Gap were used to set treatment conditions simulating environmentally relevant major ion and trace element concentrations for the study. Upstream of river kilometer (RKM) 250 mussel populations are degraded or extirpated, and in downstream sections of the river in Tennessee mussel populations are considered stable.

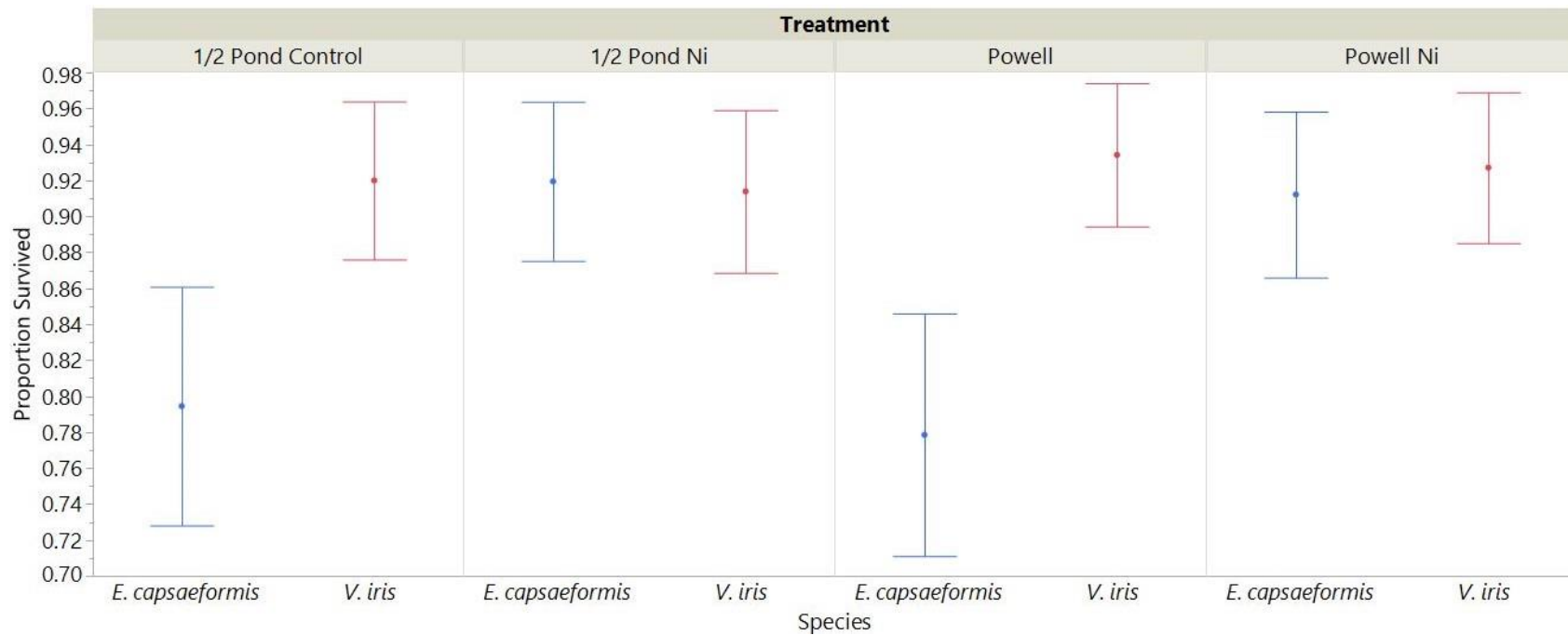


Figure 2. Mean proportion of surviving mussels by species in each treatment (n=5 replicates per treatment) at 70 days. Error bars represent 95% confidence intervals. Species had a significant effect on overall survival ( $p=0.0004$ ) with more *Villosa iris* surviving than did *Epioblasma capsaeformis*. There was no overall effect of treatment ( $p=0.62$ ).

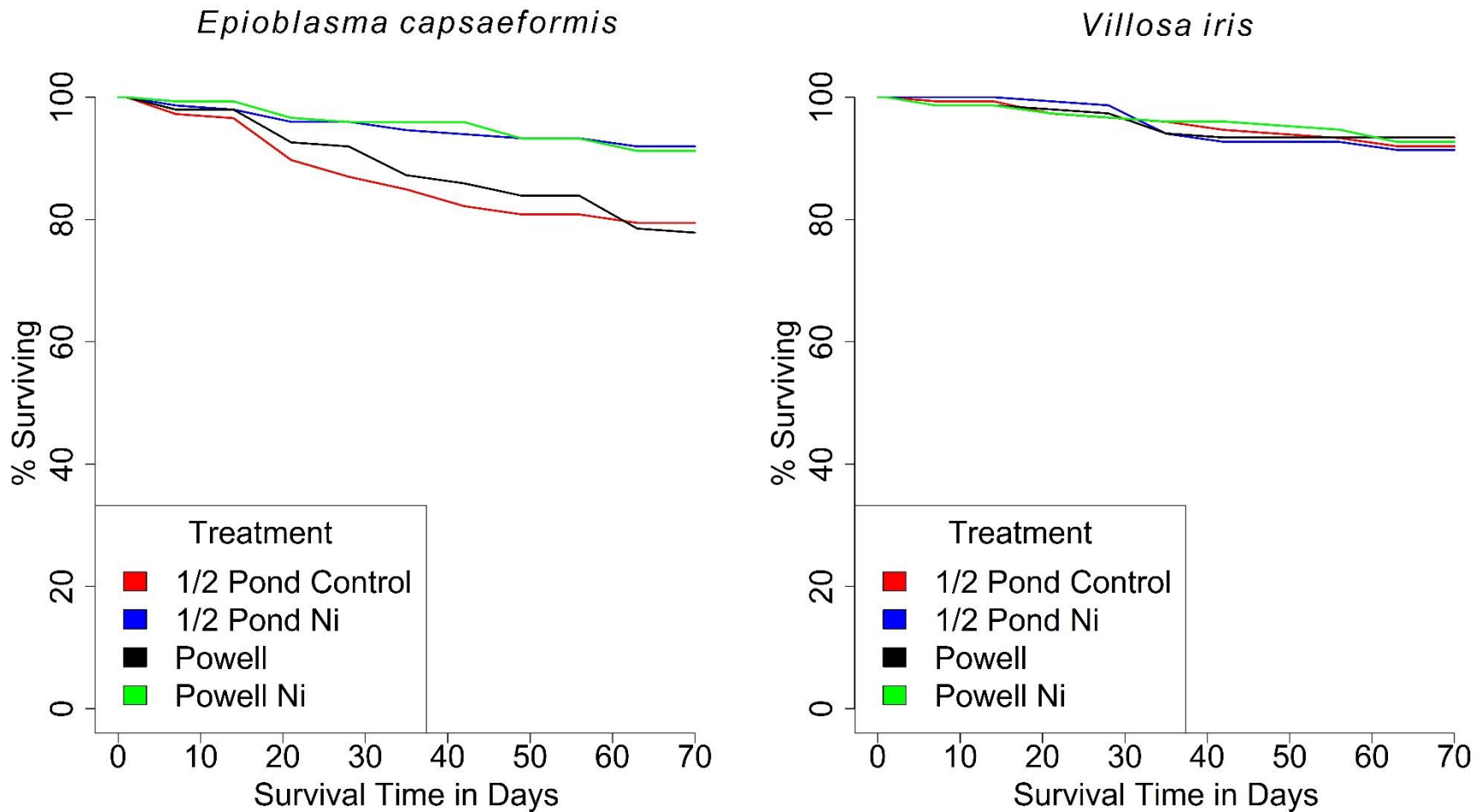


Figure 3. Percent survival in each treatment by species (n=5 replicates per treatment) over the 70-day exposure period. Survival was significantly different between species ( $p=0.0029$ ), and survival was significantly higher for *E. capsaeformis* exposed to 1/2 Pond Ni ( $p=0.02$ ) and Powell Ni ( $p=0.035$ ) treatments than it was for the 1/2 Pond Control (Figure 3). Additionally, survival was significantly higher for *E. capsaeformis* exposed to 1/2 Pond Ni ( $p=0.015$ ) and Powell Ni ( $p=0.027$ ) treatments than it was for the Powell.

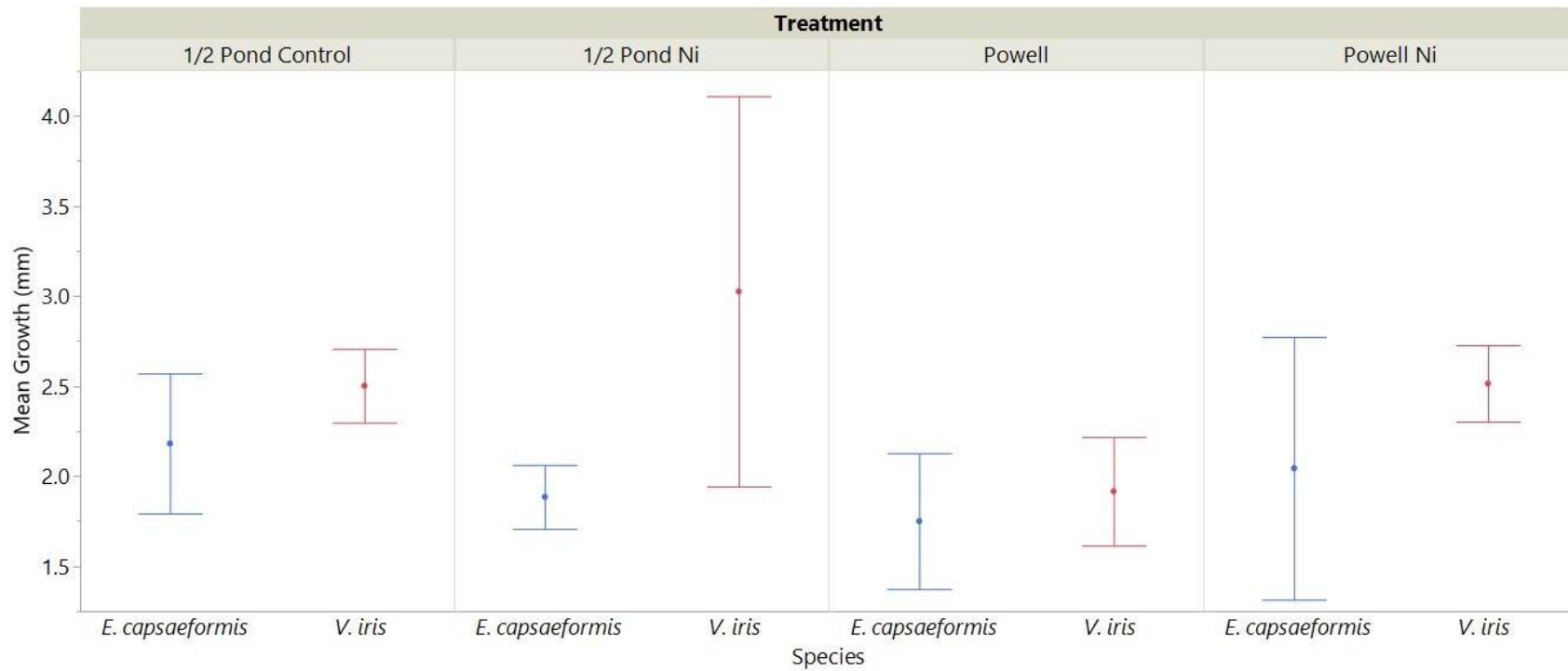


Figure 4. Mean growth of mussels by species for all treatments (n=5 replicates for each treatment) at 70 days. Significantly higher growth was observed for *Villosa iris* compared to *Epioblasma capsaeformis* for all treatments ( $p=0.009$ ). There was no effect of treatment on growth ( $p=0.16$ ). Error bars represent 95% confidence intervals.

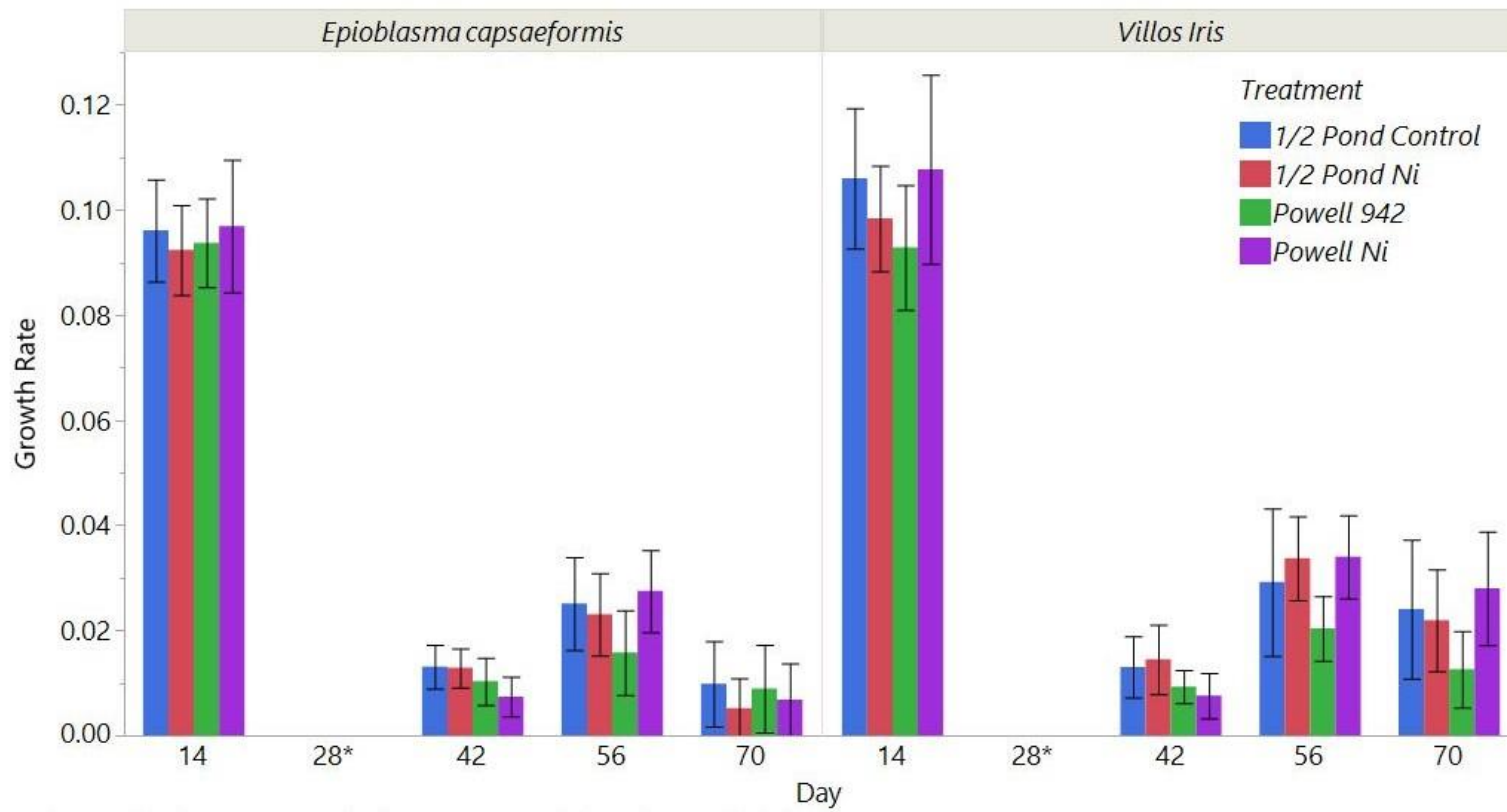


Figure 5. Mean growth rate of mussels by species in all treatments (n=5 replicates per treatment). No significant effect of treatment ( $p=0.27$ ) or significant interactions between treatment and other variables ( $p \geq 0.11$ ). However, there was a significant species effect ( $p < 0.0001$ ) and a significant interaction of day\*species ( $p < 0.0001$ ). \*Data were not used do to measurement errors due to a microscope malfunction.



## **CHAPTER 2**

### **DEPLOYMENT OF RAINBOW MUSSELS (*VILLOSA IRIS*) IN THE POWELL RIVER, VIRGINIA AND TENNESSEE, TO ASSESS EFFECTS OF COAL MINING RELATED WATER CONTAMINANTS**

## ABSTRACT

The freshwater mussel fauna of the Powell River in southwestern Virginia and northeastern Tennessee, USA has declined over the last several decades. The upper Powell River watershed in Virginia has been extensively mined for coal, causing widespread effects on water and sediment quality. The purpose of this study was to examine concentrations of major ions and trace elements in water and sediments and to assess effects on survival and growth of juvenile *Villosa iris* at eight sites in the Powell River. Two cohorts of juvenile mussels were deployed in silos: Cohort 1 was 23 months old (20-25 mm) and Cohort 2 was 7 months old (10-15 mm). On Day 106 and Day 423, juveniles were monitored for growth and survival, and tissue samples were harvested. Water samples were collected on Day 0, Day 106, and Day 423. Specific conductance was elevated throughout the Powell River, where site means ranged from 450 to 900  $\mu\text{S}/\text{cm}$ . While mortality was not significantly different between sites ( $p>0.28$ ), it was high at Day 423 across sites, averaging 58%. However, growth of juvenile mussels was significantly lower ( $p<0.001$ ) in the upper river in Virginia. Regression analysis showed significant relationships ( $p<0.001$ ) with river kilometer to temperature, specific conductance, and aqueous major ion concentrations. Principal component analysis (PC) was conducted on all water and tissue data. Growth of Cohort 1 on Day 106 was best explained by the PC dominated by aqueous major ion concentrations, and the trace elements Ba, Ni, Fe, Se, and Sr. ( $p<0.0001$ ,  $R^2= 0.65$ ) and growth of Cohort 2 on Day 106 was best explained by specific conductance ( $p<0.0001$ ,  $R^2= 0.68$ ). Growth of Cohort 2 at Day 423 was best explained by tissue trace element concentration PC1 and PC2 ( $p<0.0001$ ,  $R^2= 0.73$ ). This study suggests major ions and select trace elements (Ba, Cd, Cu, Ni, Fe, Se, Sn, Sr) in the Powell River are negatively affecting the growth of freshwater mussels and that the likely source of these contaminants is from mining in the headwaters.

## INTRODUCTION

Freshwater mussel density and diversity has declined over the last several decades in the Powell River of southwestern Virginia and northeastern Tennessee, U.S.A. This decline has been attributed to effects of coal mining in the headwaters, which releases a variety of contaminants including major ions, trace elements, and polycyclic aromatic hydrocarbons (PAHs) to tributary streams draining the area (Ahlstedt et al. 2016; Zipper et al. 2016). From 1979 to 2004, mean mussel densities (mussels/m<sup>2</sup>) at four long-term monitoring sites in Tennessee and Virginia declined by 63% (Dennis 1981; Ahlstedt et al. 2016). A quantitative survey of 22 sites from 2008-2009 in Tennessee and Virginia showed no improvement in mussel density and documented 29 extant mussel species, concluding that six were likely extirpated from the river (Johnson et al. 2012). The decline of the mussel fauna in the Powell River has been of great concern to state and federal natural-resource management agencies due to the 15 species listed as federally endangered that occur there, including some of the rarest mussel species in the country.

Coal mining in the Powell River watershed began in the late 1800's (Hibbard 1987), with surface mining in the watershed greatly increasing from 1980-2000 (Zipper et al. 2016). The extraction, processing, and burning of coal releases trace elements into streams; these include Al, As, Ba, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Mo, Ni, Pb, Sb, Se, Si, Sr, V, and Zn (Finkelman 1999). Olem (1980) documented mine seeps in the headwaters of the Powell River where Ni and Cu values exceeded by orders of magnitude acute and chronic toxicity thresholds set by the U.S. Environmental Protection Agency (USEPA). Zipper et al. (2016) showed that mining contaminants vary spatially and temporally, with the highest levels of specific conductance, major ions, trace elements, and SO<sub>4</sub><sup>-</sup> found closest to recently mined areas in the headwaters of the Powell River, and they reported that 29% of the watershed upstream of river kilometer (RKM) 288.4 has

been disturbed by surface mining. Importantly, Bernhardt et al. (2012) estimated that if >5% of a watershed area is disturbed by mining, biotic impairment occurs for benthic macroinvertebrates in headwater streams. Hence, concerns over mining and its potential long-term impacts on the mussel fauna of the Powell River are the focus of this study.

Polycyclic aromatic hydrocarbons (PAHs) are another class of contaminants that are often released in significant quantities by the mining and burning of coal. PAHs are aromatic rings of hydrogen and carbon that have mutagenic and carcinogenic properties (Baumard et al. 1999). Neff and Burns (1996) stated that PAHs can disrupt cellular processes. The release of PAHs often occurs with the burning of coal; however, even unburnt coal can contribute PAHs to the environment (Achten and Hoffman 2009).

Exposure of freshwater mussels to high concentrations of Cd, Cu, Hg, and Zn in laboratory studies have documented mortality, alterations in weight, changes in enzyme activity and filtration rate, and behavioral modifications (Naimo 1995). Most research examining effects of trace elements on freshwater mussels has been conducted in the laboratory with a focus on acute toxicity (Keller et al. 2007). Less is known about the effects of PAHs on freshwater mussels, but Wang et al. (2013) found that increasing PAH and trace element concentrations in sediment were associated with decreased survival of juvenile freshwater mussels in coal mining impacted streams. Humphries (2006) found that PAHs likely contribute to the chronic stress of freshwater mussels in certain environments.

Thus, the purpose of this study was to assess trace-element and PAH contaminant levels in the Powell River and their potential effects on freshwater mussels. Survival and growth of juvenile *Villosa iris* were assessed in the upper portion of the river from Pennington Gap, VA (RKM 250)

upstream to Appalachia, VA (RKM 294), where mussel populations have been greatly reduced or extirpated (Neves et. al 1980; Ahlstedt 1986), and at additional locations downstream to RKM 136.2 in Tennessee, including locations where mussel assemblage density and diversity is much higher (Ahlstedt et al. 2016). Specific conductance, temperature, trace element concentrations in tissue, pore water and surface water, and PAH concentrations in tissue and pore waters were measured at all sites. This study was designed to inform assessments of contaminant exposure and bioaccumulation in mussels and their influence on mussel survival and growth.

## METHODS

*Study Area* – Site selection in the Powell River was based on presence or absence of a mussel assemblage, ease of access to sites, and availability of suitable mussel habitat. Mussel assemblages in the river exhibit a spatial pattern of low mussel density in the headwaters in Virginia and higher density downstream in Tennessee (Ahlstedt et al. 2016). Two zones were defined in this study: Zone 1, which consisted of the upper portion of the river upstream of RKM 250 in Virginia, and Zone 2, which consisted of sites downstream of RKM 171.7 in Tennessee (Figure 1). Eight sites, four within each zone, were selected based on previous sampling efforts (Johnson et al. 2012) and satellite imagery from Google Earth indicating availability of suitable habitat (Figure 1; Table 1).

*Mussel Deployment* – All sites were assessed for habitat suitability and access prior to deployment of mussels in silos. Mussels were held in concrete mussel silos ~5 cm above the substrate surface. Mussels were held in 13.97 cm cylindrical holding chambers covered with 3.5 millimeter plastic mesh embedded in the silos. At each site, six silos were placed in areas with gravel substrate and moderate water velocity. Silos were placed near the thalweg to prevent

dewatering during low flow conditions. Discharge was measured using a Marsh-McBirney Model 2000 Flow-Mate flow velocity meter (Marsh McBirney, Inc., Frederick, MD) to help determine silo location. Silos were placed in similar velocity, depth, and substrate conditions at each site.

*Test Organisms* – Juvenile mussels were produced at the Freshwater Mollusk Conservation Center (FMCC), Virginia Tech, Blacksburg, VA following standard propagation procedures (Zale and Neves 1982; Carey et al. 2013). Gravid female rainbow mussels (*Villosa iris*) were collected from Copper Creek, Scott County, VA on May 14, 2014, and November 10, 2014. Host fish (rock bass) were collected from Sinking Creek, Giles County, VA and Toms Creek, Montgomery County, VA in early May 2014 and December 2015. Fish were infested with mussel glochidia on May 17, 2014 (Cohort 1) and December 1, 2015 (Cohort 2) and held in water recirculating aquaculture systems at 22 °C. Juveniles excysted from fish hosts 2-3 weeks post-infestation and were cultured in 18 L glass aquariums and trough systems at 24 °C for approximately 23 months (Cohort 1) and 7 months (Cohort 2) prior to initiating the study. Thirty mussels were placed in each silo: 10 from Cohort 1 and 20 from Cohort 2. Mean length of Cohort 1 was 22.1 mm and mean length of Cohort 2 was 8.5 mm. Six silos were placed at each site for a total of 48 Silos and 1440 mussels.

Silos were placed in the river three weeks prior to the deployment date to allow them to condition. Silos were anchored using ¾ inch rebar driven ~15–18 cm into the substrate. Heavy duty zip ties were used to attach silos to the stakes. On the day prior to deployment, mussels were randomly sorted to silo, measured and placed in individual holding chambers. Mussel deployment occurred on July 12, 2016. On the day of the deployment, mussels were taken to the river in aerated coolers and the deployment of mussels to all sites was completed in 10 hours. Once the mussels were deployed, silo locations were recorded with a Garmin GPSMAP 78GPS unit (Garmin USA,

Olathe, Kansas). Silos were monitored monthly to ensure their structural integrity and to remove large debris and sand from each silo. Cleaning the silos at flows above 150 cfs at Big Stone Gap, VA posed a safety risk and increased the possibility of silo loss and damage, so silos were cleaned at lower stream discharge — longest period without being cleaned was 60 days.

*Growth and Survival* – Mussels were marked using Hallprint shellfish tags (Hallprint Inc., 27 Commerce Crescent Hindmarsh Valley, South Australia 5211) and Bee tags (Betterbee, 8 Meader Road Greenwich, NY 12834) prior to deployment to allow for identification of individuals throughout the study. Growth was assessed using the measured maximum length. Individual mussels from Cohort 1 and Cohort 2 were measured on the day of deployment on July 12, 2016 (Day 0), and on October 26, 2016 (Day 106). Cohort 2 mussels were measured on September 7, 2017 (Day 423) (Table 2). Mussels were measured to the nearest tenth millimeter (mm) with Scienceware Dial Metric Calipers (Bel-Art Products, Pequannock, NJ). Survival was recorded during the growth sampling events. If an individual was found dead, maximum length was recorded and the shell removed from the silo. Only mussels that were alive on the sampling date were included in growth calculations for each event.

*Trace Element and PAH Tissue Sampling* – Tissue sampling for trace elements was conducted on October 26, 2016 (Day 106) and on September 7, 2017 (Day 423) (Table 2). A baseline sample consisting of individuals from both Cohort 1 and Cohort 2 ( $N=50$ ) were collected and analyzed for trace elements and polycyclic aromatic hydrocarbons (PAHs) prior to the start of the study. Trace element sampling on Day 106 consisted of removing mussels from Cohort 1 (60 mussels/site) from the silos for dissection (Table 2). Day 423 sampling consisted of harvesting all remaining live mussels from Cohort 2 for dissection (~20 mussels/site) (Table 2). All mussels were measured in the field and placed in filtered site water and transported to the laboratory in pre-

cleaned glass jars. They were left in the filtered site water for 24 hours. At the end of 24 hours, mussels from each site were dissected and the pooled tissue from all mussels was weighed. Tissue was then stored in metal-free tubes (VWR International, Radnor, PA) and frozen. A total of 8 tissue samples were prepared per event, for a total of 16 samples for the entire study.

A target tissue wet weight of 20 grams was collected from mussel tissues from Cohort 1 (40 mussels/site) for PAH analysis at Day 106. At Day 423 an insufficient amount of tissue was available to conduct PAH analysis on exposed mussels. Instead, resident Asian clams (*Corbicula fluminea*) from each site were collected and analyzed. All mussels and clams were measured in the field and then placed in filtered site water in pre-cleaned glass jars and transported to the FMCC. They were left in the filtered site water for 24 hours and then were dissected and pooled tissue was weighed. Tissues were then placed in certified clean glass containers (Environmental Express, 2345A Charleston Regional Parkway Charleston, SC) and shipped overnight to Texas A&M Geochemical and Environmental Research Group for total PAH analysis.

*Trace Element Tissue Digestion* – Tissues were freeze-dried and processed using microwave assisted acid digestion as described by Eca et al. (2014). Samples were thawed and a 5 gram wet sample was weighed, and 0.5 gram aliquots were placed in 20 ml micro centrifuge tubes that were sealed with PARAFILM (Sigma-Aldrich 3050 Spruce St. Louis, MO). Samples were then refrozen and freeze-dried (LABCONCO FreeZone 6 Liter, Labconco Corp. 8811 Prospect Avenue, Kansas City, MO) for 72 hours at -20° C. At the end of 72 hours, the samples were pooled again and weighed for a measurement of total dry mass. Approximately 0.5 dry grams of tissue was placed in a MARSXpress55 ml vessel (CEM Corporation, 3100 Smith Farm Road, Matthews, NC) and 3 ml of concentrated Nitric Acid (HNO<sub>3</sub>) (Fisher Scientific 81 Wyman Street, Waltham, Massachusetts) was added to the vessel, followed by 0.5 ml of 30% H<sub>2</sub>O<sub>2</sub> (Fisher



Scientific 81 Wyman Street, Waltham, Massachusetts). After 5 minutes, an additional 0.5 ml 30% H<sub>2</sub>O<sub>2</sub> was added for a total of 1 ml of 30% hydrogen peroxide. After 60 minutes 2 ml of deionized water was added. Following capping, vessels were placed in the MARSXpressXtraction 230/60 (CEM Corporation, 3100 Smith Farm Road, Matthews, NC). Vessels were ramped to temperature (200° C) over a 15 minute period and then held at temperature for 15 minutes. Samples were then allowed to cool for three hours and poured-off into metal-free tubes (VWR International, LLC Radnor, PA). Eight samples were processed in the microwave at a time. The eight samples consisted of six tissue samples, a blank (identical to sample composition without added tissue), and a TORT-3: Lobster Hepatopancreas Reference Material for Trace Elements (Metrology Research Centre, Ottawa, Ontario). Reference materials (TORT-3) were processed to establish recovery of elements, recovery of all elements averaged 115% with a maximum recovery of 140% and a minimum recovery of 80%. Blanks were below detection limits for all elements. Samples were analyzed for trace elements as described below.

*Continuous Monitoring* – Temp Pro V2 Hobo loggers and Hobo Conductivity loggers were placed at each site on July 12, 2016 (Onset Computer Corporation 470 MacArthur Blvd. Bourne, MA 02532). Loggers were used to record temperature and non-temperature corrected conductivity in 15 minute intervals. Loggers were deployed for the entire mussel deployment, with the following exceptions: 1) Data for month one (July 12, 2016 – August 12, 2016) of the deployment at site RKM 171.9 were lost due to logger malfunction, 2) Data for August 12, 2017 – September 7, 2017 were lost at site RKM 153.4 due to a logger battery malfunction, 3) Data for August 20, 2017 – September 7, 2017 were lost due to a logger battery malfunction at sites RKM 253.6, 153.4, 136.4. Loggers were read monthly to prevent loss of data when flows were <150 c.f.s. at Big Stone Gap, VA.

*Water Sampling* – Prior to each water sampling event, discharge was assessed in the Powell River by accessing on-line discharge data recorded by the gage at Big Stone Gap, VA (U.S. Geological Survey gage 03529500). Samples were collected at base flow conditions less than 150 c.f.s. Temperature (°C), specific conductance (µS/cm) and pH were measured at each event using a YSI 556 Multi-Probe Sensor (YSI Inc., Yellow Springs, OH) at each site. Sampling was conducted for major ions, trace elements, and PAHs over a one year period in order to assess seasonal effects. Samples were collected on three separate occasions on Day 0, Day 106 and Day 423 (Table 2).

Five surface water grab samples were collected at each site and pooled together to form one composite sample. Surface water samples were collected following methods established by the USEPA (Method 1669, 1996). Following collection, 100 ml of the 150 ml pooled sample was filtered using a GoPro 700 cm<sup>2</sup> inline 0.45 micron filter (Proactive Environmental Products Bradenton, Florida 34211). With the exception of the final filtered surface water samples which were collected on September 12, 2017 and filtered with a 0.22 micron filter (Whatman Puradisc Aqua, GE Healthcare Life Sciences, Marlborough, MA). New filters and tubing were used for each site. Samples were collected in 50 ml metal-free tubes (VWR International, Radnor, PA) and acidified following the methods outlined in Martin et al. (1999). Separate 50 ml surface water grab samples were collected and analyzed for total alkalinity (mg/L CaCO<sub>3</sub>) using a standard titration method. Total alkalinity was converted to HCO<sub>3</sub><sup>-</sup> concentrations, using HCO<sub>3</sub><sup>-</sup> = 1.22\* mg/L CaCO<sub>3</sub> (Ciparis et al. 2015).

Five pore water samples were collected at each site and pooled to form a composite sample. Samples were collected at five random locations in the river bed within suitable mussel habitat near deployed mussel silos. Samples were collected using a push point sampling device following

guidelines established by the United States Environmental Protection Agency (USEPA) (SESDPROC-513-R2 2013). At each of the five sampling locations within each site, approximately 10 ml was filtered using a GoPro 700 cm<sup>2</sup> inline 0.45 micron filter and collected in a 50 ml metal-free tube. The filter was removed and 10 ml of unfiltered pore water was collected in a 50 ml metal-free tube. Finally, 200 ml of unfiltered pore water was collected in one liter certified clean jars (Environmental Express, 2345A Charleston Regional Parkway Charleston, SC). New filters and tubing were used for each site. Filtered (50 ml) and unfiltered (50 ml) pore water composite samples for trace element analysis were acidified following the methods outlined in Martin et al. (1999). Samples were then placed in the refrigerator at 4 °C until the time of analysis. Unfiltered pore water samples for trace element analysis were placed upright overnight and allowed to settle for 24 hours prior to decanting 10 ml of water for analysis. One liter unfiltered pore water samples for PAH analysis were placed on ice immediately after sampling and shipped overnight to Texas A&M Geochemical and Environmental Research Group.

*Trace Element and PAH Analysis* – Trace element concentrations in water samples and tissue extracts were analyzed at the Department of Civil Engineering at Virginia Tech. Samples were analyzed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) for the total and dissolved concentrations of the following 27 elements: Al, As, Ba, Ca, Cd, Cl, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Se, Si, Sn, Sr, Ti, U, V, and Zn. Aliquots of 0.4 ml were taken from each dissolved tissue concentrate sample and diluted to a 2% acid to volume ratio and 10 ml were submitted for analysis. Ten ml aliquots per water sample type (Filtered Pore Water, Unfiltered Pore Water, Filtered Surface Water, and Unfiltered Surface Water) from each sampling occasion were submitted for analysis. Samples were analyzed for trace elements (Al, As, Ba, Ca, Cd, Cl, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Se, Si, Sn, Sr, Ti, U, V, and Zn) concentrations

using a Thermo Electron X-Series inductively coupled plasma mass spectrometer (ICP-MS) per Standard Method 3125-B (APHA, AWWA, and WEF, 1998). Samples and calibration standards were prepared in a matrix of 2% nitric acid by volume.

PAH tissue and water samples were processed by Texas A&M Geochemical and Environmental Research Group for total PAH concentrations using the following standard operating procedures 9807, 0001, and 9733.

*Data Analysis* – All data analyses were performed in JMP Pro 13.0.0. The relationship between mortality (proportion of dead mussels) and RKM for Cohort 1 and Cohort 2 was evaluated using logistic regression. Growth of mussels for Cohort 1 at Day 106 and Cohort 2 at Day 106 and Day 423 were regressed by RKM. Specific conductance (site mean), temperature (site mean), pH (site mean), and alkalinity (site mean) were regressed by RKM. Individual trace element and PAH concentrations in water and tissue samples were log transformed before being regressed by RKM. Tissue element concentrations were compared between Cohort 1 at Day 106 and Cohort 2 at Day 423 using a paired T-test. Assumptions of parametric statistics, including normal distribution and homogeneity of variance were verified prior to analysis using JMP distribution analysis.

*PCA Modeling Water and Tissue Trace Elements* – Correlation analysis of log transformed trace element concentrations in water and tissue revealed significant correlation ( $p < 0.05$ ) among trace elements for each sample type for each sample date. Therefore, principal components analysis (PCA) was performed using singular valued composition of the correlation matrix (centered and scaled data) with no rotation. The proportion of variance explained by each PC (Score plot) and eigenvalues ( $>1$ ) were used to determine the number of PCs retained. Three PCs

were initially retained for each sample type at each date. Eigenvector weights  $>0.20$  were considered influential to resulting principal components (PCs).

The value of the PC score for each sample site (RKM) was calculated. Exploratory correlation analysis demonstrated that only PC1 scores for water samples (all types) and PC1 and PC2 scores for tissue samples had significant relationships with mussel growth. Therefore, only PC1 for water samples and PC1 and PC2 for tissue samples were explored further.

*Relationships with growth* – Regression analysis was used to determine relationships between mussel growth of both cohorts at each sampling event and specific conductance, temperature, and sulfate concentrations. Regression analysis also was used to determine the relationships between PC scores and mussel growth for both cohorts at each sampling event when significant correlations were determined in the exploratory analysis. The PC1 scores that explained the most variability in growth of Cohort 1 and Cohort 2 at the two sampling events were selected by using the lowest AIC value and highest  $R^2$  value of all tested models. Uncorrelated principal components were included in a stepwise model selection process to determine if additional variation in the data could be explained with inclusion of scores from multiple PCs across sample types and dates.

## **RESULTS**

*Mortality* – No mortality was observed for Cohort 1 at Day 106. Mortality for Cohort 2 did not have a significant relationship with RKM at Day 106 ( $p=0.33$ ), and ranged from a maximum of 20% at RKM 293.7 to a minimum of 5% at RKM 252.6 (Figure 2). Mortality of Cohort 2 did

not have a significant relationship with RKM at Day 423 ( $p=0.28$ ), and ranged from a maximum of 75% at RKM 171.9 to a minimum of 42% at RKM 269.1 (Figure 2).

*Growth* – Maximum mean growth for Cohort 1 at Day 106 was 7.47 mm at RKM 153.4 and minimum mean growth was 1.68 mm at RKM 293.7. There was a significant negative relationship between growth and RKM, with the highest growth observed at downstream sites ( $p<0.0001$ , Slope = -0.022, and  $R^2 = 0.44$ ) (Figure 3). Maximum mean growth of Cohort 2 at Day 106 was 4.29 mm at RKM 136.2 and minimum mean growth was 0.5 mm at RKM 293.7. There was a significant negative relationship between growth and RKM, with highest growth at downstream sites ( $p<0.0001$ , Slope = -0.023, and  $R^2 = 0.45$ ) (Figure 3). Maximum mean growth of Cohort 2 at Day 423 was 12 mm at RKM 136.2, and minimum mean growth was 4.03 mm at RKM 171.5. There was a significant negative relationship between growth and RKM, with highest growth at downstream sites ( $p<0.0001$ , Slope = -0.036, and  $R^2 = 0.33$ ) (Figure 3).

*Water Quality* – Maximum and minimum mean specific conductance from Day 0 to Day 106 was 929.6  $\mu\text{S}/\text{cm}$  at RKM 293.7 and 487.4  $\mu\text{S}/\text{cm}$  at RKM 136.2. Maximum and minimum mean specific conductance from Day 0 to Day 423 was 768  $\mu\text{S}/\text{cm}$  at RKM 287.2 and 443  $\mu\text{S}/\text{cm}$  at RKM 144.2. There was a significant positive relationship between mean specific conductance and RKM, with the highest conductivities at upstream sites from Day 0 to Day 106 ( $p<0.0001$ , Slope = 2.595, and  $R^2 = 0.93$ ) and from Day 0 to Day 423 ( $p<0.0001$ , Slope = 1.88, and  $R^2 = 0.91$ ) (Figure 4).

Mean site water temperature from Day 0 to Day 106 ranged from 19.9 °C at RKM 293.7 to 23.9 °C at RKM 136.2, and from Day 0 to Day 423 mean temperature ranged from 15.2 °C at RKM 293.7 to 18.0 °C at RKM 144.2 (Figure 5). Maximum recorded temperature was 31.1 °C at

RKM 136.2 and minimum recorded temperature was 0.22 °C at RKM 269.1. There was a significant negative relationship between mean temperature and RKM from Day 0 to Day 106 ( $p=0.003$ , Slope = -0.02, and  $R^2 = 0.80$ ) and from Day 0 to Day 423 ( $p= 0.003$ , Slope = -0.013, and  $R^2 = 0.80$ ).

Mean pH of 3 samples taken at Day 0, Day 106, and Day 423 had no significant relationship to RKM ( $p=0.3458$ ). However, mean alkalinity (mg/L CaCO<sub>3</sub>) of samples taken at Day 0, Day 106, and Day 423 was significant positive relationship with RKM ( $p=0.0161$ , Slope = 0.294, and  $R^2 = 0.482$ ).

*Trace Elements and SO<sub>4</sub><sup>2-</sup> in Water Samples* – Many of the mean trace element concentrations across the three sampling events (Day 0, Day 106, and Day 423) were not statistically related to RKM. However, there were positive relationships between RKM and the following mean concentrations of Sr, U, Ca, K, Mg, Na, and SO<sub>4</sub><sup>2-</sup> for all four water sample types, ( $p\leq 0.0006$ ), Ba and Fe for filtered and unfiltered surface waters ( $p\leq 0.006$ ), and Ni for filtered pore water, filtered surface, and unfiltered surface water ( $p\leq 0.002$ ), and Se for filtered pore water, unfiltered pore water, and filtered surface water only ( $p\leq 0.007$ ) (Table 3). Concentrations of trace elements at all sites by sample type and date are reported in Appendix 1. Total mean S concentrations were converted to mean SO<sub>4</sub><sup>-</sup> concentrations for each site, which were similar among all sample types with maximum concentrations measured in the filtered surface water samples, which ranged from 377 mg/L at RKM 293.7 to 96.9 mg/L at RKM 136.2.

In comparison to USEPA aquatic water quality criteria ([www.epa.gov/wqc/national-recomended-water-quality-criteria-aquatic-life-criteria-table](http://www.epa.gov/wqc/national-recomended-water-quality-criteria-aquatic-life-criteria-table)), only one sample exceeded acute or chronic standards in the filtered pore and filtered surface water samples. The concentration of

dissolved Al (filtered surface water) measured at RKM 252.6 at Day 106 was 915.2 µg/L (Acute 750.0 µg/L). Concentrations of Al in filtered surface water at Day 0 and Day 106 at RKM 252.6 were 6.9 and 2.1 µg/L, respectively. Two other trace element samples were somewhat elevated; Zn in the filtered pore water samples at RKM 293.7 and RKM 287.2 on Day 423 were 197.4 and 144.1 µg/L, respectively. However, the concentrations were well below the hardness-adjusted acute water quality Zn criterion (same as chronic criterion) at each site, which were 402 and 393.4 µg/L, respectively. Previously measured Zn concentrations at these two sites at Day 0 and Day 423 were lower at 22.6 and 8.9 µg/L, and 1.7 and 15.4 µg/L, respectively. Comparisons of measured concentrations of trace elements by site, sample type, date and EPA water quality criteria are reported in Appendix 1. Trace element concentrations were consistently higher in filtered and unfiltered pore water samples for several elements of interest, including for Cu, Se, and Zn (Table 4, and Appendix 1).

*Trace Elements in Tissues* – Tissue trace element concentrations were regressed by RKM for each mussel cohort and sampling event. At Day 106, Cl had a significant positive relationship with RKM ( $p=0.007$ , Slope = 0.007, and  $R^2 = 0.73$ ) and at Day 423; Cu ( $p=0.02$ , Slope = 0.003, and  $R^2 = 0.63$ ), Se ( $p=0.01$ , Slope = 0.003, and  $R^2 = 0.67$ ); and Sr ( $p=0.03$ , Slope = 0.004, and  $R^2 = 0.56$ ) had significant positive relationships with RKM. Concentrations of the following elements were significantly higher at Day 423 for Cohort 2 compared to Day 106 for Cohort 1 for all sites: Cd ( $p<0.0001$ ), Cu ( $p<0.0001$ ), K ( $p=0.0104$ ), Mg ( $p=0.0019$ ), Mn ( $p=0.0026$ ), Mo ( $p=0.0025$ ), Ni ( $p=0.0331$ ), P ( $p=0.0016$ ), Se ( $p<0.0001$ ), and Sn ( $p<0.0001$ ). Concentrations of all trace elements sampled by date and RKM are reported in Appendix 2.

*PAHs in Pore Water* – The relationship between concentrations of any PAHs in pore water samples and RKM was not significant ( $p>0.5$ ) for Day 106 or 423. Concentrations of PAH from



pore water samples at Day 106 and Day 423 are reported in Figure 6 and Appendix 3, with the majority below detection limits. The compound C1-Naphthalene had the highest recorded concentrations in the pore water samples collected at Day 106, with concentrations ranging from 28.6 ng/L at RKM 144.2 to 4.5 ng/L at RKM 136.2 (Figure 6). The compound 2, 6-dimethylnaphthalene had the second highest recorded concentrations at Day 106 ranging from 4.2 ng/L at RKM 144.2 to 1.1 ng/L RKM 136.2. The C1-Naphthalenes (42.6, 39.6 ng/L) and C2-Naphthalenes (42.4, 39.4 ng/L) at RKM 293.7 and 287.2, respectively, were the two compounds with the highest concentrations in sampled pore water at Day 423. The C1-Naphthalenes ranged from 42.6 to 15.3 ng/L at RKM 293.7 and RKM 252.9, respectively, and the C2-Naphthalenes ranged from 42.4 to 15.9 ng/L at RKM 293.7 and RKM 153.4, respectively.

*PAHs in Tissues* – The majority of compounds tested were below detection limits and concentrations of those that were detected had no correlation with RKM ( $p>0.5$ ) (Figure 6; Appendix 3). Fluoranthene was the highest recorded compound collected from both mussel (14.4 ng/g RKM 293.7) and *Corbicula* (25.8 ng/g RKM 287.2) tissues. Pyrenes ranged from 14.2 ng/g at RKM 287.2 in *Corbicula* and 10.4 ng/g at RKM 293.7 in mussel tissue. The C1-Naphthalenes ranged from 6.3 ng/g to non-detectable at RKM 269.1 and RKM 136.2, respectively for *Corbicula*, and in mussel tissues, they ranged from 8.7 to 3.1 ng/g at RKM 269.1 and RKM 144.2, respectively.

*PCA Modeling of Trace Elements in Water and Tissue Samples* – Eigenvalues for Principal Component 1 (PC1) for all water sample types other than unfiltered pore water at all dates were  $\geq 8.4$  (Table 4). Percent of variance explained by PC1s for all water sample types other than unfiltered pore water at all dates was  $\geq 46.7\%$  (Table 4). PC1 for all water sample types on all dates except for unfiltered pore water samples was significantly influenced by the following suite of

elements: Ca, K, Mg, Na, S, and Sr (Table 4). PC1 for all dates for unfiltered pore water samples were comprised of more evenly distributed eigenvectors among the majority of trace elements tested (Table 4). Eigenvalues for PC1s for unfiltered pore water at all dates were  $\geq 8.7$  (Table 4). Percent of variance explained by PC1s for unfiltered pore water at all dates was  $\geq 48.2\%$  (Table 4). There was significant correlation among all PC1s for all water sample types ( $p > 0.05$ ).

Percent of variance explained by PC1 for concentrations of trace elements in tissues at Day 106 and Day 423 was  $\geq 56.1\%$  (Table 5). Percent of variance explained by PC2 for concentrations of trace elements in tissues at Day 106 and Day 423 was  $\geq 16.1\%$  (Table 5). Eigenvalues for PC1 for concentrations of trace elements in tissues at Day 106 and Day 423 was  $\geq 15.7$  (Table 5). Eigenvalues for PC2 for concentrations of trace elements in tissues at Day 106 and Day 423 were  $\geq 4.5$  (Table 5). The score plot for PC1 by PC2 at Day 423 revealed that RKM 171.9 and 252.6 had the highest positive scores for PC1 and that RKM 287.2 and 293.7 had the highest negative scores for PC2 (Figure 7). Increased PC1 scores for RKM 171.9 and 252.6 were the result of increased concentrations of the following elements at the two sites As, Ba, Mg, Mo, Ni, P, S, Sr, Ti, Zn. Loading values by trace element for PC1 and PC2 at Day 106 and Day 423 were not similar and are included in Table 5. Correlation analysis revealed that the principal components were not highly correlated within either sampling date ( $p < 0.05$ ).

*Growth Model Summary* – For Cohort 1 at Day 106 there were significant relationships between growth and all tested variables, including specific conductance, temperature, and PC scores for water and tissue samples (Table 6). The best predictor of mussel growth of Cohort 1 was the PC1 score for filtered surface water on Day 0, followed by mean specific conductance from Day 0 to Day 106, and the PC1 score for unfiltered surface water on Day 0 (Table 6). For Cohort 2 at Day 106 there were significant relationships between growth and mean specific

conductance from Day 0 to Day 106 and temperature only, with similar model fit for both variables and there were no significant relationships between any PC scores (Table 6). For Cohort 2 at Day 423 there were significant relationships between growth and all tested variables, including specific conductance, temperature, and PC scores for water and tissue samples (Table 6). The best single-variable predictor of mussel growth for Cohort 2 at Day 423 was the PC1 score for filtered surface water on Day 0, followed by temperature, and mean specific conductance from Day 0 to Day 423 (Table 6). However, the best overall model for predicting growth of Cohort 2 at Day 423 was produced when both PC1 and PC2 scores for concentrations of elements in tissue were included (Table 6). The higher tissue PC1 scores at RKM 171.2 and 252.6, and lower PC2 scores at upper 2 sites are related to the lower than predicted growth at these sites.

## **DISCUSSION**

Concentrations of mining related contaminants were higher in the Virginia section of the Powell River and showed a spatial distribution with concentrations decreasing downstream in Tennessee. In Virginia average specific conductance values exceeded the benchmarks set forth for aquatic life in the Appalachian region. Individual elements in the Powell River such as potassium, magnesium, and copper are at levels nearing those that negatively impact aquatic life. Mortality of juvenile mussels was high at all sites for Cohort 2. This widespread mortality indicates that contaminants are impacting survival throughout the river. This is supported by the decline in mussel populations throughout the Powell River in Virginia and Tennessee (Johnson et al. 2012; Phipps et al. 2018). Growth of both Cohort 1 and Cohort 2 showed a strong relationship with RKM and was higher in the Tennessee section of the river. Growth of both Cohorts was negatively correlated with the concentration of trace elements and major ions.

It appears that multiple contaminants may be acting together to cause diminished mussel growth in the upper Powell River. The strongest predictors of mussel growth for Cohort 1 were surface-water PC1 and specific conductance. For Cohort 2 the strongest predictors of mussel growth were tissue PC1 and PC2 and surface-water PC1. All of these predictors express combined effects of multiple elements. Although numerous individual elements are also correlated negatively with mussel growth, most of those relationships are less significant than relationships of the combination variables. Also, comparison of my results to those of other studies, as they concern potential influence by individual elements, show little consistency among studies for individual elements (see section Influence of Specific Conductance, Major Ions, and Trace Elements on Mussel Growth below). This lack of consistency among studies likely occurs because of water chemistry differences among the solutions in which individual-element effects were tested by various studies, suggesting that multiple contaminants in the test-water solutions applied by the various studies may be acting together to cause influence.

*Mortality and Growth* – While there was no mortality observed for Cohort 1 at Day 106, Cohort 2 experienced proportional mortality as high as 20% at Day 106 and 75% at Day 423. The higher mortality in Cohort 2 indicates that younger mussels may be more susceptible to the factors causing mussel declines in the Powell River. Surveys of the mussel assemblage in the Powell River upstream of RKM 250 support this conclusion. Johnson et al. (2012) and Phipps et al. (2018) recorded the presence of adult mussels at low abundances at sites upstream of RKM 250 and the absence of recruitment. These recent mussel surveys and the high mortality observed in Cohort 2 that was not correlated with RKM suggest that impacts to the mussel assemblage are ongoing throughout the river. Further, Growth of both Cohort 1 and Cohort 2 at Day 106 trended spatially and was lower at upstream sites. This trend in growth for Cohort 1 at Day 106 indicates that even

though no mortality was observed, river conditions appear to be negatively affecting mussel growth at upstream sites.

*Influence of Specific Conductance, Major Ions, and Trace Elements on Mussel Growth –* Mean specific conductance at all sites in this study were well above EPA aquatic life benchmark criteria of 300  $\mu\text{S}/\text{cm}$  for the central Appalachian region, a level below which 95% of benthic macroinvertebrate taxa are assumed to be safe from harm (USEPA 2011). As mean specific conductance of sites in the Powell River declined moving downstream and approached this benchmark, mussel growth improved.

Kunz et al. (2013) observed significant effects on survival and growth of *L. siliquoidea* during laboratory trials at specific conductances of 504 and 565  $\mu\text{S}/\text{cm}$ , while Ciparis et al. (2015) observed no significant effects on survival and growth of *V. iris* during laboratory trials at specific conductances of 570 and 1190  $\mu\text{S}/\text{cm}$ . Similarly, in Chapter 1 I found no significant effect on survival and growth of *V. iris* at a specific conductance of 1200  $\mu\text{S}/\text{cm}$ . However, in this study growth of *V. iris* was significantly correlated with mean specific conductance, with the lowest mean growth occurring in the upper river where mean specific conductance was highest.

Kunz et al. (2013) noted that test waters containing a higher percentage of  $\text{SO}_4^-$  decreased survival of *L. siliquoidea*. Concentrations of  $\text{SO}_4^-$  were lower in the test water conditions of Ciparis et al. (2015) and Chapter 1 (~452 mg/L), compared to much higher concentrations (~1020 and 1580 mg/L) in Kunz et al. (2013). Wang et al. (2018) found a chronic LC20 of  $\text{SO}_4^-$  for *Lampsilis abrupta* to be 1759 mg/L for mortality, 639 mg/L dry weight effect, and 696 mg/L biomass effect. Mean  $\text{SO}_4^-$  concentrations at the four upper sites in Virginia ranged from 377 mg/L to 224 mg/L in surface filtered water samples, whereas concentrations at downstream sites ranged from 105 to

96 mg/L. Growth was significantly lower in the upstream sites, relative to downstream, despite the fact that measured  $\text{SO}_4^{2-}$  levels were far lower than those found to be associated with growth impairments by Wang et al. (2016). Growth was significantly higher at these sites where  $\text{SO}_4^{2-}$  was lower; however,  $\text{SO}_4^{2-}$  concentrations alone are unlikely to be the main stressor responsible for the observed differences in growth given the presence of multiple other contaminant elements in the water.

Although temperature is also correlated with mussel growth, positively, we consider temperature differences unlikely to be the only cause of the mussel growth differences observed. Historical data demonstrate that freshwater mussels were far more common in the upper river segments, those with lowest temperatures and with lowest growth, historically than they are today (Phipps et al. 2018).

Mean potassium (K) concentrations in filtered surface waters samples were higher in Virginia (~5.0 mg/L), with a maximum mean concentration in filtered surface water recorded at RKM 293.7 (5.6 mg/L). In contrast, mean K concentration in Tennessee was ~2.9 mg/L. Maximum mean concentration of K in filtered pore water was recorded at RKM 293.7 (5.5 mg/L). Morris et al. (2008) reported reduced densities of *Lampsilis fasciola* at sites in the Sydenham River, Ontario, Canada with K concentrations above 6 mg/L, and Wang et al. (2018) reported a 28-day chronic EC20 of 8.7 mg/L K for biomass of juvenile *Lampsilis siliquoidea*. Target K concentrations in tests water for Kunz et al. (2013) and Ciparis et al. (2015) were 4.0 and 6.0 mg/L, respectively; Kunz et al. (2013) observed growth impairments while Ciparis et al. (2015), despite the higher K concentrations, did not. Concentrations of K in my Chapter 1 study were similar to those in Ciparis et al. (2015). In this study juvenile *V. iris* held in silos in the Powell River exhibited lower growth at sites in Zone 1 in Virginia where K concentrations were higher.

Mean  $\text{HCO}_3^-$  ranged from 239.9 mg/L at RKM 293.7 to 196.0 at RKM 136.4, concentrations that are well below an LC50 of 1120 mg/L for *Lampsilis siliquoidea* established by Harper et al. (2014). I am aware of no studies demonstrating thresholds for growth impairments by  $\text{HCO}_3^-$ . The highest mean dissolved Cl concentration recorded in the Powell River, occurring at RKM 269.1, was 6.2 mg/L, which is far lower than the LC50s of 1130–1430 mg/L established for glochidia of four mussel species recorded by Gillis et al. (2011). Limited research has been conducted for  $\text{Mg}^{2+}$  toxicity to aquatic organisms, but De March (1988) established a 96-hour LC50 of 65 mg/L using a freshwater amphipod (*Gammarus lacustris*). The highest recorded value in filtered pore water samples in the Powell River was 47.7 mg/L at RKM 293.7, and highest  $\text{Mg}^{2+}$  concentration in filtered surface water was 45.5 mg/L at RKM 287.2. Thus,  $\text{HCO}_3^-$ , Cl, and  $\text{Mg}^{2+}$  all occur at Powell River sites with observed growth impairments at levels lower than growth-impairment thresholds found by other studies.

While mean Copper (Cu) concentrations were low in both filtered and unfiltered surface water samples (<1  $\mu\text{g/L}$ ). They were much higher in filtered pore water samples. Mean concentration of Cu was 3.53  $\mu\text{g/L}$  across all sites in filtered pore water, with a maximum mean concentration of 5.93  $\mu\text{g/L}$  at RKM 179.9 at a mean hardness of 239.2 mg/L. Although Cu concentrations in filtered pore water samples were not significantly different among sites and did not exhibit a spatial trend, RKM 179.9 had the highest mortality of any site and significantly lower growth than the three other downstream sites. Wang et al. (2007) recorded a 10 day EC50 shell length value of 8.6  $\mu\text{g/L}$  for two-month old *V. iris*, which was 57% lower than the 2 day EC50 shell-length of 20  $\mu\text{g/L}$  for Cu, and *V. iris* had the lowest EC50 shell length of the six species tested. Newly transformed juveniles (2 weeks old) of six mussel species were tested in Wang et al. (2007), and of the six species tested *Epioblasma capsaeformis* and *Lampsilis fasciola* had the

lowest 10 day EC50 shell lengths at 5.9 and 4.8  $\mu\text{g/L}$  of Cu, respectively. These values were 41% and 93% lower than the 2 day EC50 of 10 and 73  $\mu\text{g/L}$  of Cu. It is worth noting that this trend of decreasing EC50s held for all species. Wang et al. (2010) found the 28 day median juvenile mussel survival EC50s for Pb, Cd, and Zn to be 20, 8.1, and 228  $\mu\text{g/L}$ , respectively. These values are generally higher than any of the dissolved values in either pore or surface waters recorded in this study.

*PCA Growth Models* – Principal Component 1 for all water sample type scores were higher at upstream sites and were highly correlated with growth of Cohort 1 at Day 106 and growth of Cohort 2 at Day 423. The PC1s for all water sample types except the unfiltered pore water samples were driven by the following suite of major ions:  $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^{2+}$ ,  $\text{SO}_4^{2-}$ , and Sr. Although, Cu, Ni and Zn did not heavily influence the PC1s for water samples, they were significant in the development of PC1 and PC2 for tissue of Cohort 1 and Cohort 2. PC1 scores for tissues of Cohort 1 and Cohort 2 were higher at upstream sites and had a negative correlation with growth. RKM 171.9 and 251.6 had the highest PC1 scores of any sites and lower growth than predicted by the regression model.

The trace element constituents of the PC1s and PC2s in this study are similar to trace element suites observed in other coal mining effected waters. Merriam et al. (2015) identified the following suite of elements as a signature of surface mining ( $\text{Ca}^{2+}$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{SO}_4^{2-}$ ,  $\text{HCO}_3^-$ , and Se), and abandoned mine lands (Cd, Mn, Ni, and Zn). Rogers et al. (2018) found that mussel growth was negatively correlated with concentrations of Mn,  $\text{Na}^+$ , Fe,  $\text{K}^+$ , Al, and  $\text{SO}_4^{2-}$  in the Clinch River in Virginia and Tennessee. Johnson et al. (2014) showed that  $\text{HCO}_3^-$ ,  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{F}^-$ ,  $\text{Mg}^{2+}$ , and  $\text{SO}_4^{2-}$  in water samples and Cd, P,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^+$  in sediment in the Clinch River Virginia and Tennessee were significantly negatively correlated with mussel species richness, total



recruitment, and lack of imperiled species. Johnson et al. (2014) also documented negative correlations among patterns of mussel declines and concentrations of dissolved  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{Cl}^-$ , and  $\text{F}^-$  and total Fe and Mn in filtered surface waters for a zone of mussel decline in the Clinch River. Mussel growth in this study was significantly negatively correlated with filtered surface water concentrations of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{SO}_4^{2-}$ .

In the Powell River, PC1 for filtered surface water was the best predictor of mussel growth at Day 106, suggesting that mussels were exposed the most to and affected by contaminants contained in the surface flow. The PC1 and PC2 of tissue samples of Cohort 2 was the best predictor of mussel growth at Day 423, suggesting that a longer exposure period than 106 days may be needed to observe significant accumulations in tissues or that the older Cohort 1 mussels were more capable of purging excess trace elements. The results of the PCA analysis suggest that examining concentrations of suites of trace elements was a better way of predicting the growth of juvenile *V. iris* than individual trace element concentrations.

*PAHs* – PAH concentrations varied greatly between Day 106 and Day 423, concentrations of PAHs was higher at Day 423. This is consistent with the results of Cope and Jones (2016) who observed variation in concentration and types of PAHs among sampling events. The various PAH compounds and concentrations were similar in both native *Corbicula fluminea* and Cohort 1 tissues at Day 106 and Day 423. However, PAH concentrations in both tissues and pore waters did not trend spatially and were not correlated with growth. Fluoranthenes and pyrenes were the highest recorded PAHs sampled in tissues at RKM 293.7, Fluoranthenes and naphthalenes were the highest PAHs recorded in water samples. The concentrations of PAHs of all types were <45 ng/L and below concentrations considered toxic to aquatic organisms. The highest concentration of naphthalenes were well below the chronic level of 1100 ng/L set forth by the state of California

for surface waters. Composition of PAHs was consistent with those associated with un-burnt coal particles which are prevalent in the substrate in the Powell River (Ribeiro 2011). Johnson et al. (2014) documented PAHs in bed sediments in the Clinch River but did not observe a spatial trend. While Cope and Jones (2016) observed spatial trends in occurrence of PAHs in the Clinch River; however, neither Johnson et al. (2014) or Archambault et al. (2017) observed correlations between total PAH concentrations and mussel metrics.

*Influence of Mining in the Powell River* – More than 30% of the watershed area of the upper Powell River has been disturbed by mining up to 2011 (Zipper et al. 2016). Bernhardt et al. (2012) estimated that if 5.4% of the watershed area was disturbed by mining, a biotic impairment threshold is reached for benthic macroinvertebrates in headwater streams. Specifically, pH, specific conductance, and dissolved solids and sulfate concentrations are elevated in mining-influenced streams, especially by surface coal mining (Hartman et al., 2005; Pond et al., 2008; Pond et al., 2014; Timpano et al. 2015). Zipper et al. (2016) developed a decay-weight function that predicts decreasing inputs of major ions and trace elements from the time of land disturbance of mining sites. Peak release of major ions and trace elements typically occurs in response to initial geologic disturbance, and water concentrations decline with continued leaching (Sena et al., 2014; Orndorff et al., 2015). Column-leaching experiments, conducted with Appalachian coal mine spoils including samples taken from the upper Powell River watershed, demonstrate high rates of leaching of these soluble ions, especially during and immediately following initial water exposures after fracturing (Orndorff et al. 2015; Clark et al. 2018). Zipper et. al (2016) noted increasing trends in pH, specific conductance, dissolved solids, and sulfate concentrations over their study period from 1984–2011 and predicted major ions and trace element concentrations would increase if severe mining disturbance continued.

My study found that concentrations of major ions and trace elements were higher at upstream sites nearer to mined areas. Again growth of exposed juvenile *V. iris* was suppressed at these same upstream sites, suggesting that mined sites are the most likely sources of contamination in the Powell River. Further, other potential source of contaminants, such as residences, small industry, sewage treatment, and urban runoff, occur in the watershed; hence, coal mining is not the only source of water contaminants. However, it is well known that the major ions that are major contributors to specific conductance (Ca, Mg, K, Na,  $\text{SO}_4^{2-}$ ,  $\text{HCO}_3^-$ ,  $\text{Cl}^-$ ) are elevated in waters draining from Appalachian mine sites, compared to watersheds with minimal disturbance (Pond et al. 2008, 2014; Timpano et al. 2018). These major ions and certain trace elements were associated positively with surface-water filtered PC1 and tissue PC1 and PC2 (e.g. Fe, Ni, Se), and are known to be elevated in mining-influenced Appalachian streams and/or released at high concentrations from Appalachian mine spoils with initial exposures (Pond et al. 2008, 2014; Clark et al. 2018). Although Sr, which contributed positively to filtered surface-water PC1s, is not typically measured in such studies because it is not known to be toxic to aquatic organisms even at concentrations far higher than those that occur typically in natural waters and those measured in this study (McPherson et al. 2014). However, Sr has been measured at elevated concentrations in streams influenced by Appalachian mining (Lindberg et al. 2011; Vengosh et al. 2013) and is a common tracer for coal residuals (Ruhl et al. 2014).

*Conclusions and Implications* – Principal components of water and tissue trace element concentrations were the best predictors of growth of juvenile *V. iris* in the Powell River. These PCs were driven by suites of major ions and trace elements associated with coal mining. PAHs do not appear to be exerting significant effects on mussel growth in the Powell River. Survival of juvenile *V. iris* was low at all sites indicating impacts to mussels are ongoing and widespread in

the Powell River. Significant mining impacts in the headwaters of the Powell River are likely a major source of contaminants to the river. Patterns of juvenile mussel growth in this study corresponded with survey data from the Powell River, showing degraded mussel populations in the upper river from Appalachia, VA downstream to Jonesville, VA and larger more diverse populations further downstream in Tennessee. Long-term monitoring of concentrations of trace elements and of specific conductance would generate information critical to understanding the dynamic role these contaminants play as influences on mussel survival and growth. Longer exposure times for *in-situ* caged mussels are needed to better assess the effects of certain contaminant exposure pathways. The use of multiple cohorts and multiple species in these longer-term exposures is needed as well.

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## TABLES

Table 1. Site names, river kilometer (RKM) locations, and longitude and latitude of study sites sampled in the Powell River in Wise and Lee Counties, VA and Hancock County, TN in 2016 and 2017.

<b>Site Number</b>	<b>Site Name</b>	<b>State</b>	<b>Zone</b>	<b>River Kilometer</b>	<b>Latitude</b>	<b>Longitude</b>
1	Appalachia	Virginia	Zone 1	293.7	36.904834	-82.7813
2	Big Stone Gap	Virginia	Zone 1	287.2	36.863494	-82.7855
3	Dryden	Virginia	Zone 1	269.1	36.838605	-82.8271
4	Pennington Gap	Virginia	Zone 1	252.6	36.735584	-82.9993
5	McDowell	Tennessee	Zone 2	171.9	36.57649	-83.3639
6	Upper Brooks Bridge	Tennessee	Zone 2	153.4	36.560758	-83.3933
7	Oakley Property	Tennessee	Zone 2	144.2	36.53509	-83.4417
8	Yellow Shoals	Tennessee	Zone 2	136.2	36.535747	-83.4681

**Table 2. Sampling dates, number of days to sampling event, description of samples taken, and analysis performed at the eight river kilometer sites in the Powell River in Wise and Lee Counties, VA and Hancock County, TN in 2016 and 2017.**

Date	Day	Sample Types	Analysis
<b>Water Sampling</b>			
7/13/2016	0	<ul style="list-style-type: none"> <li>• Pore Water Filtered</li> <li>• Pore Water Unfiltered</li> <li>• Surface Water Filtered</li> <li>• Surface Water Unfiltered</li> </ul>	<p>Trace Elements</p> <p>Trace Elements</p> <p>Trace Elements</p> <p>Trace Elements</p>
10/26/2016	106	<ul style="list-style-type: none"> <li>• Pore Water Filtered</li> <li>• Pore Water Unfiltered</li> <li>• Surface Water Filtered</li> <li>• Surface Water Unfiltered</li> </ul>	<p>Trace Elements</p> <p>Trace Elements, PAHs</p> <p>Trace Elements</p> <p>Trace Elements</p>
9/7/2017	423	<ul style="list-style-type: none"> <li>• Pore Water Filtered</li> <li>• Pore Water Unfiltered</li> <li>• Surface Water Filtered</li> <li>• Surface Water Unfiltered</li> </ul>	<p>Trace Elements</p> <p>Trace Elements, PAHs</p> <p>Trace Elements</p> <p>Trace Elements</p>
<b>Mussel Sampling</b>			
7/12/2016	0	<ul style="list-style-type: none"> <li>• Mussels deployed and maximum length measured</li> <li>• 10 individuals from Cohort 1 (23 months old) and 20 individuals from Cohort 2 (7 months old) placed in each silo</li> <li>• Six silos deployed at each site.</li> </ul>	
10/26/2016	106	<ul style="list-style-type: none"> <li>• Mortality and growth of Cohort 1 and 2 recorded</li> <li>• Tissue collected from Cohort 1 (N=60 per site)</li> </ul>	<p>Trace Elements for Cohort 1 Tissues</p> <p>PAHs for Cohort 1 Tissues</p>
9/7/2017	423	<ul style="list-style-type: none"> <li>• Mortality and growth of Cohort 2 recorded</li> <li>• Tissue collected from Cohort 2 (all remaining live mussels) and <i>Corbicula fluminea</i>(N=~20 per site)</li> </ul>	<p>Trace Elements for Cohort 2 Tissues</p> <p>PAHs for <i>Corbicula fluminea</i> Tissues</p>

**Table 3. Results of statistical analysis of average trace element concentrations in four water sample types regressed by site river kilometer for sites sampled in the Powell River in Wise and Lee Counties, VA and Hancock County, TN in 2016 and 2017. Only statistically significant p-values and R<sup>2</sup> values are reported. “–” represents non-significant results.**

Element	Pore Filtered			Pore Unfiltered			Surface Filtered			Surface Unfiltered		
	P-value	Slope	R <sup>2</sup>	P-value	Slope	R <sup>2</sup>	P-value	Slope	R <sup>2</sup>	P-value	Slope	R <sup>2</sup>
Ba	–	–	–	–	–	–	0.01	0.003	0.25	0.01	0.0008	0.61
Ca	<0.0001	0.003	0.72	0.006	0.003	0.29	<0.0001	0.003	0.76	<0.0001	0.003	0.60
Fe	–	–	–	–	–	–	0.006	0.008	0.30	0.0005	0.005	0.43
K	<0.0001	0.004	0.89	<0.0001	0.004	0.76	<0.0001	0.004	0.91	<0.0001	0.004	0.84
Mg	<0.0001	0.004	0.76	<0.0001	0.004	0.66	<0.0001	0.005	0.81	<0.0001	0.005	0.79
Na	<0.0001	0.007	0.85	<0.0001	0.007	0.81	<0.0001	0.008	0.87	<0.0001	0.007	0.81
Ni	0.002	0.002	0.35	–	–	–	0.0007	0.002	0.42	<0.0001	0.002	0.74
SO <sub>4</sub> <sup>2-</sup>	0.004	0.01	0.32	0.005	0.01	0.31	<0.0001	0.009	0.89	<0.0001	0.008	0.84
Se	0.007	0.007	0.28	0.003	0.005	0.33	0.004	0.008	0.33	–	–	–
Sr	<0.0001	0.007	0.91	<0.0001	0.007	0.82	<0.0001	0.008	0.93	<0.0001	0.007	0.90
U	<0.0001	0.006	0.84	0.003	0.003	0.33	<0.0001	0.007	0.92	<0.0001	0.006	0.91

**Table 4. Mean concentrations (ppb) of trace elements measured across all three sampling events (Day 0, 106, and 423) and EPA acute and chronic criteria for four water sample types by zone at eight sites in the Powell River in Wise and Lee Counties, VA and Hancock County, TN in 2016 and 2017.**

Trace Element	Pore Filtered		Pore Unfiltered		Surface Filtered		Surface Unfiltered			
	Acute	Chronic	Zone 1	Zone 2	Zone 1	Zone 2	Zone 1	Zone 2	Zone 1	Zone 2
Ag	11.0		0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
Al			9.2	19.2	4717.6	4464.6	86.0	8.7	27.0	32.6
As	340.0	150.0	0.3	0.4	2.5	2.3	0.2	0.3	0.2	0.3
Ba			57.6	53.0	160.7	223.7	85.8	45.5	49.7	44.4
Ca			72719.2	52587.5	92429.2	59187.5	75784.2	51803.3	68321.7	48590.8
Cd	7.5	1.8	0.0	0.0	0.3	0.3	0.0	0.0	0.0	0.0
Cl	860000.0	230000.0	5446.9	4609.9	5204.0	3816.6	5599.4	4900.8	5170.5	4521.3
Co			0.2	0.1	14.1	15.5	0.1	0.1	0.1	0.1
Cr			0.4	0.4	19.1	12.1	0.5	0.4	0.3	0.3
Cu	23.1	14.6	2.8	4.3	29.3	16.7	0.7	0.7	0.4	0.3
Fe			32.5	14.0	16960.1	11517.0	48.7	19.5	113.9	63.0
K			4849.8	2926.3	4790.4	3039.3	4975.9	2892.7	4468.9	2676.2
Mg			36985.0	21531.7	37026.7	21991.7	39870.8	22490.8	36498.3	20915.8
Mn			188.8	68.2	1586.8	2073.3	13.4	9.1	18.9	16.0
Mo			0.7	0.6	0.9	0.5	0.5	0.4	0.3	0.3
Na			57118.3	23509.2	50255.0	21380.0	65745.0	24940.0	54496.7	22638.3
Ni	296.6	33.0	1.7	1.3	31.1	21.2	1.2	1.0	1.2	0.9
Pb	248.3	28.2	0.3	0.4	27.4	34.3	0.0	0.0	0.1	0.2
Se	20.0	5.0	0.9	0.4	0.7	0.3	1.0	0.3	0.6	0.3
Si			2555.4	2096.0	9793.3	7572.7	1970.3	1415.0	1863.9	1532.5
Sr			1127.2	437.3	1191.6	466.5	1249.6	457.1	1124.6	445.4
U			0.8	0.4	1.2	0.8	0.9	0.3	0.8	0.4
V			0.2	0.4	9.7	11.3	0.2	0.3	0.2	0.3
Zn	190.7		44.0	23.8	381.5	278.3	10.0	2.4	1.2	3.1

**Table5. Principal Component 1 (PC1) eigenvector values from PCA analysis for trace elements in four water samples types: pore filtered, pore unfiltered, surface filtered, surface unfiltered from sites sampled in the Powell River in Wise and Lee Counties, VA and Hancock County, TN in 2016 and 2017. “—” signifies non-significant eigenvector values that were <0.200.**

Trace Element	Pore Filtered			Pore Unfiltered			Surface Filtered			Surface Unfiltered		
	Day 1	Day 106	Day 423	Day 1	Day 106	Day 423	Day 1	Day 106	Day 423	Day 1	Day 106	Day 423
	PC1	PC1	PC1	PC1	PC1	PC1	PC1	PC1	PC1	PC1	PC1	PC1
Al	-0.26	—	—	0.24	0.29	0.32	0.20	—	—	—	—	-0.27
Ba	—	0.25	—	0.25	0.28	0.32	0.27	0.24	0.27	0.26	0.27	—
Ca	0.31	0.32	0.32	0.26	—	—	0.29	0.31	0.30	0.31	0.31	0.30
Cl	—	—	—	—	—	-0.26	—	—	—	—	—	—
Cu	-0.22	—	—	0.26	0.22	0.26	—	—	—	—	—	—
Fe	—	—	0.30	0.27	0.26	0.32	0.27	0.25	0.26	0.22	0.28	0.21
K	0.32	0.32	0.31	0.25	—	—	0.30	0.31	0.29	0.32	0.32	0.30
Mg	0.31	0.32	0.32	0.24	-0.23	—	0.30	0.30	0.30	0.31	0.31	0.30
Mn	0.23	—	—	0.26	0.29	0.31	0.29	—	—	—	0.23	—
Na	0.32	0.32	0.31	0.21	-0.22	—	0.31	0.31	0.30	0.33	0.32	0.30
Ni	0.30	—	0.28	0.27	0.23	0.32	—	0.27	0.29	0.28	0.29	0.28
P	—	—	-0.22	0.25	0.29	0.31	—	—	—	—	—	—
Pb	—	—	0.29	0.21	0.33	0.31	—	—	0.14	—	—	-0.28
S	0.31	0.32	—	0.20	-0.23	—	0.31	0.31	0.30	0.32	0.32	0.30
Se	—	0.26	0.20	0.23	-0.28	—	0.29	0.31	—	—	—	0.25
Si	—	0.21	—	0.27	0.23	0.31	—	0.28	0.29	0.30	—	0.29
Sr	0.32	0.32	0.29	0.22	-0.19	—	0.31	0.31	0.29	0.32	0.32	0.29
Zn	—	—	0.28	—	—	—	—	—	0.26	—	—	—
Eigenvalue	8.4	9.2	9.1	12.9	8.7	9.4	10.3	10.2	11.1	9.4	9.4	11.1
% Variance Explained	46.7	51.4	50.5	71.6	48.2	52.4	57.4	56.8	65.0	52.4	52.1	61.4



Table6. Eigenvector values from principal component analysis of juvenile *Villosa iris* at Day 106 on October 10, 2017 and Day 423 on September 7, 2017 for tissue trace element Principal Component 1 (PC1) and Principal Component 2 (PC2) at sites sampled in the Powell River in Wise and Lee Counties, VA and Hancock County, TN. “—” signifies non-significant eigenvector values that were <0.200.

	Day 106	Day 106	Day 423	Day 423
	PC1	PC2	PC1	PC2
<b>Ag</b>	0.24	—	—	0.22
<b>Al</b>	—	-0.27	—	0.30
<b>As</b>	—	0.36	0.22	—
<b>Ba</b>	—	0.24	0.21	—
<b>Ca</b>	—	0.30	—	—
<b>Cd</b>	0.24	—	—	-0.30
<b>Co</b>	—	—	—	—
<b>Cr</b>	—	—	—	0.30
<b>Cu</b>	0.22	—	—	-0.24
<b>Fe</b>	0.23	—	—	—
<b>Mg</b>	0.24	—	0.23	—
<b>Mn</b>	0.24	—	—	—
<b>Mo</b>	0.25	—	0.23	—
<b>Ni</b>	0.21	—	0.22	—
<b>P</b>	—	0.36	0.22	—
<b>Pb</b>	—	—	—	0.32
<b>S</b>	0.21	—	0.22	—
<b>Se</b>	0.22	—	—	-0.24
<b>Si</b>	—	-0.21	—	0.33
<b>Sn</b>	0.24	—	—	-0.28
<b>Sr</b>	0.21	—	0.20	—
<b>Ti</b>	—	0.27	0.20	—
<b>U</b>	0.22	—	—	—
<b>V</b>	—	-0.33	—	0.30
<b>Zn</b>	0.23	—	0.23	—
<b>Eigenvalue</b>	15.7	4.6	18.0	4.5
<b>% Variance Explained</b>	56.1	16.5	64.5	16.1

Table7. Results of regression models for specific conductance, temperature, and Principal Component 1 (PC1) and Principal Component 2 (PC2) for pore water and surface water samples regressed against growth of juvenile *Villosa iris* Cohort 1 and 2 at Day 106 on July 13, 2016 and Day 423 on October 26, 2017 at sites sampled in the Powell River in Wise and Lee Counties, VA and Hancock County, TN.

Cohort	Day	Variable	P-Value	Slope	AICc	R <sup>2</sup>
1	106	Specific conductance	<0.0001	-0.012	185.36	0.6097
		Temperature	<0.0001	1.24	193.271	0.5398
		Pore Filtered for PC1	<0.0001	-0.68	192.063	0.5512
		Pore Unfiltered for PC1	<0.0001	-0.43	211.138	0.3323
		Surface Filtered for PC1	<0.0001	-0.67	180.475	0.6475
		Surface Unfiltered for PC1	<0.0001	-0.68	185.607	0.6077
		Tissue for PC2	0.0015	0.52	219.874	0.1990
2	106	Specific conductance	<0.0001	-0.008	135.35	0.6749
		Temperature	<0.0001	0.88	138.911	0.6499
		Pore Filtered for PC1	0.6278			
		Pore Unfiltered for PC1	0.4229			
		Surface Filtered for PC1	0.8292			
		Surface Unfiltered for PC1	0.762			
2	423	Specific conductance	<0.0001	-0.013	202.33	0.5495
		Temperature	<0.0001	1.54	201.645	0.5562
		Pore Filtered for PC1	<0.0001	-0.72	212.786	0.4345
		Pore Unfiltered for PC1	<0.0001	0.64	221.141	0.3219
		Surface Filtered for PC1	<0.0001	-0.75	201.603	0.5566
		Surface Unfiltered for PC1	<0.0001	-0.76	206.194	0.51
		Tissue for PC1	<0.0001	-0.52	208.008	0.4903
		Tissue for PC1 & PC2	<0.0001	0.76	181.703	0.7269

## FIGURES

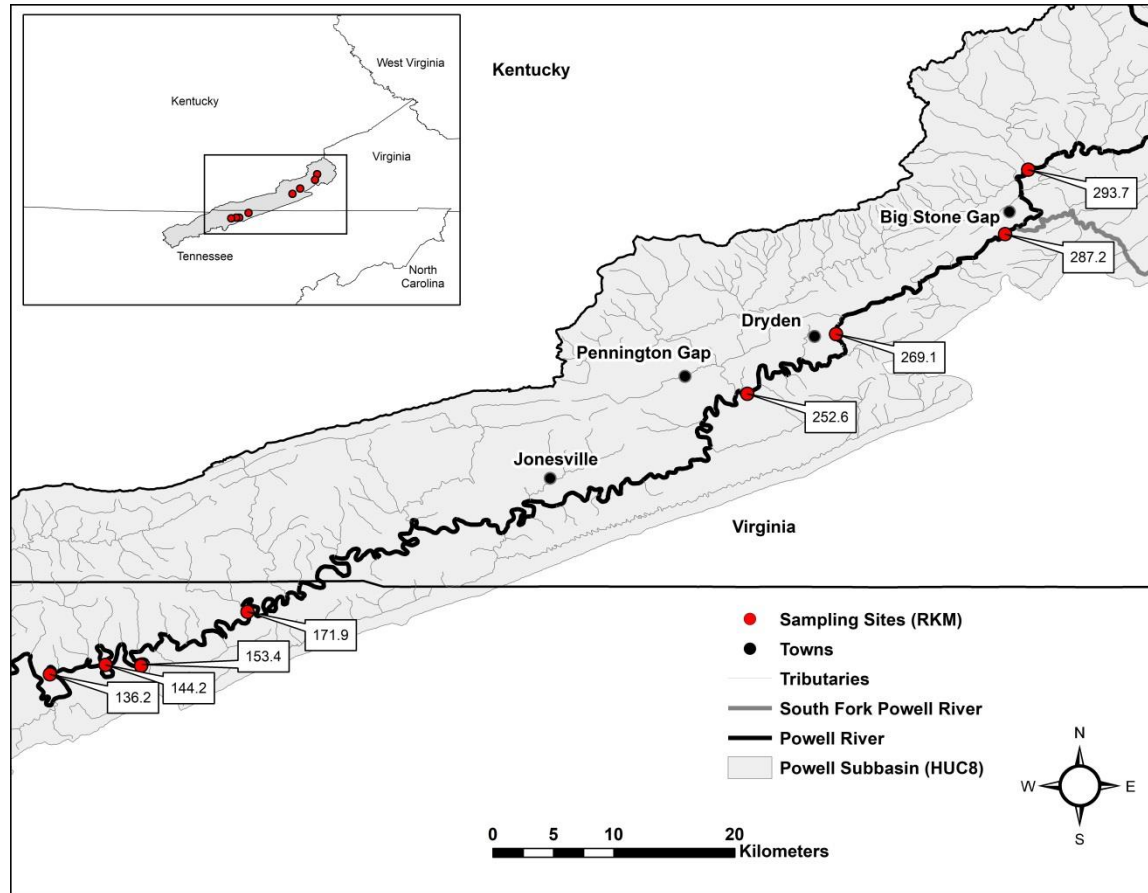
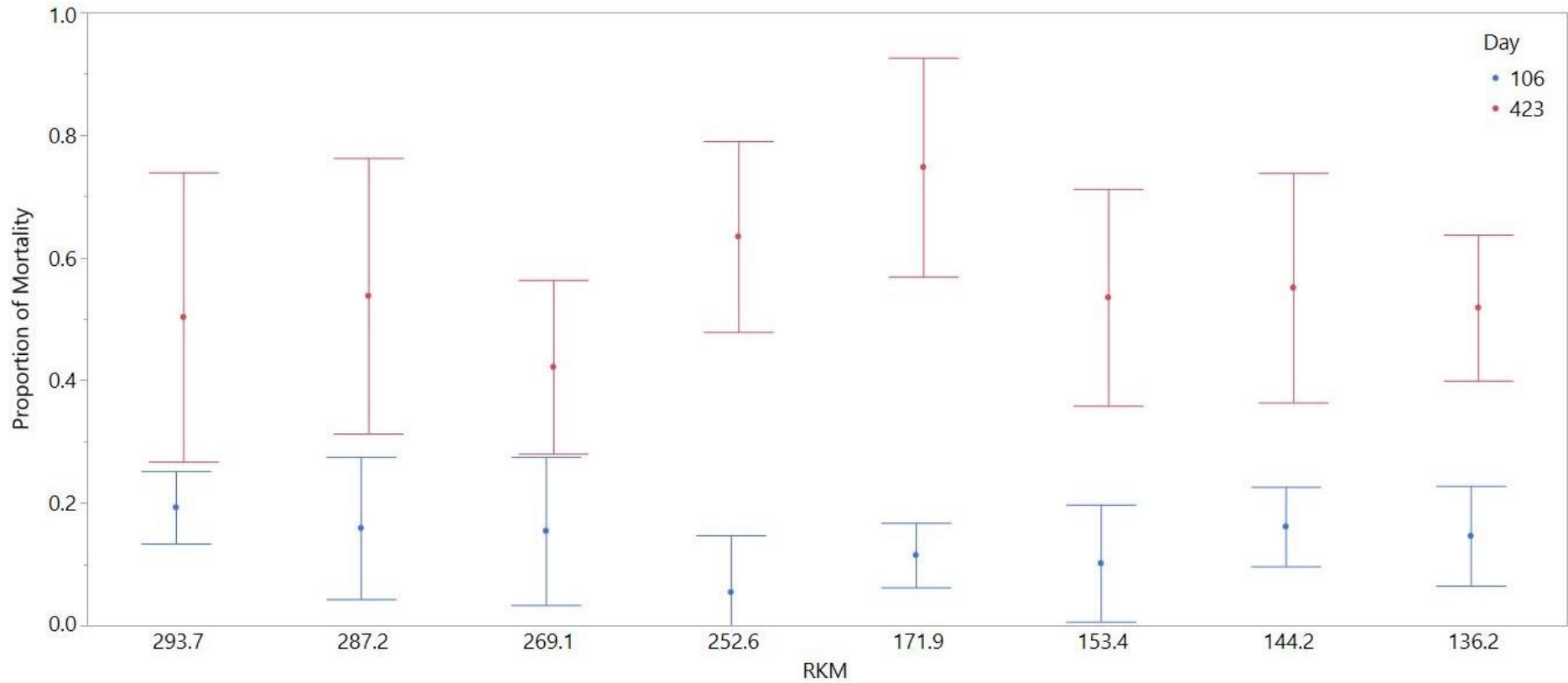
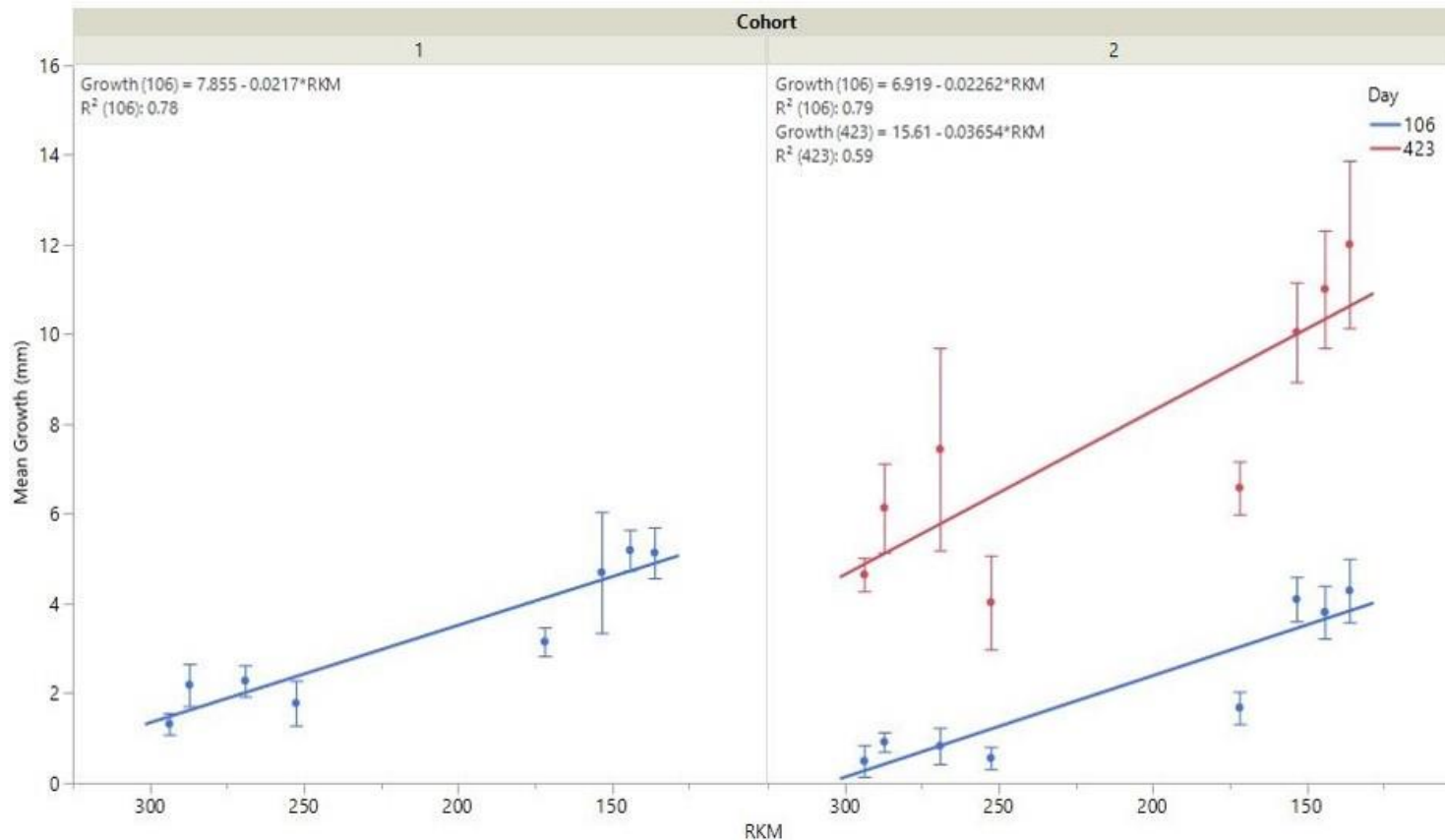


Figure 3. Site river kilometer (RKM) locations sampled in the Powell River in Wise and Lee Counties, VA and Hancock County, TN in 2016 and 2017.



**Figure 4** Mean proportion mortality of *Villosa iris* juveniles of Cohort 2 at Day 106 from July 12 to October 26, 2016 and at Day 423 from July 12, 2016 to September 7, 2017. Mussels were held in silos (see methods) in the Powell River at eight river kilometer (RKM) locations in Wise and Lee Counties, VA and Hancock County, TN. Error bars represent 95% confidence intervals. Mean mortality did not vary significantly among sites for Day 106 ( $p=0.33$ ) or for Day 423 ( $p=0.28$ ).



**Figure 5.** Mean growth (mm) of *Villosa iris* juveniles of Cohorts 1 and 2 at Day 106 from July 12 to October 26, 2016 and Cohort 2 at Day 423 from July 12, 2016 to September 7, 2017. Mussels were held in silos (see methods) in the Powell River at eight river kilometer (RKM) locations in Wise and Lee Counties, VA and Hancock County, TN. Error bars represent 95% confidence intervals. Mean growth for Cohort 1 at Day 106 had a significant negative relationship with RKM ( $p < 0.0001$ , Slope = -0.022, and  $R^2 = 0.44$ ). Mean growth for Cohort 2 at Day 106 ( $p < 0.0001$ , Slope = -0.023, and  $R^2 = 0.45$ ) and Day 423 ( $p < 0.0001$ , Slope = -0.036, and  $R^2 = 0.33$ ) had a significant negative relationship with RKM.

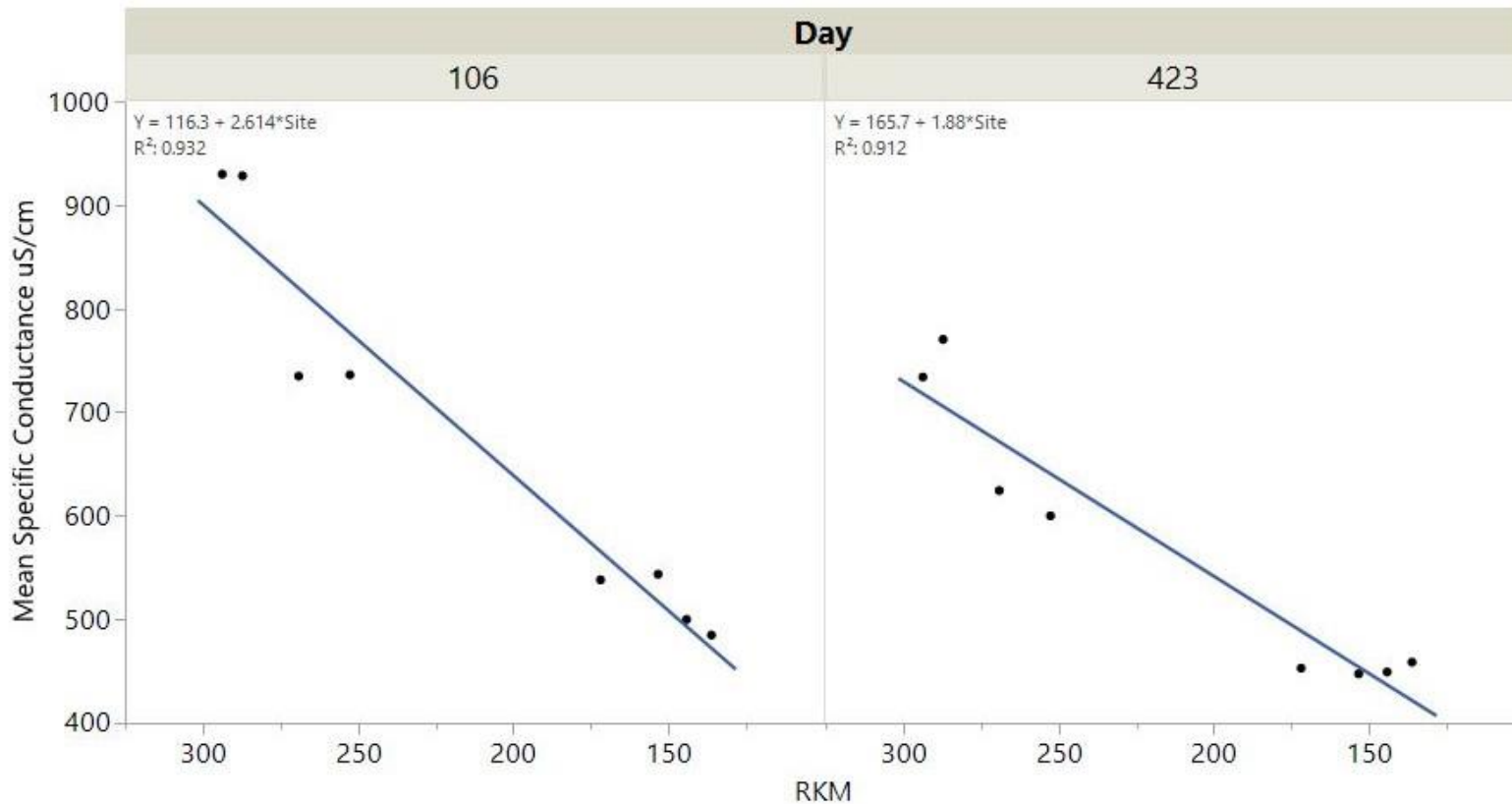
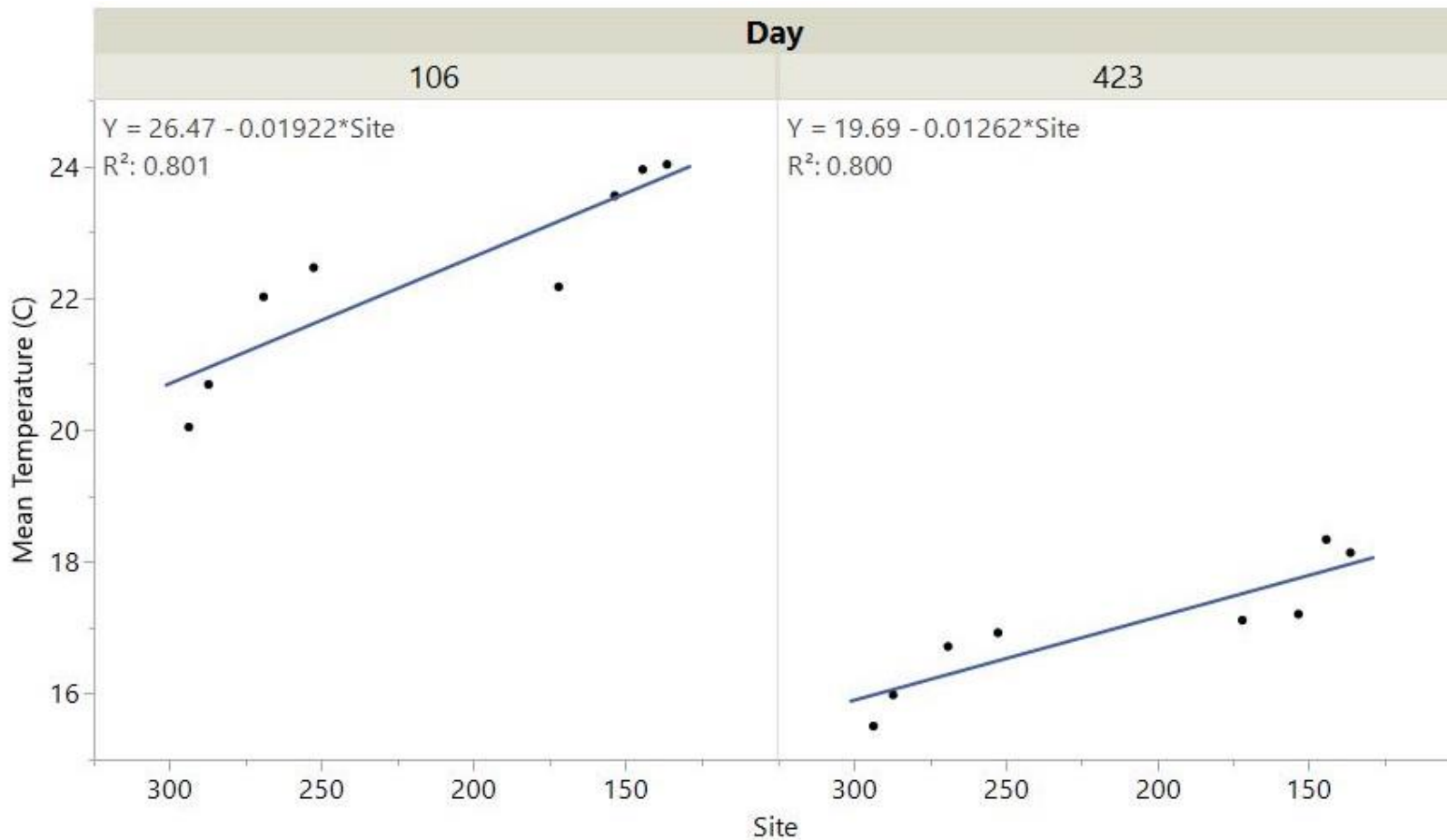


Figure 6. Mean specific conductance ( $\mu\text{S}/\text{cm}$ ) at eight river kilometer (RKM) locations in the Powell River, Wise and Lee Counties, VA and Hancock County, TN from Day 0 to Day 106 from July 12 to October 26, 2016 and from Day 0 to Day 423 from July 12, 2016 to September 7, 2017. Error bars represent 95% confidence intervals. Mean specific conductance was significantly different among sites at Day 106 ( $p < 0.0001$ , Slope = 2.59, and  $R^2 = 0.93$ ) and Day 423 ( $p < 0.0001$ , Slope = 1.88, and  $R^2 = 0.91$ ).

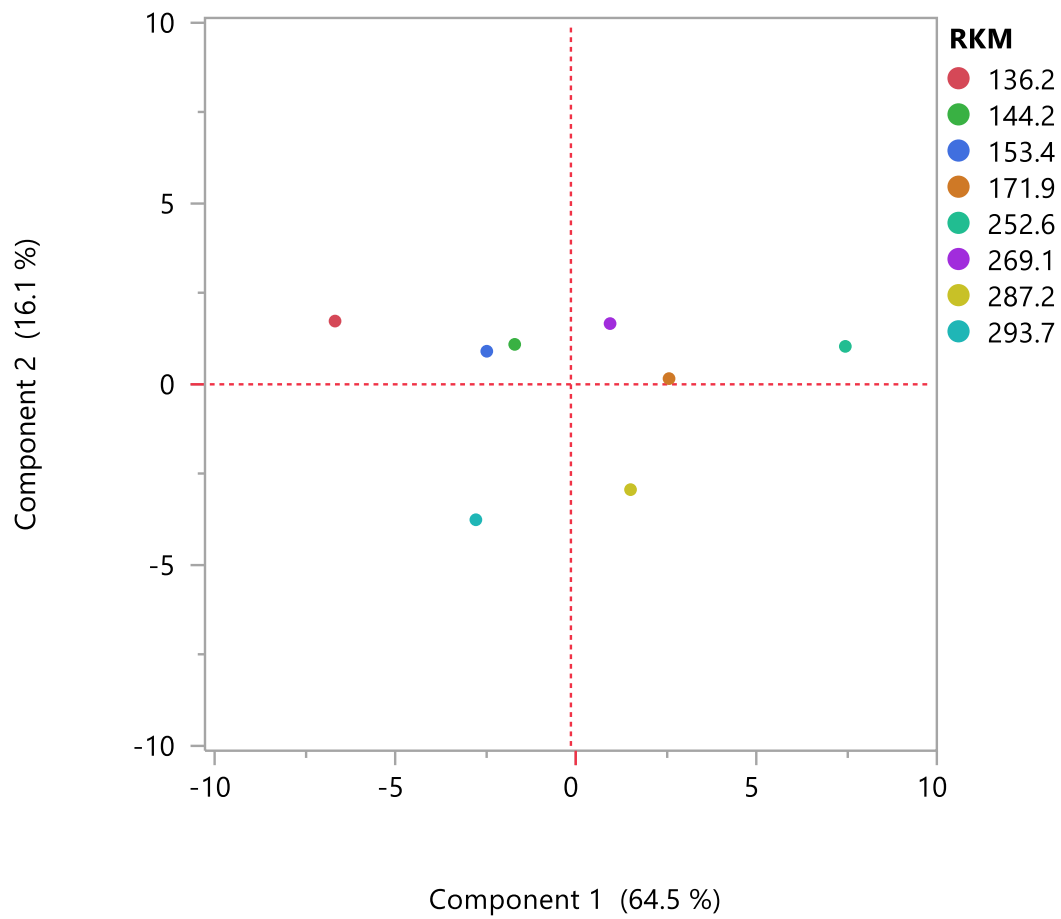


**Figure 7.** Mean temperature (°C) at river kilometer (RKM) locations in the Powell River, Wise and Lee Counties, VA and Hancock County, TN from Day 0 to Day 106 from July 12 to October 26, 2016 and from Day 0 to Day 423 from July 12, 2016 to September 7, 2017. Mean temperature (°C) was significantly negatively correlated with RKM at Day 106 ( $p=0.003$ , Slope = -0.02, and  $R^2 = 0.80$ ) and Day 423 ( $p=0.003$ , Slope = -0.013, and  $R^2 = 0.80$ ). Error bars represent 95% confidence intervals.



**Figure 8.** Polycyclic aromatic hydrocarbon (PAH) concentrations (ng/g) in tissues of juvenile *Villosa iris* of Cohort 1 at Day 106 on October 10, 2016 and *Corbicula fluminea* at Day 423 on September 7, 2017 and pore water PAH concentrations (ng/L) among river kilometer (RKM) locations in the Powell River, Wise and Lee Counties, VA and Hancock County, TN. There were no significant correlations between any PAH tissue or pore water concentrations to RKM ( $p>0.05$ ).





**Figure 9.** Score plot of tissue trace element Principal Component 1 by Principal Component 2 at the Day 423 sampling event at the eight Powell River RKM locations in Wise and Lee Counties, VA and Hancock County, TN.





## Appendix 2

Concentrations ( $\mu\text{g/g}$ ) of 28 trace elements in mussel tissues sampled on three occasions: Day 0 on July 7, 2016 (Baseline), Day 106 on October 26, 2016 (Cohort 1), and Day 423 on September 7, 2017 (Cohort 1) at eight river kilometer (RKM) sites in the Powell River in Virginia and Tennessee.

RKM	Date	Ag	Al	As	Ba	Ca	Cd	Cl	Co	Cr	Cu	Fe	K	Mg	Mn	Mo	Na	Ni	P	Pb	S	Se	Si	Sn	Sr	Ti	U	V	Zn
<b>136.2</b>	10/26/2016	0.19	278.28	7.47	205.56	73314.73	0.4	0.46	1.16	0.7	13.37	1667.29	1490.18	1684.74	1499.57	0.39	1255.9	1.69	20188.39	1.13	21.14	4.54	647.27	0.48	443.32	66.98	0.12	0.55	243.59
	9/8/2017	0.57	436.93	4.07	96.75	22124.39	0.64	0.48	1.09	0.95	18.88	1155.6	1577.35	1692.57	1300.74	0.47	1199.21	1.71	19978.22	1.56	19.37	8.11	763.13	0.67	143.16	61.05	0.21	0.45	210.09
<b>144.2</b>	10/26/2016	0.3	538.89	5.61	108.53	20666.38	0.5	0.61	0.86	0.86	14.66	1191.08	1735.59	1567.12	1088.76	0.39	1303.84	1.47	17993.36	1.58	19.2	5.6	1089.3	0.55	246.14	65.89	0.12	0.98	217.43
	9/8/2017	0.75	738.11	4.63	89.75	21472.35	1.11	1.05	1.05	1.08	29.23	1559.85	2128.49	1891.66	1281.33	0.58	1413.56	2.13	20515.17	1.94	21.55	11.42	1114.82	1.11	222.89	64.14	0.2	0.99	262.98
<b>153.4</b>	10/26/2016	0.23	373.55	6.07	118.77	19226.65	0.38	0.44	0.94	0.89	13.03	1413.63	1646.44	1664.82	1085.11	0.36	1163.65	1.45	18929.83	1.87	21.71	5.59	824.63	0.42	245.53	63.06	0.12	0.74	245.13
	9/8/2017	0.69	384.52	4.89	104.98	25783.28	0.81	1.44	1.38	1.08	23.54	1152.63	1854.18	1871.83	1373.37	0.58	1574.61	2.25	22340.56	1.7	21.69	9.45	596.59	0.84	159.94	73.03	0.25	0.38	252.72
<b>171.9</b>	10/26/2016	0.41	571.36	6.06	123.03	17837.71	0.56	0.62	1.3	0.98	18.67	1564.68	2011.46	1862.05	1186.16	0.5	1457.28	2.13	18136.04	1.41	22.65	8.53	1172.55	0.61	306.44	62.86	0.11	1.03	267.3
	9/8/2017	0.93	501.27	5.86	148.91	35023.33	1.14	0.9	1.42	1.37	31.38	1442.16	2192.84	2505.51	1808.31	0.78	1723	2.32	28835.05	1.71	24.67	11.65	794.76	1.17	249.78	86.99	0.34	0.44	308.23
<b>252.6</b>	10/26/2016	0.3	417.64	6.02	94.91	14712.33	0.51	1.67	1.15	1.38	16.11	1609.67	1675.17	1494.86	925.17	0.53	1381.59	2.39	16726.03	1.65	21.82	7.56	826.03	0.57	240.39	54.22	0.22	0.98	232.96
	9/8/2017	1.31	825.45	5.9	183.55	39025.28	1.45	1.37	2.09	1.77	41.55	2170.96	2230.21	2846.9	2318.18	1.01	1706.5	3.12	31290.42	2.59	26.14	14.54	1335.48	1.5	433.28	97.27	0.38	0.96	351.24
<b>269.1</b>	10/26/2016	0.32	550.72	4.41	110.61	15190.71	0.41	0.73	1.03	0.81	19.4	1490.06	1803.84	1751.14	1061.8	0.45	1378.9	2.48	17329.81	1.69	21.5	7.93	1065.79	0.45	281.89	57.68	0.16	1.04	241.15
	9/8/2017	0.72	914.66	5.1	108.65	23615.56	0.95	1.19	1.29	1.37	32.54	1800.94	2320.7	2187.72	1428.35	0.73	1563.07	2.56	22952.08	1.74	23.28	12.51	1412.7	0.97	270.4	70.37	0.23	1.28	272.31
<b>287.2</b>	10/26/2016	0.27	147.06	9.3	109.34	20985.68	0.47	1.94	1.43	0.83	14.83	1787.11	1848.65	1544.31	1070.01	0.51	1476.91	1.45	19043.26	1.16	24.27	6.74	275.37	0.56	253.77	61.24	0.12	0.49	292.03
	9/8/2017	0.59	429.12	5.7	118.72	24288.28	1.44	1.22	1.15	0.95	35.03	1772.06	2167.51	2248.47	1916.08	0.67	1517.42	2.55	24066.51	1.58	25.51	14.72	643.41	1.46	294.72	68.22	1.3	0.51	291.25
<b>293.7</b>	10/26/2016	0.2	261.62	5.05	64.26	11163.45	0.43	1.33	1.1	0.62	14.72	1336.98	1632.12	1419.21	669.65	0.32	1305.54	1.63	14942.16	0.72	21	6.33	468.86	0.47	183.39	47.32	0.08	0.61	186.85
	9/8/2017	0.52	321.91	4.79	103.86	23558.2	1.32	0.9	0.99	0.76	38.43	1480.42	1860.86	2080.67	1618.79	0.55	1222.76	2.06	21411.05	1.01	22.28	12.95	440.88	1.33	282.6	61.68	0.18	0.22	248.31
<b>Baseline</b>	7/12/2016	0.04	188.75	5.07	49.4	5638.59	0.06	0.74	0.71	0.54	8.69	821.16	1838.52	1069.73	238.77	0.17	1118.43	0.92	17067.78	0.75	16.56	2.63	382.29	0.12	131.6	56.36	0.04	0.53	150.72

### Appendix 3

Concentrations ( $\mu\text{g/g}$ ) of PAHs in mussel tissues and pore water sampled on three occasions: Day 0 on July 7, 2016 (Baseline), Day 106 on October 26, 2016 (Cohort 1), and Day 423 on September 7, 2017 (Cohort 1) at eight river kilometer (RKM) sites in the Powell River in Virginia and Tennessee.

Site (RKM)	Sample Type	Day	C1-Chrysenes	C1-Dibenzothiophenes	C1-Fluoranthenes/Pyrenes	C1-Fluorenes	C1-Naphthalenes
293.7	Tissue	106	—	—	—	—	3.57
		423	—	—	—	—	7.34
	Pore Water	106	—	7.35	9.29	11.08	42.61
287.2	Tissue	423	—	—	—	—	—
		106	1.33	0.66	3.11	—	1.86
	Pore Water	423	4.1	1.45	—	—	3.34
269.1	Tissue	106	—	8.21	—	—	39.59
		423	—	—	—	—	—
	Pore Water	106	1.84	—	—	—	6.27
252.6	Tissue	423	6.14	1.62	—	—	8.69
		106	—	7.29	—	—	34.93
	Pore Water	423	—	—	—	—	—
171.9	Tissue	106	2.51	—	—	—	3.02
		423	3.67	—	—	—	5.64
	Pore Water	106	—	6.8	—	8.85	15.29
153.4	Tissue	423	—	—	—	—	—
		106	1.45	—	—	—	—
	Pore Water	423	—	1.49	4.17	—	7.32
144.2	Tissue	106	—	4.54	—	—	25.99
		423	—	—	—	—	—
	Pore Water	106	3.37	—	—	—	2.33
136.2	Tissue	423	—	—	—	—	—
		106	3.9	—	—	—	—
	Pore Water	423	—	—	—	—	—
Baseline	Tissue	106	—	3.97	—	—	3.05
		423	—	—	—	—	—
	Pore Water	106	—	7.14	—	—	24.53
Baseline	Tissue	423	—	—	—	—	—
		0	—	—	—	—	—
	Pore Water	423	—	—	—	—	—

Appendix 3 continued.

Site (RKM)	Sample Type	Day	C2-Naphthalenes	C2-Phenanthrenes/Anthracenes	C3-Chrysenes	1,6,7-Trimethylnaphthalene	1-Methylnaphthalene
293.7	Tissue	106	2.13	2.65	—	—	—
		423	1.01	—	—	—	2.88
	Pore Water	106	42.38	20.07	—	—	19.97
		423	—	—	—	0.31	—
287.2	Tissue	106	1.02	3.25	—	—	—
		423	2.38	1.65	—	—	—
	Pore Water	106	39.43	14.9	—	—	19.93
		423	—	—	—	0.35	—
269.1	Tissue	106	3.75	2.5	—	—	2.49
		423	5.8	3.43	—	—	3.44
	Pore Water	106	26.74	12.71	—	—	17.32
		423	—	—	—	0.69	—
252.6	Tissue	106	1.81	—	—	—	1.15
		423	4.08	1.29	—	—	2.31
	Pore Water	106	23.07	9.32	—	—	6.76
		423	—	—	—	0.33	—
171.9	Tissue	106	1.02	—	—	—	—
		423	4.52	3.92	—	—	3.09
	Pore Water	106	22.01	8.61	—	—	12.82
		423	—	—	—	0.49	—
153.4	Tissue	106	1.32	—	—	—	—
		423	2.17	1.17	—	—	—
	Pore Water	106	15.91	7.85	—	—	9.27
		423	—	—	—	1.19	—
144.2	Tissue	106	—	—	—	—	—
		423	2.34	2.29	—	—	—
	Pore Water	106	20.25	6.77	—	—	12.11
		423	—	—	—	0.35	—
136.2	Tissue	106	—	—	—	—	—
		423	2.71	—	—	—	2.21
	Pore Water	106	25.58	11.33	—	—	11.21
		423	—	—	—	0.32	—
Baseline	Tissue	0	—	—	—	—	—

Appendix 3 continued.

Site (RKM)	Sample Type	Day	Anthracene	Benzo(a)anthracene	Benzo(a)pyrene	Benzo(b)fluoranthene	Benzo(e)pyrene
293.7	Tissue	106	2.25	—	—	—	—
		423	2.02	—	—	—	—
	Pore Water	106	—	4.01	—	—	—
287.2	Tissue	423	—	—	—	—	—
		106	3.76	1.9	—	—	0.82
	Pore Water	423	—	0.69	—	—	—
269.1	Tissue	106	4.19	3.01	—	—	—
		423	—	—	—	—	—
	Pore Water	106	3.56	2.88	—	—	—
252.6	Tissue	423	—	—	—	—	—
		106	0.92	—	—	—	—
	Pore Water	423	—	2.08	—	—	1.69
171.9	Tissue	106	3.61	—	—	—	—
		423	—	—	—	—	—
	Pore Water	423	—	2.57	—	—	2.34
153.4	Tissue	106	—	—	—	—	—
		423	—	—	—	—	—
	Pore Water	106	2.02	—	—	—	—
144.2	Tissue	423	—	—	—	—	—
		106	—	—	—	—	—
	Pore Water	423	—	—	—	—	—
136.2	Tissue	106	—	—	—	—	—
		423	—	—	—	—	—
	Pore Water	106	—	—	—	—	—
Baseline	Tissue	423	—	—	—	—	—
		0	—	—	—	—	—

Appendix 3 continued.

Site (RKM)	Sample Type	Day	C3-Phenanthrenes/Anthracenes	C4-Chrysenes	C4-Naphthalenes	C4-Phenanthrenes/Anthracenes	1,6,7-Trimethylnaphthalene
293.7	Tissue	106	1.99	—	—	—	—
		423	—	—	—	—	—
	Pore Water	106	14.61	—	15.9	—	4.16
287.2	Tissue	423	—	—	—	—	—
		106	2.02	—	2.56	—	—
	Pore Water	106	—	—	12.5	—	7.23
269.1	Tissue	423	—	—	—	—	—
		106	0.97	—	—	—	—
	Pore Water	106	2.18	—	3.32	—	2.1
252.6	Tissue	423	—	—	—	—	—
		106	—	—	—	—	—
	Pore Water	106	—	—	12.29	—	6.37
171.9	Tissue	423	—	—	—	—	—
		106	—	—	—	—	—
	Pore Water	106	3.85	—	—	—	4.62
153.4	Tissue	423	—	—	—	—	—
		106	—	—	—	—	—
	Pore Water	106	—	—	—	—	—
144.2	Tissue	423	—	—	—	—	—
		106	—	—	—	—	—
	Pore Water	106	2.25	—	8.56	—	4.23
136.2	Tissue	423	—	—	—	—	—
		106	—	—	—	—	—
	Pore Water	106	—	—	11.95	—	—
Baseline	Tissue	0	—	—	—	—	—



## Appendix 3 continued.

Site (RKM)	Sample Type	Day	C1-Naphthalenes	C2-Naphthalenes	C3-Naphthalenes	Chrysene	Dibenzo(a,h)anthracene
293.7	Tissue	106	—	—	—	3.11	—
		423	—	—	—	—	—
	Pore Water	106	—	—	—	4.26	—
287.2	Tissue	423	16.7	—	—	—	—
		106	—	—	—	5.9	—
	Pore Water	106	—	—	—	3.25	1.39
269.1	Tissue	423	20.2	—	—	—	—
		106	—	—	—	1.85	—
	Pore Water	106	—	—	—	3.33	—
252.6	Tissue	423	23.2	—	—	—	—
		106	—	—	—	0.88	—
	Pore Water	106	—	—	—	2.63	3.02
171.9	Tissue	423	9.92	—	—	—	—
		106	—	—	—	—	—
	Pore Water	106	—	—	—	4.68	2.43
153.4	Tissue	423	14.7	—	—	—	—
		106	—	—	—	—	—
	Pore Water	106	—	—	—	—	—
144.2	Tissue	423	13.5	—	—	—	—
		106	—	—	—	—	—
	Pore Water	106	—	—	—	2.92	—
136.2	Tissue	423	28.6	—	—	—	—
		106	—	—	—	—	—
	Pore Water	106	—	—	—	1.51	2.74
Baseline	Tissue	0	4.54	—	—	—	—

Appendix 3 continued.

Site (RKM)	Sample Type	Day	C1-Phenanthrenes/Anthracenes	C2-Chrysenes	C2-Dibenzothiophenes	C2-Fluoranthenes/Pyrenes	C2-Fluorenes
293.7	Tissue	106	2.9	—	—	—	—
		423	1.32	—	—	—	—
	Pore Water	106	19.52	—	11.43	9.98	13.31
287.2	Tissue	423	—	—	—	—	—
		106	3.89	—	0.78	2.11	—
	Pore Water	106	16.23	—	10.44	—	12.65
269.1	Tissue	423	—	—	—	—	—
		106	2.87	—	—	1.84	—
	Pore Water	106	13.87	2.82	—	—	9.96
252.6	Tissue	423	—	—	—	—	—
		106	1.05	—	—	—	—
	Pore Water	106	11.59	—	—	—	15.46
171.9	Tissue	423	—	—	—	—	—
		106	0.74	—	—	—	—
	Pore Water	106	10.34	—	—	3.03	—
153.4	Tissue	423	—	—	—	—	—
		106	0.8	—	—	—	—
	Pore Water	106	6.86	—	3.63	—	—
144.2	Tissue	423	—	—	—	—	—
		106	0.79	—	—	—	—
	Pore Water	106	7.43	—	3.62	—	—
136.2	Tissue	423	—	—	—	—	—
		106	—	—	—	—	—
	Pore Water	106	10.31	—	5.69	—	—
Baseline	Tissue	0	—	—	—	—	—

Appendix 3 continued.

Site (RKM)	Sample Type	Day	1-Methylphenanthrene	2,6-Dimethylnaphthalene	2-Methylnaphthalene	Acenaphthene	Acenaphthylene
293.7	Tissue	106	0.7	—	2.16	—	1.86
		423	—	2.65	4.46	—	—
	Pore Water	106	4.74	10.78	22.64	—	10.3
287.2	Tissue	423	—	2.91	—	—	—
		106	0.94	—	1.07	0.96	2.71
	Pore Water	106	4.47	9.79	19.66	—	9.3
269.1	Tissue	423	—	3.45	—	—	—
		106	0.74	1.12	3.78	—	1.1
	Pore Water	106	5.62	9.37	17.61	—	6.89
252.6	Tissue	423	—	2.66	—	—	—
		106	—	0.64	1.87	—	0.6
	Pore Water	106	5.06	6.52	8.53	—	6.2
171.9	Tissue	423	—	1.34	—	—	—
		106	—	—	0.9	—	—
	Pore Water	106	3.03	6.49	13.17	—	4.93
153.4	Tissue	423	—	1.58	—	—	—
		106	—	—	1.43	—	—
	Pore Water	106	—	—	9.2	—	2.95
144.2	Tissue	423	—	0.61	—	—	—
		106	—	—	—	—	—
	Pore Water	106	0.63	6.11	12.42	—	6.23
136.2	Tissue	423	—	4.2	—	—	—
		106	—	—	—	—	—
	Pore Water	106	3.03	8.36	16.22	—	6.73
Baseline	Tissue	0	—	—	—	—	—

Appendix 3 continued.

Site (RKM)	Sample Type	Day	Benzo(g,h,i)perylene	C3-Dibenzothiophenes	C3-Fluoranthenes/Pyrenes	C3-Fluorenes	C3-Naphthalenes
293.7	Tissue	106	—	—	—	—	—
		423	—	—	—	—	—
	Pore Water	106	—	7.7	—	23.25	29.35
287.2	Tissue	423	—	—	—	—	—
		106	—	0.46	—	—	—
	Pore Water	106	—	8.46	—	—	25.47
269.1	Tissue	423	—	—	—	—	—
		106	—	—	—	—	2.33
	Pore Water	106	—	—	—	—	6.46
252.6	Tissue	423	—	—	—	—	—
		106	—	—	—	—	2.33
	Pore Water	106	—	—	—	—	6.46
171.9	Tissue	423	—	—	—	—	—
		106	—	—	—	—	20.02
	Pore Water	106	—	—	—	—	20.02
153.4	Tissue	423	—	—	—	—	—
		106	—	—	—	—	2.87
	Pore Water	106	—	—	—	—	2.87
144.2	Tissue	423	—	—	—	—	—
		106	—	—	—	—	18.83
	Pore Water	106	—	—	—	—	18.83
136.2	Tissue	423	—	—	—	—	—
		106	—	—	—	—	—
	Pore Water	106	—	—	—	—	4.06
Baseline	Tissue	423	—	—	—	—	—
		106	—	—	—	—	—
	Pore Water	106	—	—	—	—	14.99

Appendix 3 continued.

Site (RKM)	Sample Type	Day	Perylene	Phenanthrene	Pyrene	Benzo(k)fluoranthene	Biphenyl
293.7	Tissue	106	1.3	4.01	6.88	—	—
		423	—	12.71	10.36	—	3.02
	Pore Water	106	—	21.22	—	5.89	3.95
		423	—	—	—	—	—
287.2	Tissue	106	0.34	5.64	14.21	—	—
		423	0.97	1.98	—	—	—
	Pore Water	106	—	24.51	—	—	4.74
		423	—	—	—	—	—
269.1	Tissue	106	0.55	3.03	3.17	—	—
		423	1.58	4.18	2.82	—	3.17
	Pore Water	106	—	24.37	—	—	6.67
		423	—	—	—	—	—
252.6	Tissue	106	0.4	1.3	0.99	—	—
		423	2.7	2.28	—	1.65	—
	Pore Water	106	—	24.02	2.14	—	4.41
		423	—	—	—	—	—
171.9	Tissue	106	—	0.83	—	—	—
		423	2.91	6.59	6.97	1.51	—
	Pore Water	106	—	36.56	—	—	4.07
		423	—	—	—	—	—
153.4	Tissue	106	0.37	0.83	—	—	—
		423	—	1.71	—	—	—
	Pore Water	106	9.76	16.9	—	—	4.41
		423	—	—	—	—	—
144.2	Tissue	106	—	0.9	—	—	—
		423	—	1.72	—	—	—
	Pore Water	106	—	9.94	—	—	5.1
		423	—	—	—	—	—
136.2	Tissue	106	—	1.38	—	—	—
		423	—	1.75	—	—	2.31
	Pore Water	106	—	13.31	—	—	8.6
		423	—	—	—	—	—
Baseline	Tissue	0	—	—	—	—	—

## Appendix 3 continued.

Site (RKM)	Sample Type	Day	Dibenzothiophene	Fluoranthene	Fluorene	Indeno(1,2,3-c,d)pyrene	Naphthalene
293.7	Tissue	106	—	11.74	—	—	2.84
		423	—	14.44	1.79	—	3.43
	Pore Water	106	5.33	4.77	8.73	—	14.76
287.2	Tissue	423	—	—	—	—	—
		106	0.42	25.81	0.98	—	1.38
	Pore Water	106	5.71	6.03	8.33	—	17.8
269.1	Tissue	423	—	—	—	—	—
		106	—	7.45	—	—	2.56
	Pore Water	106	4.49	4.29	8.95	—	13.16
252.6	Tissue	423	—	—	—	—	—
		106	0.13	1.99	—	—	1.62
	Pore Water	106	3.67	3.63	10.36	—	8.07
171.9	Tissue	423	—	—	—	—	—
		106	—	1.19	—	—	1.55
	Pore Water	106	4.22	3.92	12.66	—	17.86
153.4	Tissue	423	—	—	—	—	—
		106	—	0.84	0.28	—	1.65
	Pore Water	106	2.37	—	6.19	—	8.33
144.2	Tissue	423	—	—	—	—	—
		106	—	1.01	—	—	1.02
	Pore Water	106	—	—	—	—	14.47
136.2	Tissue	423	—	—	—	—	—
		106	—	—	—	—	—
	Pore Water	106	2.03	—	6.06	—	19.61
Baseline	Tissue	0	—	—	—	—	—