Assessment of Mussel Declines in the Clinch and North Fork Holston Rivers Using Histological Evaluations of Vital Organs

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Abstract

The Clinch River (CR) and North Fork Holston River (NFHR) contain some of the most diverse freshwater mussel assemblages in the United States; however, both rivers are experiencing declines in mussel populations. The first component of this study used histological evaluations and water quality data to determine whether mussels were negatively impacted in the CR zone of decline (ZD) and to inform future management of freshwater mussels in the river. In the 91 kilometer (km) section from Carbo, Virginia (CRKM 431) downstream to Speers Ferry, Virginia (CRKM 340), referred to as the ZD, mussel density decreased >90% from 1979 to 2014 at key sites such as Semones Island (CRKM 378.3) and Pendleton Island (CRKM 364.2). Laboratory propagated mussels were placed in cages in the river for one year from June 2012 to May 2013 at four sites within the ZD and four sites in reaches where mussel populations remain stable or are increasing, a zone of stability (ZS). The survival, growth and histological results indicated that there are continuing impacts to mussels in the ZD. Research investigating impacts to the ZD and methods to improve water quality in this zone are needed.

The laboratory component of this study examined sublethal effects of potassium (K^+), chloride (Cl^-), and un-ionized ammonia (NH_3-N) on mussel tissues at concentrations relevant to those found in the NFHR. Historical industrial activities at Saltville, Virginia, as well as continued pollution of the NFHR from chemical waste ponds at this location, are believed to be significant contributors to mussel declines. Contaminant seepages from the waste ponds that include Cl^-, K^+, and NH_3-N have been shown to be toxic to adult and juvenile mussels. A three-month laboratory study was conducted to assess impacts to organ tissues (gills, digestive glands, kidneys, and gonads) of adult *Villosa iris* exposed to environmentally relevant concentrations of K^+ (4 and 8 mg/L), Cl^- (230 and 705 mg/L), and NH_3-N (0.014 and 0.15 mg/L) using histological evaluations. No detectable differences were observed among the histological endpoints from mussels held in treatments and control (p>0.05). The study design was modified and repeated

using increased concentrations of K^+ (8, 16, and 32 mg/L) and Cl^- (705, 1410, and 2820 mg/L) for a two-month exposure period. Due to issues with maintaining NH_3-N in mussel holding chambers, the second study did not the second study did not include NH_3-N exposures. Control mussels in both studies had a higher abundance of lipofuscin in kidneys and degraded cytoplasm in the digestive gland diverticula compared to baseline mussels, indicating that captivity influenced mussel tissues. Future studies are needed to more thoroughly address these captivity effects. Both survival and histological data in the second test showed a significant negative effect of the increased concentrations of Cl^- and K^+, which were representative of those found at some sites in the NFHR downstream of Saltville, Virginia.

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Chapter 1

Monitoring and Assessment of Mussel Declines in the Clinch River, Virginia Using Histological Evaluations of Vital Organs

Abstract

The Clinch River (CR) contains one of the most diverse freshwater mussel assemblages in the United States, with 46 extant species, 20 of which are listed as federally endangered. In the 91 kilometer (km) section from Carbo, Virginia (CRKM 431) downstream to Speers Ferry, Virginia (CRKM 340) mussel density decreased >90% from 1979 to 2014 at key sites such as Semones Island (CRKM 378.4) and Pendleton Island (CRKM 364.2). This study used histological evaluations and water quality data to determine whether mussels were negatively impacted in the zone of decline (ZD) and to inform future management of freshwater mussels in the river. Laboratory propagated mussels were placed in cages in the river for one year from June 2012 to May 2013 at four sites within the ZD and four sites in reaches where mussel populations remain stable or are increasing, a zone of stability (ZS). Mean shell growth (as measured by gain in total length in mm) of mussels in the ZS (4.6 mm) was significantly greater than mussels in the ZD (2.4 mm) (p<0.0001). There also was significantly higher survival in the ZS (65.42%) than in the ZD (43.82%) (p=0.0175). Histological differences in gonad and digestive gland tissues were not found between zones; however, fractions of kidney diverticula cells containing lipofuscin (FKDL), an indicator of exposure to contaminants in mussels, was significantly less from mussels held in the ZS ($\overline{x}$=0.24) compared to those held in the ZD ($\overline{x}$=0.27, p=0.0265). The survival, growth and FKDL histological results indicate that there are continuing impacts to mussels in the ZD. Research investigating impacts to the ZD and methods to improve water quality in this zone are needed.

Keywords: Freshwater Mussels, Histology, *Villosa iris*, Clinch River, Mussel Declines

Introduction

An estimated 840 freshwater mussel species are distributed worldwide, but with approximately 300, the greatest diversity is found in North America (Bogan 1993; Graf and Cummings 2007). However, mussels are experiencing drastic declines in North America, with about 10% already extinct and more than 70% in need of protection (Williams et al. 1993; Neves et al. 1997; Strayer et al. 2004; Haag and Williams 2013). These declines are most often attributed to a suite of impacts, including habitat destruction caused by dams, dredging and channelization, which drastically change the riverine and riparian environments, and also eliminate host fish species that are essential to mussel reproduction (Strayer et al. 2004; Haag 2012). Additionally, sedimentation from deforestation, agriculture, and urbanization causes degradation of stream water and sediments. Chemical contamination, primarily derived from agriculture and industry, often introduces heavy metals, pesticides, and mine drainage into the riverine system (Ortmann 1909; Ellis 1931; Cope et al. 2008). Commercial harvesting of freshwater mussels and introduction of exotic species (Asian clam, *Corbicula fluminea*, and zebra mussel, *Dreissena polymorpha*) also have been shown to negatively affect mussel populations (Claassen 1994; Strayer 1999; Strayer et al. 2004).

Freshwater mussels provide important ecosystem services, and with over 70 species federally listed as threatened or endangered, the United States Endangered Species Act of 1973 drives conservation actions to monitor and protect remaining mussel populations and the ecosystem services that they provide. These benthic invertebrates stabilize the lotic substrates and sequester suspended particles, nutrients and some pollutants by filtering stream water (Strayer et al. 1994; Vaughn and Hakenkamp 2001). They also are a source of food to animals such as muskrats, otters, raccoons, sportfish, and ducks (Helfrich et al. 1986).

The Clinch River (CR) contains one of the most diverse mussel assemblages in the United States, with 46 extant mussel species, 20 of which are listed as federally endangered (Jones et al. 2014). The CR has been a target for both research and restoration efforts due to its ecological importance for mussel diversity and conservation (Zipper et al. 2014). Located in the upper Tennessee River Valley drainage system and originating in Tazewell, Virginia, the CR flows freely through southwestern Virginia and northeastern Tennessee for 320 kilometers (km) until it meets Norris Reservoir (Locke et al. 2006; van der Schalie 1938; Neves 1991; Chaplin et

al. 2000; Zipper et al. 2014), a Tennessee Valley Authority impoundment constructed between 1933 and 1936 for flood-control and hydroelectric power (Tennessee Valley Authority 2012). The tributary watersheds of the CR are predominately forested; however, areas of agriculture, mining and urban development are found along the CR and throughout the watershed (Locke et al. 2006).

With 56 species documented historically, the current estimate of 46 extant species, (Ortmann 1918; Stansbery 1973; Locke et al. 2006; Jones et al. 2014) represents a serious decline in mussel diversity in the CR. Declines in both species richness and abundance have been observed. In the 91 km section from Carbo, Virginia (Clinch River Kilometer [CRKM] 431) downstream to Speers Ferry, Virginia (CRKM 340) mussel density decreased >90% from 1979 to 2014 at key sites such as Semones Island (CRKM 378.4), Pendleton Island (CRKM 364.2) (Jones et al. 2014). This reach of the upper CR, referred to here as the "zone of population decrease (ZD)," has been severely impacted by a variety of factors, including: wastewater effluents; chemical spills; and runoff and sediment from agriculture, deforested lands, mining influences and urbanization (Ahlstedt 1984; Dennis 1987; Jones et al. 2001; Zimmerman 2003; Ahlstedt et al. 2005; Zipper et al. 2014). A majority of land throughout the CR watershed is forested; however, permitted mining is more prevalent in the ZD than in other areas of the river. The Guest River flows through these heavily mined areas, bringing water with elevated conductivity directly into the ZD (Johnson et al. 2014).

Additionally, several spills have been blamed for mussel declines in the ZD. In 1967, a coal ash settling pond failed and spilled into Dumps Creek, a Clinch River tributary at Carbo, Virginia, the location of a coal-powered electricity generation plant. This spill released nearly 200 million m^3 of coal ash slurry with a pH around 12.5 into the river (Cairns et al. 1971; Ahlstedt et al. 2009) and resulted in elimination of mussels for >11 miles downstream of Carbo (Raleigh et al. 1978; Ahlstedt et al. 2009). A second spill from the Carbo power plant in 1970 released an unknown amount of sulfuric acid into the river. Mussels, snails, fishes and macroinvertebrates were impacted for 21 km downstream to St. Paul, Virginia (Ahlstedt et al. 2009).

In other reaches of the CR, recent surveys have shown that mussel populations are stable or actually increasing (Jones et al. 2014). These upper (from Nash Ford [CRKM 450] to the town of Carbo [CRKM 431]) and lower (Speers Ferry [CRKM 340] downstream to Wallen Bend

[CRKM 309]) reaches will be referred to here as the "zones of population stability (ZS)" because both reaches support mussel populations showing recruitment and with a high density and richness as compared to the ZD (Jones et al. 2014). The ZS encompasses reaches that are not influenced as heavily as the CR reach in the ZD by mine drainage, wastewater effluents, and other unknown impacts (Zipper et al. 2014). Tributaries entering the ZS from the Valley and Ridge physiographic province are unaffected by mining, as opposed to tributaries entering the ZD from the Appalachian Plateau (Johnson et al. 2014). The Freshwater Mollusk Conservation Center (FMCC), Department of Fish and Wildlife Conservation, Virginia Tech, Blacksburg, along with agencies in Tennessee and Virginia, have targeted sites in ZS reaches for restoration of mussel populations.

Many studies of toxicant effects on mussels have focused on growth and mortality as primary endpoints. However, there is a lack of research focusing on sub-lethal bio-markers or indicators of mussel health, which can be valuable tools in assessing mussel physiological condition. Histological evaluations showing adverse tissue outcomes may provide an indication that health of mussels may be impaired by specific local conditions, and can potentially demonstrate non-lethal, chronic effects of contaminants.

Studying the impacts of contaminants on mussel organ tissues in the ZS and ZD can provide baseline data for monitoring mussel health in presumptively "impacted" and "unimpacted" sites in the CR. The variables used in this study to evaluate condition of mussels at sites in the CR included evaluations of gonad, digestive gland, gill, and kidney tissues, and have been used in previous studies to demonstrate effects of contaminants on vital organ tissues of marine bivalves (Bayne et al. 1981; Seiler and Morse 1988; Au 2004). Recent studies using histological evaluations have shown atrophy of digestive gland cells, erosion of gill cilia, and differences in gamete production and resorption of oocytes to be indicators of exposure to contaminants (Henley 2007; Henley et al 2008a; 2008b). Organ tissues are sites of absorption, sequestration, accumulation and excretion of contaminants, all of which can cause cellular alterations to occur (Seiler and Morse 1988; Domouhtsidou and Dimitruadis 2000).

For example, acini within the gonads are the site of gamete development in mussels, which includes a natural cycle of maturation, spawning and resorption of gametes (Kennedy and Battle 1964; Pipe 1987; Dorange and Pennec 1989; Barber 1996; Henley 2010). Various metal and organic contaminants have been shown to affect this process by suppressing development of

gametes in bivalves (Gosling 2003). Resorption of gametes in the acini is a natural occurrence after a mussel spawns; however, untimely resorption has been observed as a response to stress and exposure to contaminants (Bayne and Thompson 1970; Bayne et al. 1981; Tay et al. 2003; Henley 2010).

The bivalve digestive gland contains diverticula with epithelial layers made up of secretory and digestive cells. These diverticula cells are the site of intra- and extra-cellular digestion, nutrient absorption, lipid and glycogen storage, and contaminant detoxification (Owen 1970; Lobo-da Cunha 1999; Petrović et al. 2001; Henley 2010). Contaminant exposure can cause cellular alteration in the digestive gland including degradation of cytoplasm in the diverticula cells (Lowe et al. 1981; Au 2004; Usheva et al. 2006; Henley et al. 2013).

Bivalve gills are paired ciliated organs involved in respiration, osmoregulation, and food capture and transport. Because they are the first uptake site in the mussel, gill cells are exposed to many contaminants found in water (Gómez-Mendikute et al. 2005). Loss of cilia along with fusion of gill filaments, inflammation, necrosis, epithelial cell sloughing, and increased mucus production are all histopathological alterations that have been linked to contaminant exposure (Domouhtsidou and Dimitruadis 2000; Lajtner et al. 2003; Gómez-Mendikute et al. 2005; Supanopas et al. 2005; Henley et al. 2013).

The bivalve kidney is responsible for ultrafiltration of hemolymph, ion exchange, and excretion (Dietz et al. 2000; Fahrner and Haszprunar 2002). The kidney also is responsible for sequestering contaminants by excretion of lipofuscin granules. The abundance of lipofuscin can increase with age, but also has been related to contaminant exposure in mussels (Riveros et al. 2002; Kagley et al. 2003).

The purpose of this study was to use histological evaluations combined with water quality data to determine whether freshwater mussels continue to be negatively impacted in the Clinch River in the ZD and to inform future management of freshwater mussels in the river. The specific objectives of the project were as follows:

1. To assess the condition of vital organ tissues from adult rainbow mussels, *Villosa iris*, held in cages at eight sites in the CR (four sites in the ZS and four sites in the ZD) using microscopical evaluations of histologically prepared gill, digestive gland, kidney, and gonad tissues.

2. To evaluate the relation of histological endpoints to water quality conditions measured at the same CR sites.
3. To synthesize histological and water quality findings for use in future mussel conservation and management strategies.

Materials and Methods

Site Selection and Mussel Deployment

Mussels were placed in cages at eight sites in the Clinch River. Sites were chosen throughout three reaches within the two zones. The ZD (CRKM 431.3 to 234.8), was a continuous reach and included four sites, Carterton (CRKM 389.5), Semones (CRKM 378.4), Pendleton Island (CRKM 364.2), and Clinchport (CRKM 342.8) (see map in Fig. 1). The ZS included two separate reaches: the upper reach included two sites upstream of the ZD, Cleveland Islands (CRKM 435.8) and Artrip (CRKM 441), and the lower reach included two sites downstream of the ZD, Horton Ford (CRKM 318.7) and Wallen Bend (CRKM 309) (Fig. 1). Both the upper and lower reaches were defined to form one zone, the ZS, while the ZD was found between them.

Mussels were deployed in cages that were ¾ filled with river substrate from the placement location at each site and consisted mostly of sand and gravel (~1-60 mm). This allowed the mussels to live in their natural stream habitat while facilitating ease of recovery. Forty-eight cages were constructed for use in the field study, plus an additional three cages for backup. The cages were constructed from polyethylene industrial containers with mesh sides and base, and with dimensions of 406.4 mm x 304.8 mm x 114.3 mm (Schaefer Systems, 52356, US Plastic Corporation, Lima, Ohio). Plastic mesh (6.34 mm) was affixed on the inside of the cages on the top, sides, and bottom to insure that mussels could not escape and to allow for water flow through cages (Fig. 2).

In early May of 2012, all sites were evaluated by Virginia Tech and U.S. Fish and Wildlife Service employees to determine the exact location for placement of the cages. Cage placement was optimized for habitat with a substrate mixture of gravel and sand (Otsby et al. 2014) and known locations of historical mussel populations based on published literature and expert consensus (Jones et al. 2014).

Over three days in late May 2012, six cages at each of the predetermined locations at a site were buried in the river bottom. The cages were placed in the river the week before mussels were deployed to ensure that any water soluble or volatile chemicals were removed from the cages (American Society for Testing and Materials 2002). The cages were filled with substrate and buried so that the top of the cages were flush with the sediment-water interface. Pieces of 35 cm steel rebar stakes were hammered into the substrate about 15 cm upstream of each cage (Fig. 3). Large (91 cm) nylon cable ties were attached to both the cage and rebar to anchor the cages in place. The locations of the cages were recorded using a GPS unit.

On June 5 and 6, 2012, captively propagated adult *V. iris* were deployed in all cages. The mussels used in this study were produced by standard host fish infection techniques and grown at the FMCC in water recirculating systems using pond water (Carey et al. 2013). At the time of deployment, the mussels were about 17 months old and ranged from about 15 to 40 mm. Using a random number table, 18 adult mussels were randomly selected for deployment in each cage, for a total of 108 per site and 864 for the entire project. During deployment, organ tissues of 20 mussels were harvested and immediately fixed in 10% formalin to be used as reference specimens. Histological data from these mussels served as a baseline during subsequent statistical comparisons.

Once mussels were deployed, cages were monitored on a monthly basis to confirm that cages were intact. Cages were also cleaned by fanning the water directly above the cage to remove any fine sediment deposition. Every three months, cages were opened to check for mortality and to perform a more thorough cleaning. If over 50 percent of mussels in a cage were found alive, the cage was left in place. Any cages with greater than 50 percent mortality were removed. After the November cage check, high water flow conditions in the CR generally prohibited monthly monitoring.

Histological Sampling

Before mussels were removed from the cages, two histological cassettes per mussel were labeled and organized into containers for each site and sampling event. Each label consisted of a specific code for each mussel (including sample event number, site identification code, cage number, and mussel number, as well as organ type of the tissue to be contained in the cassette; for example, SE1ArtC1M1 Gill would be gill tissue from mussel one in cage one at Artrip

sampled during the winter sample event). Cassette codes were used to track the two cassettes containing tissues from the same mussel throughout histological processing and microscopical evaluations of stained tissues. One cassette held gill tissue, and the other held gonad, digestive gland, and kidney tissues. Bottles of 10% neutral buffered formalin also were prepared and labeled (one bottle per site per sampling event) for organ tissues to be fixed at the time of collection (Bancroft and Gamble 2008).

To perform histological evaluations on gonad tissue and determine effects of site conditions on reproduction, sampling events were planned so that mussels would be collected at a time when active gametogenesis was occurring. Periods of gametogenesis can vary from year to year, depending on environmental factors, but active gametogenesis in this species is known to occur sometime during the winter to early spring (Henley 2010).

Conducting field work in the CR in winter can be problematic. Water discharge typically rises to levels that make access and sampling the river not feasible. To plan around these conditions and also to make sure that mussels were collected during active gametogenesis, sampling events were planned for December 2012, January or March 2013, and May 2013. It was uncertain whether December would be too early for mussels to be in active gametogenic stages; however, this earlier sampling event was scheduled to ensure at least one sampling event was conducted before river conditions potentially became too hazardous to sample again in winter. The final sampling event was May 2013, which allowed mussels a full year of exposure in the river.

The first sampling event occurred in January 2013 (Day 221). During this sampling event, cages were opened underwater and all mussels (live and dead) were placed into mesh bags. Mussels were sampled from one cage at a time and taken to a designated working area on the river bank. Four mussels were randomly selected for collection of their organs from four of the six cages at all eight sites using a random number table. The shell length (millimeter [mm]) of sampled mussels was measured and recorded. To preserve the mussels, the entire body mass was removed from their shell and then sliced in half through the dorsal to ventral mid-section of the body and placed in the appropriate labeled cassette. Cassettes containing tissues were immediately placed in a formalin bottle labeled for the collection site. Remaining mussels were returned to cages and the lids were secured with cable ties.

The final sampling event occurred in May 2013 (Day 344). At each site, cages were removed from the river and carried to a designated working area on the shore. All mussels were removed and four mussels were randomly selected for collection of their organ tissues from four of the six cages at each site and then preserved in the same method as used in the January sampling event. Mussel mortality was recorded for each cage based on recovery of empty shells. The remaining live mussels were collected, measured (nearest mm), and placed in a separate labeled formalin bottle for each site.

Histological Processing

At the FMCC laboratory, mussels were removed from their shells, cut in half and placed in a histological cassette labeled with the sampling date, as previously described. After being fixed in 10% formalin for at least one week, tissues were rinsed with deionized water to remove formalin and placed in a container of 70% ethanol for no more than one week. The gills of each individual were dissected from the visceral mass and placed into separate labeled histology cassettes. The cassettes containing gills, and the cassettes containing the gonad, digestive glands, and kidney, were placed back in the container of 70% ethanol. The tissues underwent a process of dehydration through a progressive series of concentrations of ethyl alcohol and then cleared in 100% xylene in preparation for paraffin embedding. This process had a different time sequence for the visceral tissues and gill tissues due to the sensitivity of the gills. Once embedded in paraffin wax, the tissues were sectioned and mounted on microscope slides.

Tissue sections were cut from paraffin-embedded tissue blocks using a rotary microtome (Leica RM2125RT, Leica Microsystems Incorporated, Wetzlar, Germany). Sections were cut at approximately 50% of the tissue depth and mounted on glass microscope slides. Tissues from one mussel were placed on three microscope slides; one slide containing gill tissues, and the other two slides containing gonad, digestive gland, and kidney tissues. Gill tissues and one slide containing visceral organs were stained with hematoxylin and eosin for microscopic evaluations. The duplicate slides containing visceral organs were stained using the Long Ziehl-Neelsen method (Bancroft and Gamble 2008) for elaboration and evaluation of lipofuscin in kidney tissues. Coverslips were mounted over all stained tissues. After staining, the slides were evaluated using light microscopy (Olympus BX 41 light microscope, Olympus America, Incorporated, Center Valley, Pennsylvania).

Histological Evaluations

Gonad, digestive gland, kidney, and gill tissues of *V. iris* sampled during this study were microscopically evaluated using five histologically-based dependent variables. The dependent variables were fractions of reproductive acini containing mature and/or developing gametes (FAMD); fractions of acini containing resorbing gametes (FAR); fractions of digestive gland diverticula cells containing degraded cytoplasm (FDGDC); fractions of kidney diverticula cells containing lipofuscin (FKDL); and fractions of gill filament termini with cilia (FGFTC).

Quantitative evaluations of organ tissues were performed by light microscopy using a point count method (Chalkey 1943). Six dots were drawn on the ocular piece of the microscope so they appeared over the slides containing mussel tissues (Fig. 4). Evaluations were only made on target tissues directly under the dots; thus, up to six evaluations were made at each location of the slide. Once tissue under all six dots was evaluated or determined to not be target tissue, the slide was randomly moved to another area of tissue until all evaluations were conducted for each dependent variable. Fifty evaluations per mussel were conducted for the two dependent variables associated with gonad tissue. One hundred evaluations per mussel were conducted for the dependent variables associated with the kidney, digestive gland, and gills. Data were recorded using a dichotomous dependent variable index using 1s (for presence of a particular variable) and 0s (for absence) (Table 1). Ten percent of all slides were evaluated by a second skilled evaluator for quality assurance and control.

Relation of Mussel Histology to Water Quality

Water quality data were collected during a concurrent study on the CR by staff at The Nature Conservancy, Virginia Department of Environmental Quality, North Carolina State University, and Tennessee Department of Environment and Conservation. All eight sites of this histologically-based study on the CR also served as sampling sites for this concurrent study investigating toxicological effects of contaminant stressors (metals and inorganics) to mussels in the water and sediment in the ZS and ZD. Water samples were collected on two sampling events (August 22 and November 16, 2012). The samples were analyzed for a suite of contaminants at the U.S. Environmental Protection Agency (EPA) Laboratory at Fort Meade, Maryland.

Additional *in situ* water chemistry data were collected on six occasions throughout the year using a Yellow Springs Instruments (YSI) multi-probe system handheld meter (Yellow Springs Instruments, Incorporated, Yellow Springs, Ohio) and consisted of temperature (degrees Celsius [°C]), dissolved oxygen (milligrams per liter [mg/L]), percent dissolved oxygen (% of saturation), pH, and conductivity (millisiemens per centimeter [mS/cm]). The YSI was calibrated prior to each sampling event.

Prioritizing Sites for Managing Mussels and Water Quality

Assessing water quality and the health of mussels deployed in cages at selected sites allowed comparisons to be made to prioritize sites for future management strategies. Sites were identified as impacted if mussel survival, growth, and histological evaluations showed declining condition of organ tissues and/or water quality parameters were measured at levels known or likely to be harmful to mussels based on established threshold criteria values in the scientific literature. Sites where mussels did not demonstrate a decline in organ tissue condition will serve as potential areas for restoring common and endangered mussel species, depending upon the suitability of habitat and other riverine conditions. Sites identified as problem sites will be evaluated to determine future management strategies. This information will help provide early-warning indications of possible ongoing impacts to sites and mussel populations and guide management decisions throughout the river system.

Data Analysis

The majority of statistical analyses were performed in JMP or SAS (SAS Institute, Inc., Cary, NC). The SAS code for histological variables and water quality analyses were modified from a previous histological study (Henley et al. 2013) in consultation with staff at Virginia Tech's Laboratory for Interdisciplinary Statistical Analysis (LISA). Results from histological evaluations were correlated with water quality data. Statistical test results in this study were considered significantly different when p-values were <0.05.

Growth- Growth, as measured by increase in total length (mm) was determined for mussels in each cage by subtracting the mean initial length of mussels from the mean length at the final sampling event on Day 344. An analysis of variance (ANOVA) was performed in JMP to determine potential differences in growth (mm) between riverine zones and sites.

Survival and Recovery- Survival was determined for all six replicate cages at each site. Four of the six cages were randomly selected at each site to be sampled during the January 2013 sampling event (SE1). The number of mussels removed in January (four if a cage was selected for sampling or zero if a cage was not selected for sampling) was subtracted from the total mussels deployed at the start of the study. Every cage started with 18 mussels, but not all mussels were recovered. Those not recovered from cages were recorded as missing and not assumed to be dead; therefore, they were also subtracted from the total number of mussels deployed (18). The following equation was used to calculate percent survival:

$$\frac{18 - \#\ of\ mussels\ sampled\ in\ SE\ 1\ (4\ or\ 0) - \#\ missing - \#\ dead}{18 - \#\ of\ mussels\ sampled\ in\ SE\ 1\ (4\ or\ 0) - \#\ missing} \times 100$$

An ANOVA was performed in JMP to determine whether significant differences in mussel survival existed among sites or between zones.

Histological Evaluations- Statistical analyses of histological data were performed using a generalized linear mixed model (GLIMMIX) in SAS for binomial data. Dependent variables (gills, digestive glands and kidneys) were first compared between zones. Within the mixed models for these analyses, sample event, zone, and site were fixed factors while cage was a random factor. The interactions between site and zone and sample event and zone were tested and removed if found to be non-significant (p>0.05). Over-dispersion of models was corrected using a residual term. Due to significant differences between zones related to the gills and no significant interaction between zones and sites, further analysis was not performed at the site level for this variable.

When no significant differences occurred between zones or when there was a significant interaction between zone and site, additional analyses were run to compare sites. Within the mixed models for these analyses, sample event and site were fixed factors while cage was a random factor. The interaction between sample event and zone was tested and removed if found to be non-significant. Over-dispersion of models was again corrected using a residual term. If the effect of site or sample event was significant, least-square means were compared using a Tukey-Kramer post-hoc test for multiple comparisons.

GLIMMIX was also used to analyze sites versus histological evaluations of baseline mussel tissues for the gills, digestive glands, and kidneys. Site was the fixed factor and cage was the random factor for these mixed models. When the effect of site was significant, least-squares

means among sites were compared using a Dunnett post-hoc test to compare individual sites to the baseline.

Variables evaluating the gonads were split by sex. Statistical analyses were not necessary because of lack of variation in both the fractions of acini containing mature or developing gametes and fractions of acini containing resorbing gametes. Mussels were labeled as indeterminate (I) if the evaluator was unable to determine the sex. Those mussels with both male and female acini were labeled as hermaphrodites (H).

Quality assurance of histology was performed using an ANOVA in SAS to compare results of two independent evaluators (two co-authors of this study). Ten percent of slides were randomly chosen for evaluations before evaluations of all slides were conducted. Disagreements were discussed and addressed for the remaining slides.

Water Quality Data- For water samples analyzed by the U.S. EPA laboratory, any analyte that was not detected at any site was removed from the dataset. Analytes that were detected at less than 50 percent of sites, with random occurrences at sites, were also removed from the dataset. Analytes reported to be below the method detection limit were included in data analysis by giving them a value equal to one-half the method detection limit. Ammonia, nitrite, nitrate, Total Kjeldahl Nitrogen, and total phosphorus and nitrogen were all removed from data analysis because sample holding times exceeded US EPA protocols. Analytes and water chemistry measured, as well as units and detection limits for each analyte are presented in Table 2. All analytes and water chemistry data, with the exception of pH and temperature, were log-transformed to help meet assumptions that variables were normally distributed prior to statistical analysis.

A Principal Components Analysis (PCA) was conducted with the log transformed variables and pH data. Dissolved calcium (Ca^{2+}), potassium (K^{+}), Na^{+}, and Mg, and dissolved oxygen saturation were all removed because they were highly correlated ($R > 0.80$) with total Ca^{2+}, K, Na^{+}, and Mg, and dissolved oxygen concentration, respectively. After highly correlated variables were removed, the PCA was repeated. The resulting PC scores from the final PCA which explained greater than 5% of variance, as well as individual analytes were used for correlations with histological variables and survival data. Histological data from gill and digestive gland from both SE1 and SE2 were used for correlations; however, only kidney data from SE2 were used for correlations because of a significantly greater presence of lipofuscin in

kidneys in SE2. Eigenvector weights greater than 0.20 were considered influential to principal components.

Site Prioritization for Management

Sites or zones found to have lower growth or survival, or significantly higher proportions of degraded mussel tissues or lipofuscin than other sites or zone were identified as problem sites or a problem zone. Sites found to have water quality parameters measured at significantly higher levels or parameters found to be highly correlated with impacted mussel tissues ($p<0.05$) were also identified as problem sites.

Results

Sampling Events

Weather and river conditions did not allow sampling in December 2012. On January 12-13, 2013 (Day 221 of deployment), flow conditions in the river were low enough to sample all sites and complete the first sampling event. Due to weather and high flow conditions in the river, a second winter sampling event was not conducted. The final sampling event was May 2013 (Day 344 of deployment), which provided the mussels with nearly a full year of exposure in the river.

Mussel Growth

Mean growth of mussels in the ZS (4.7 mm) was significantly greater than mussels in the ZD (2.5 mm) ($p=0.0006$). When compared by sites, mean growth of mussels at Artrip (3.8 mm), Cleveland (4.9 mm), Carterton (5.5 mm), Horton Ford (4.8 mm) and Wallen Bend (5.2 mm) did not differ significantly among sites, but each site showed a significantly greater mean growth than mussels at Pendleton (0.6 mm) ($p<0.05$) (Fig. 5). Mussels at Cleveland, Carterton, Horton Ford, and Wallen Bend showed significantly greater mean growth than mussels at Semones (1.7 mm) ($p<0.05$) (Fig. 5). Mean growth at Semones was not significantly different from mean growth at Pendleton ($p=0.3022$). Growth of mussels at Clinchport (2.9 mm) did not differ significantly from any other site. Growth also did not differ between the upper ($\bar{x}=4.3$ mm) and lower ($\bar{x}=5.9$ mm) reaches of the ZS ($p=0.2567$).

Mussel Survival and Recovery

Recovery of mussels (dead and living combined) ranged from 82-95%, and out of the mussels that were recovered at each site, survival ranged from 27-86% (Fig. 6). Horton Ford had the lowest survival (27.1%), but all mussels from two of the six cages died after a few months of deployment due to heavy deposition of sediment over the cages. Survival in the four cages not covered by sediment at Horton Ford was 40.7%. Carterton, Clinchport, and Cleveland all had one cage with zero survival after several months, each of which was also covered by sediment. The survival in the remaining five cages at each of those sites was 56.8%, 50.5%, 86.6%, respectively. Mussels at Wallen Bend had significantly greater survival (85.8%) than those at Horton Ford (p=0.0127). Survival at all other sites did not differ significantly. Mean survival did not differ between the upper (75%) and lower (55%) reaches of the ZS (p=0.1162). There was significantly higher survival in the ZS (65.42%) than in the ZD (43.82%) (p=0.0175).

Histological Evaluations

Histological evaluations were performed on tissues from 273 mussels (20 baseline, 128 from SE1, and 125 from SE2). One cage sampled at Clinchport during SE2 only had one live mussel, bringing the total number of mussels down from 128 to 125.

Digestive Gland- Fractions of digestive gland diverticula cells containing degraded cytoplasm (FDGDC) were significantly greater in mussels caged at Artrip ($\overline{x}$=0.25) (p<0.0001), Cleveland ($\overline{x}$=0.28) (p<0.0001), Carterton ($\overline{x}$=0.26) (p<0.0001), Semones ($\overline{x}$=0.21) (p=0.0001), Pendleton ($\overline{x}$=0.27) (p<0.0001), Clinchport ($\overline{x}$=0.22) (p=0.0001), Horton ($\overline{x}$=0.24) (p<0.0001), and Wallen ($\overline{x}$=0.22) (p<0.0001) compared to the baseline mussels ($\overline{x}$=0.05) (Fig. 7; compare Figs 9A and 10B). The FDGDC in mussels in the ZS ($\overline{x}$=0.242) was not significantly different from mussels in the ZD ($\overline{x}$=0.237) (p=0.7962) (Table 3); although a significant difference occurred between sampling events, with FDGDC significantly higher in SE1 ($\overline{x}$=0.28) than SE2 ($\overline{x}$=0.20) (p<0.0001). Higher FDGDC values in SE1 than in SE2 were observed at every site. Cleveland and Pendleton showed the largest differences in FDGDC values within a site between sampling events, and Horton and Clinchport showed the smallest differences. Because of the significant differences between SE1 and SE2, additional analysis was performed to determine

site differences. No significant differences in FDGDC occurred among sites (p>0.05 for all comparisons) in SE1 or SE2.

Gill- When mussels held at each site were compared to baseline mussels, mean fraction of gill filament termini with cilia (FGFTC) in baseline mussels were not significantly different from mussels at any site except Cleveland (compare Figs. 9C with 9D). The FGFTC was significantly lower in mussels held at Cleveland ($\overline{x}$=0.7998) than in baseline mussels ($\overline{x}$=0.95) (p=0.0018) (Fig. 7). The FGFTC was significantly higher in mussels in the ZD ($\overline{x}$=0.9082) than in the ZS ($\overline{x}$=0.8646) (p=0.0160) (Table 3).

Kidney- Fractions of kidney diverticula cells containing lipofuscin (FKDL) were significantly higher in mussels held at Artrip ($\overline{x}$=0.19) (p<0.0001), Cleveland ($\overline{x}$=0.24) (p<0.0001), Carterton ($\overline{x}$=0.26) (p<0.0001), Semones ($\overline{x}$=0.24) (p<0.0001), Pendleton ($\overline{x}$=0.29) (p<0.0001), Clinchport ($\overline{x}$=0.30) (p<0.0001), Horton ($\overline{x}$=0.29) (p<0.0001), and Wallen ($\overline{x}$=0.25) (p<0.0001) compared to the baseline mussels ($\overline{x}$=0.06) (Table 4; Figs. 7 and 10A). The FKDL in mussels from the ZD ($\overline{x}$=0.27) was significantly higher than in mussels from the ZS ($\overline{x}$=0.24) (p=0.0265) (Table 3). A significant difference occurred between sampling events, with FKDL being significantly lower in SE1 ($\overline{x}$=0.24) than SE2 ($\overline{x}$=0.28) (p=0.0117). Mussels at Wallen, Clinchport, and Cleveland actually showed a slight decrease in lipofuscin from sampling event one to two. However, Artrip, Horton, Semones, Pendleton, and Carterton all showed an increase in lipofuscin between sampling events. There was also a significant interaction between zone and site (p=0.0202), so further analysis was performed to determine differences between sites within zones. A significant difference within the ZS was found between Artrip and Horton (p=0.0059), so additional analysis was performed to determine differences between all sites, regardless of zone. No significant differences occurred in FKDL among mussels caged at Cleveland, Carterton, Semones, Pendleton, Clinchport, Horton, and Wallen or between Artrip and Cleveland, Carterton, Semones, and Wallen. However, mussels held at Artrip had a significantly lower FKDL than mussels held at Pendleton (p=0.0164), Clinchport (p=0.0090), and Horton (p=0.0217) (Table 4).

Gonad- Of the mussels evaluated for histology, 98 were male, 91 were female, and 2 were hermaphrodites (Figs. 8, 10B, 10C, and 10D). In 61 mussels no gametes were observed, and their sex was therefore assigned as indeterminate. Data from mussels with indeterminate sex

were not used in statistical analyses. Fractions of reproductive acini containing mature and/or developing gametes (FAMD) and acini containing resorbing gametes (FAR) in both males and females had such small variation that they were not analyzed (Table 5).

Other Histological Observations- While evaluating tissues for the histological variables, other abnormalities also were noted. The most common observation was the presence of parasites (Fig. 11A), which were found in 38 mussels (2 at Cleveland, 2 at Carterton, 8 at Semones, 2 at Pendleton, 4 at Clinchport, 9 at Horton, and 11 at Wallen). There were no parasites found in baseline mussels or from mussels held at Artrip. Fusion of gill filaments was observed in 10 mussels (3 at Cleveland, 2 at Carterton, 1 at Pendleton, 1 at Clinchport, 2 at Horton, and 1 at Wallen) (Fig. 11B).

Quality Assurance- Evaluations of ten percent of slides conducted by two independent evaluators showed no significant differences for any variables between evaluators.

Assessment of Water Quality and Contaminants

Conductivity, temperature, pH, and concentrations of dissolved oxygen, nickel (Ni) and calcium (Ca^{2+}) were similar at all sites. Some analytes showed spatial trends. For example, alkalinity and chloride (Cl^-) concentrations appeared to decrease from upstream to downstream sites, while total dissolved solids (TDS) and magnesium (Mg) concentrations appeared to increase going downstream. Sulfate (SO_4^{2-}), aluminum (Al), iron (Fe), manganese (Mn), and sodium (Na^+), showed a unimodal pattern, with the highest concentrations found in the ZD and the lowest concentrations found at upstream and downstream ZS sites. Means for analytes and water quality parameters are found in Tables 6 and 7.

The first five Principle Components (PC) explained 96.97% of the variance in water quality data, with PC1, PC2, PC3, PC4, and PC5 explaining 36.5%, 24.9 %, 17.3 %, 11.9%, and 6.4% of variance, respectively (see eigenvalues, proportions of variance explained, and eigenvector weights for the PCs in Table 8). Total dissolved solids, alkalinity, SO_4^{2-}, Ca^{2+}, Mg, Mn, K^+, and dissolved Mn were considered influential to PC1. Total dissolved solids, Cl^-, Al, Fe, Mg, Mn, Ni, Na^+, dissolved Ni, pH, and dissolved oxygen were considered influential to PC2. Alkalinity, Cl^-, Al, Fe, Ni, Na^+, dissolved Ni, and pH were considered influential to PC3. Total dissolved solids, Ca^{2+}, K^+, dissolved Ni, and dissolved oxygen were considered influential to PC4. Dissolved Mn, Ni, and pH were considered influential to PC5. No significant

correlations were found among individual water quality data or PC scores and histological or survival data.

Site Prioritization for Management

Histological evaluations of kidneys showed significantly higher abundances of lipofuscin in the ZD than the ZS. Survival and growth were also significantly lower in the ZD. Therefore, sites in the ZS (Artrip, Cleveland, Horton Ford, and Wallen Bend) should be priority sites for management objectives relating to stocking mussels to augment existing mussel populations. Results from water quality data collected during this study did not indicate parameters that are negatively impacting the health of mussel tissues at any sites. Sites in the ZD should be priority sites for further water quality monitoring to determine what is causing the significantly lower survival and greater histological impacts to kidney tissues.

Discussion

Differences in growth, survival and histological variables were found when comparing the ZS to the ZD. Lower growth and survival and higher abundances of tissue lesions and lipofuscin in mussels indicated that site conditions negatively affected mussel health in the ZD. Exactly what is causing mussel condition to decline is still unclear, but results from this study and water quality data provided insight to at least partially explain these differences in mussels deployed at the ZD sites.

Mussels caged in the ZS grew significantly more than mussels caged in the ZD. This demonstrated that there are differences between the zones, such as the presence of contaminants or other stressors found in the ZD. While the exact stressors are unknown, higher survival and higher growth indicated that site conditions within the ZS are more suitable to support mussel populations. One potential explanation is conductivity. Studies have shown that growth rate can be impacted by conductivity. For example, *Corbicula fluminea* held in river reaches with higher levels of conductivity showed slowed or stagnant growth when compared to *C. fluminea* held in river reaches with low conductivities (Mincy, 2012). The conductivity data collected during this study did not show significant differences in conductivity among sites, however, conductivity was only measured on six events over an entire year. More than 40,000 measurements of

conductivity were collected using continuous samplers deployed *in situ* from 2009 through 2011 in an intensive water quality study conducted in the CR by Johnson et al. (2014). They measured higher conductivity in the ZD and the Guest River, a tributary flowing into the ZD, compared to the ZS and Copper Creek, a tributary flowing into the ZS downstream of the ZD (Johnson et al. 2014). Higher measured conductivities, indicating increased total dissolved solid concentrations, have been found to reduce growth and survival of mussels in several laboratory tests (Bodkin et al. 2007; Cope et al. 2008; Wang et al., 2013).

Survival also was significantly greater in the ZS than in the ZD, indicating that factors influencing mussel survival differed between the zones. It is possible that certain contaminants present at higher concentrations in the ZD had lethal impacts on mussels, leaving only healthier and stronger mussels for histological evaluations. Hence, evaluation of remaining potentially healthier mussels have positively biased the histological results. Sulfate, Al, Fe, Mn, and Na^+ were all found in higher concentrations in the ZD than the ZS in this study. A variety of studies have shown that elevated concentrations of Al, Fe, and Mn have impacted mussel survival during acute exposures, which suggests that chronic exposures to even lower concentrations, as seen in the ZD could have adverse impacts. For example, Taskinen et al. (2011) found elevated concentrations of Al (0.25-1.0 mg/L) and Fe (0.5-2.0 mg/L) to negatively impact survival of glochidia and juveniles of *Margaritifera margaritifera* in an acute 72 hour exposure. Morgan et al. (1986) found Mn to have an acute lethal value of 30 mg/L in tests on larvae of *Mytilus edulis*. While Mn was not found at concentrations near this value in the CR, the concentrations in the ZD (ranging from 10-25 µg/L) may still impact mussels due to increased toxicity of metals in freshwater and impacts of chronic exposures (Chapman et al. 1982).

The fraction of digestive gland diverticula cells containing degraded cytoplasm was not significantly different between zones. However, there was a significant difference between sampling events. Histological evaluations showed that digestive glands contained significantly more degraded cytoplasm in the January sampling event than in the May sampling event. These diverticula cells are the site of intra- and extra-cellular digestion, nutrient absorption, lipid and glycogen storage, and contaminant detoxification (Owen 1970; Lobo-da Cunha 1999; Petrović et al. 2001; Henley 2010).

More degraded digestive diverticula in January with healthier diverticula in May indicate that there are possible seasonal or handling effects that occurred at all sites. *Villosa iris* are

suspension and deposit feeders that rely heavily on a diet of organic detritus, algae and bacteria that they siphon from the water column and sediment (Nichols et al. 2005). With colder water temperatures, bivalve metabolic rate decreases, thus decreasing the amount of food filtered (Vanderploeg et al. 1995; Vaughn and Hakenkamp 2001). Thompson et al. (1974) found a similar reduction of cytoplasm in digestive cells of *M. edulis* after a 4-week starvation period. The decrease in food processed by mussels in the winter is a potential explanation for the significantly more degraded cytoplasm found in digestive gland diverticula throughout both the ZD and ZS in the winter sampling event. The effects of handling and relocation can also be problematic, and may result in sublethal responses (Cope and Waller 1995). Significantly higher abundances of lesions in digestive cells observed during winter could be from the impact of handling and relocating mussels from the laboratory to cages. These impacts, in addition to reduced filtration for food, could have been observed in mussels sampled in January. Thompson et al. (1974) reported that digestive tubules in *M. edulis* impacted by stress events were able to recover once the stressor was eliminated. It is possible that *V. iris* also were able to recover from stressors impacting digestive tubules, such as impacts of relocation or decreased feeding, and would explain the significant decrease in degraded cytoplasm in the spring sampling event.

Gill tissues from mussels in the ZS were found to be significantly more impacted with a loss of cilia than gill tissues in the ZD. However, when broken down by site, Cleveland was the only site that differed from baseline mussels. This indicates that it was not so much of a zone difference but something occurring at Cleveland impacting gill tissue. Due to no significant differences in the water quality data among sites collected during this study, these data did not provide insight into possible contamination effects at Cleveland to explain the histological differences. Because loss of cilia has been linked to contaminant exposure (Domouhtsidou and Dimitruadis 2000; Lajtner et al. 2003; Gómez-Mendikute et al. 2005; Supanopas et al. 2005; Henley et al. 2013), it is possible that other contaminants are present at Cleveland that were not measured or identified in this study.

A lack of significant difference in histological evaluations of gill tissues at all other sites beyond Cleveland does not necessarily mean that the gill tissues were not adversely impacted. Fisher et al. (1991) examined the effects of K^+ on gill tissue of *Dreissena polymorphia* and were unable to detect histological differences between treatment and controls when using similar light microscopy methods to those used in this study. However, when electron microscopy was used

for histological evaluations, lesions and vacuolation were evident in gill tissue of mussels exposed to K^+ (Fisher et al. 1991).

The abundance of lipofuscin has been related to contaminant exposure in marine mussels (Riveros et al. 2002; Kagley et al. 2003). The pattern of significantly higher lipofuscin observed in SE2 versus SE1, and ZD versus ZS indicates that over time mussels in the ZD were exposed to higher concentrations of contaminants than mussels in the ZS. As mentioned previously in relation to survival, conductivity data were collected from 2009 through 2011 and it was higher in the ZD and the Guest River compared to the ZS and Copper Creek (Johnson et al. 2014). Increased ion concentrations, as indicated by higher conductivities and other associated contaminants, such as those found in higher concentrations in the ZD (SO_4^{2-}, Al, Fe, Mn, and Na^+), could potentially cause an increase in lipofuscin abundance as mussels have to work harder to sequester contaminants.

Consistent trends among water quality parameters were not observed in the study area. However, some metal analytes exhibited a unimodal trend, with higher concentrations in the ZD, while others showed increasing concentrations either in the downstream or upstream direction. The results of the PCA and correlations did not show any significant contributions of individual or groups of contaminants to histological impacts on mussel tissues. There were only two sampling events during this study, with data variation between sampling events. A larger set of water quality data would have been ideal to truly determine water quality trends at each of the sites. Other studies with more intensive monitoring have shown significant differences in water quality parameters in the ZD and have concluded that mussel declines in the ZD can be attributed to water and sediment contaminants (Hampson et al. 2000; Johnson et al.2014; Price et al. 2014; Zipper et al. 2014). Some of the trends found in this study, such as higher Mn, Na^+, Fe, Al, and SO_4^{2-} in the ZD, matched results from these other studies. It is possible that some of these analytes occurring at higher concentrations can have additional negative impacts on mussels that are not evident in the histological evaluations of this study.

Some histological results from this study indicated that there was a significant effect of mussel deployment regardless of site. Significant differences were present between histological variables at all sites and baseline mussels, showing a general decline in health of all mussels during the year that they were deployed in the river and an effect of the cages containing the mussels. The cages were visited on a monthly basis when weather conditions allowed. On

several occasions, however, the cages were filled with fine sediment or covered with sand. It is possible that the cage design did not allow enough water flow for the mussels to properly siphon to receive an adequate supply of food. Simon and James (2007) found that spiny lobster juveniles, *Jasus edwardsii* (Hutton 1875) fed a more nutritious diet in holding systems not only had better growth, but histological evaluations also revealed healthier digestive gland tissues. Lack of adequate food could have impacted the overall health of mussels in this study.

It was noted on several occasions that sediment in cages was turning black. This indicated that sediment was anoxic and oxygen supply to mussels was inadequate. During months that cages were not cleaned, a buildup of anoxic fine sediment occurred in the cages. Oxygen depletion is a potential explanation for decline in condition of the digestive glands and kidneys at field sites compared to baseline mussels. *Villosa iris* are likely intolerant of oxygen depletion because their natural habitat is in riffles where high concentrations of dissolved oxygen are available. For example, when exposed to water with lower levels of DO, tissues of *V. iris* displayed elevated glucose levels, demonstrating a stress response (Chen et al. 2001). Cages filled with sediment also caused mussels to attempt to migrate to the surface of the sediment within cages. The cages were not meshed along the entire top, so the mussels could have been further hindered from siphoning due to being crowded against a solid area of the lid. Some benthic animals are known to migrate to the surface when substrate reaches a level of low oxygen (Jorgensen 1980). When cages were checked, the majority of mussels were found sitting near the surface of the substrate, indicating a lack of oxygen in the substrate. Jorgenson (1980) found that freshwater mussels could survive anoxic conditions for one to two weeks, but then would start to die.

Since *V. iris* and most other mussel species exhibit a seasonal pattern of vertical migration, with the majority of mussels surfacing in spring and burrowing themselves again in October (Watters et al. 2001), it is possible that confinement in cages inhibited their natural surface-subsurface migration, preventing mussels from fully burrowing themselves during the winter months leaving them more exposed and stressed.

Conclusions and Recommendations

Assessing health of mussels deployed in cages at eight sites in the CR allowed comparisons to be made to prioritize areas in the river for future management options. As was originally hypothesized, tissues from mussels deployed at sites in the ZS had fewer tissue abnormalities and lower levels of lipofuscin and importantly, had higher growth and survival than from sites in the ZD. This indicated that there continues to be ongoing effects to mussel tissues in the ZD. Sites in the ZS should continue to be used for restoring common and endangered mussel species, while those sites in the ZD should be further studied to identify potential causal factors contributing to poor conditions and determine how to remediate impacts.

Histological methods could be modified for future studies, including use of electron microscopy to evaluate gill tissues, which would allow for ultrastructural analysis to provide greater resolution of effects on organ tissues. For example, mussels exhibit sexual dimorphism in the structure and function of marsupial gills (McElwain & Bullard 2014). Hence, statistically analyzing histological evaluations by sex or evaluating only non-marsupial gills could result in more accurate comparisons. The structure and function of mantle tissue has recently been thoroughly described (McElwain & Bullard 2014). Thus, evaluating health of mantle tissue should be considered.

The cages potentially caused histological effects and adversely impacted survival and growth. If cages are used for future studies, the design should be modified to allow for better water flow through interiors of the cages. Alternatively, mussels could be pit tagged and released uncaged at sites (Kurth et al. 2007; Hua et al. 2014); however, controlling for loss of individuals from the release site can be more challenging than mussels deployed in cages.

Mussels in this study were not individually tagged so only a cage mean for beginning and end length were used to determine growth. Not all mussels were recovered and others were sampled during the first sample event, thus only those mussels remaining at the final sample event were used to calculate the mean end length for each cage. Future studies should consider tagging all mussels to track individual growth.

Relocation of mussels for restoration has been found to be most effective when handling stress is minimized, and can be accomplished by moving mussels during a time of low reproductive stress and while mussels' metabolic rate is high enough to burrow in substrate

(Peredo et al. 2006). When designing this study, the major factor in determining timing of mussel deployment was river condition, as opposed to the mussels' physiological condition, but the latter should be more thoroughly studied.

Freshwater mussels can spend a majority of their time feeding from sediment and pore water, allowing them to be exposed to contaminants beyond what is found in surface water. It is important to consider testing both the sediment and pore water to understand potential impacts to mussel populations. Collaborators from The Nature Conservancy, Virginia Department of Environmental Quality, Tennessee Department of Environmental Conservation, U.S. Fish and Wildlife Service, and the U.S. Environmental Protection Agency (Maryland) began a study in July 2012 that is examining these impacts and will provide further information on the conditions affecting the mussel populations in the CR. Restoring mussel populations in the zone of decline is possible with current conservation techniques. However, continued research is needed to understand the potential limiting effects of water and sediment quality in this zone.

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Table 1. Dependent variables used to assess the condition of vital organ tissues from adult *Villosa iris.* Fifty observations were acquired from gonadal tissues, and 100 observations were obtained from digestive gland, kidney, and gill tissues of each evaluated mussel. Each datum was recorded as either a one or zero, as described in this table.

Dependent Variable	Recorded as One	Recorded as Zero
Fractions of acini containing mature or developing gametes	acini contains mature or developing gametes	acini does not contain mature or developing gametes
Fractions of acini containing resorbing gametes	acini contains resorbing gametes	acini does not contain resorbing gametes
Fractions of digestive gland diverticula cells containing degraded cytoplasm	diverticula contains degraded cytoplasm	diverticula does not contain degraded cytoplasm
Fractions of gill filaments with cilia	cilia present on gill filament	cilia not present on gill filament
Fractions of kidney cells containing lipofuscin	kidney cell contains lipofuscin	kidney cell does not contain lipofuscin

Table 2. Analytes with units and detection limits measured in the Clinch River by staff at The Nature Conservancy, Virginia Department of Environmental Quality, North Carolina State University, and Tennessee Department of Environmental Conservation during a concurrent study at the same sites where *Villosa iris* were deployed in cages. *Indicate analytes that were below detection limit.

Analyte	Unit	Detection Limit	Analyte	Unit	Detection Limit
Carb Alk*	mg/L	20	TDS	mg/L	10
Chloride	mg/L	0.3	Tot Alk	mg/L	20
Dissolved Al*	µg/L	10.0	Total Al	µg/L	10.0
Dissolved As*	µg/L	1.0	Total As*	µg/L	1.0
Dissolved Ca	µg/L	500	Total Ca	µg/L	500
Dissolved Cd*	µg/L	1.0	Total Cd*	µg/L	1.0
Dissolved Cr*	µg/L	1.0	Total Cr*	µg/L	1.0
Dissolved Cu*	µg/L	1.0	Total Cu*	µg/L	1.0
Dissolved Fe*	µg/L	100	Total Fe	µg/L	100
Dissolved Hg*	µg/L	0.2	Total Hg*	µg/L	0.2
Dissolved K	µg/L	500	Total K	µg/L	500
Dissolved Mg	µg/L	500	Total Mg	µg/L	500
Dissolved Mn	µg/L	1.0	Total Mn	µg/L	1.0
Dissolved Na	µg/L	1000	Total Na	µg/L	1000
Dissolved Ni	µg/L	1.0	Total Ni	µg/L	1.0
Dissolved Pb*	µg/L	1.0	Total Pb*	µg/L	1.0
Dissolved Se*	µg/L	1.0	Total Se*	µg/L	1.0
Dissolved Th*	µg/L	1.0	Total Th*	µg/L	1.0
Dissolved Zn*	µg/L	2.0	Total Zn*	µg/L	2.0
SO_4^{2-}	mg/L	1.0	TSS*	mg/L	10

Table 3. Least squares means (± standard errors) for fractions of digestive gland diverticula cells containing degraded cytoplasm (FDGDC), kidney diverticula cells containing lipofuscin (FKDL), and gill filament termini with cilia (FGFTC) from *Villosa iris* held in cages in each zone in the Clinch River. LETTERS Indicate significant differences between zones ($p<0.05$) (comparisons made within columns).

Variable	Zone of Stability	Zone of Decrease
FDGDC	0.24 (0.01)^{A}	0.24 (0.01)^{A}
FKDL	0.24 (0.01)^{A}	0.27 (0.01)^{B}
FGFTC	0.86 (0.01)^{A}	0.91 (0.01)^{B}

Table 4. Least squares means (± standard errors) for fractions of digestive gland diverticula cells containing degraded cytoplasm (FDGDC) and kidney diverticula cells containing lipofuscin (FKDL) from *Villosa iris* held in cages at each site in the Clinch River. LETTERS Indicate significant differences between sites (p<0.05) (comparisons made across rows).

Variable	Artrip	Cleveland	Carterton	Semones	Pendleton	Clinchport	Horton	Wallen
FDGDC	0.24 ^{A}	0.27 ^{A}	0.26 ^{A}	0.21 ^{A}	0.26 ^{A}	0.21 ^{A}	0.23 ^{A}	0.22 ^{A}
	(0.02)	(0.03)	(0.03)	(0.02)	(0.03)	(0.03)	(0.02)	(0.02)
FKDL	0.19 ^{A}	0.24 ^{AB}	0.26 ^{AB}	0.24 ^{AB}	0.29 ^{B}	0.30^{B}	0.29^{B}	0.26 ^{AB}
	(0.02)	(0.02)	(0.02)	(0.02)	(0.02)	(0.02)	(0.02)	(0.02)

Table 5. Arithmetic means (± standard errors) for fractions of reproductive acini containing mature and/or developing gametes (FAMD) and acini containing resorbing gametes (FAR) by sex of *Villosa iris* held in cages at each site in the Clinch River.

Variable	Sex	Artrip	Cleveland	Carterton	Semones	Pendleton	Clinchport	Horton	Wallen
FAMD	♂	0.96 (.042)	0.98 (.014)	0.97 (.024)	0.9 (.068)	0.89 (.088)	0.99 (.005)	0.95 (.032)	0.99 (.005)
	♀	1.0 (0.0)	1.0 (0.0)	0.98 (.023)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	0.99 (.004)
	H	-	1.0 (0.0)	-	-	-	-	-	-
FAR	♂	0.0 (0.0)	0.07 (.071)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.01 (.014)	0.0 (0.0)
	♀	1.0 (0.0)	1.0 (0.0)	0.9 (.078)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	1.0 (0.0)	0.99 (.004)
	H	-	0.17 (.07)	-	-	-	-	-	-

Table 6. Means for water quality measurements taken at sites in the Clinch River. Samples were taken in August and November of 2012. [1]Original measurement units were µg/L, means converted to mg/L.

Parameter	Unit	Artrip	Cleveland	Carterton	Semones	Pendleton	Clinchport	Horton	Wallen
TDS	mg/L	192.5	210.0	206.5	206.5	209.5	218.0	209.0	215.0
Tot Alk	mg/L	139.5	141.0	143.5	122.5	121.5	129.0	127.5	126.0
Cl	mg/L	11.2	11.4	12.0	10.6	10.8	11.0	9.4	9.2
SO_4^{2-}	mg/L	22.1	22.1	27.3	47.7	47.1	60.0	41.6	40.2
Total Al	µg/L	37.1	53.6	64.7	45.1	91.1	18.2	62.9	49.0
Total Ca[1]	mg/L	41.4	43.0	43.4	38.8	40.5	41.6	41.0	40.7
Total Cr	µg/L	0.5	0.5	0.9	0.5	0.5	0.5	0.8	0.8
Total Fe	µg/L	83.0	108.5	112.5	121.0	194.5	50.0	99.5	75.5
Total Mg[1]	mg/L	12.2	12.4	12.8	13.7	13.7	14.3	13.9	13.9
Total Mn	µg/L	6.3	9.6	11.1	16.1	24.7	10.5	12.4	9.6
Total Ni	µg/L	2.0	1.9	2.0	1.9	2.0	1.8	1.9	2.0
Total K[1]	mg/L	2.0	1.9	2.1	2.1	2.2	2.2	2.0	2.0
Total Na[1]	mg/L	12.0	12.0	15.9	14.4	14.1	14.1	11.5	11.3
Dissolved Al	µg/L	5.0	5.0	7.6	42.9	8.3	49.8	5.0	5.0
Dissolved Ca[1]	mg/L	40.4	41.4	41.0	38.4	38.7	40.6	40.1	40.5
Dissolved Mn	µg/L	3.2	4.6	5.2	18.2	13.1	12.7	5.7	3.9
Dissolved Ni	µg/L	1.8	1.9	1.9	1.9	1.8	1.8	1.7	1.8
Dissolved K[1]	mg/L	1.8	1.9	2.0	2.1	2.1	2.2	2.0	2.0
Dissolved Na[1]	mg/L	11.7	11.6	15.1	14.1	13.8	13.5	11.4	11.2

Table 7. Overall arithmetic means (± standard errors) for water quality measurements with hand-held meter at each site in the Clinch River (multi-probe system, YSI Incorporated, Yellow Springs, Ohio).

Parameter	Unit	Artrip	Cleveland	Carterton	Semones	Pendleton	Clinchport	Horton	Wallen
Temperature	°C	18.9 (2.3)	18.8 (2.2)	19.5 (2.1)	20.2 (2.1)	19.5 (1.8)	19.1 (1.6)	19.1 (1.7)	19.1 (1.6)
Conductivity	mS/cm	348.5 (7.7)	348.7 (8.3)	369.0 (13.1)	355.4 (10.9)	320.8 (22.2)	344.3(19.1)	335.5 (16.7)	339.3 (14.5)
Dissolved Oxygen	%	107.3 (1.9)	96.5 (3.9)	102.8 (4.4)	112.7 (1.9)	107.7 (3.2)	100.5 (2.1)	99.8 (2.4)	96.9 (3.7)
Dissolved Oxygen	mg/L	10.1 (0.5)	9.1 (0.6)	9.5 (0.7)	10.2 (0.4)	9.9 (0.5)	9.4 (0.4)	9.3 (0.5)	9.0 (0.6)
pH	log [H+]	8.5	8.5	8.0	8.6	8.4	8.2	8.4	8.4

Table 8. First five principal components (PC) from analysis of analyte concentrations and water quality measurements from all sites on both sampling events, including eigenvalues, the proportion of variance explained, and eigenvector (EV) weights. Eigenvector weights >0.20 (in bold) were considered influential to the component.

	PC				
	PC1	PC2	PC3	PC4	PC5
Eigenvalue	5.84	3.98	2.77	7.90	1.38
Variance Explained	0.36	0.25	0.17	0.12	0.06
Analyte/Measurement	EV	EV	EV	EV	EV
Total Dissolved Solids	**0.21**	**-0.23**	-0.02	**0.52**	-0.12
Alkalinity	**-0.36**	0.10	**0.21**	0.18	0.07
Cl^-	-0.12	**0.26**	**0.46**	0.07	-0.19
SO_4^{2-}	**0.38**	-0.20	0.03	0.00	0.03
Al	0.03	**0.37**	**-0.36**	0.11	0.17
Ca^{2+}	**-0.28**	0.02	0.17	**0.45**	0.08
Fe	0.10	**0.45**	**-0.21**	-0.03	-0.09
Mg	**0.35**	**-0.24**	-0.08	0.10	0.13
Mn	**0.35**	**0.22**	-0.10	0.08	-0.12
Ni	0.04	**0.33**	**-0.24**	0.14	**0.58**
K^+	**0.36**	0.04	0.16	**0.20**	0.18
Na^+	0.17	**0.24**	**0.44**	0.12	0.17
Dissolved Mn	**0.36**	0.05	0.19	-0.08	**-0.28**
Dissolved Ni	0.13	**0.33**	**0.23**	**0.22**	-0.13
pH	0.06	**-0.22**	**0.35**	-0.18	**0.62**
Dissolved oxygen	0.11	**0.25**	0.19	**-0.55**	0.03

Figure 1. Map of sites in the Zone of Stability (ZS) and Zone of Decline (ZD) in the Clinch River where cages were deployed from June 2012 to May 2013 (modified from map created by Brett Ostby).

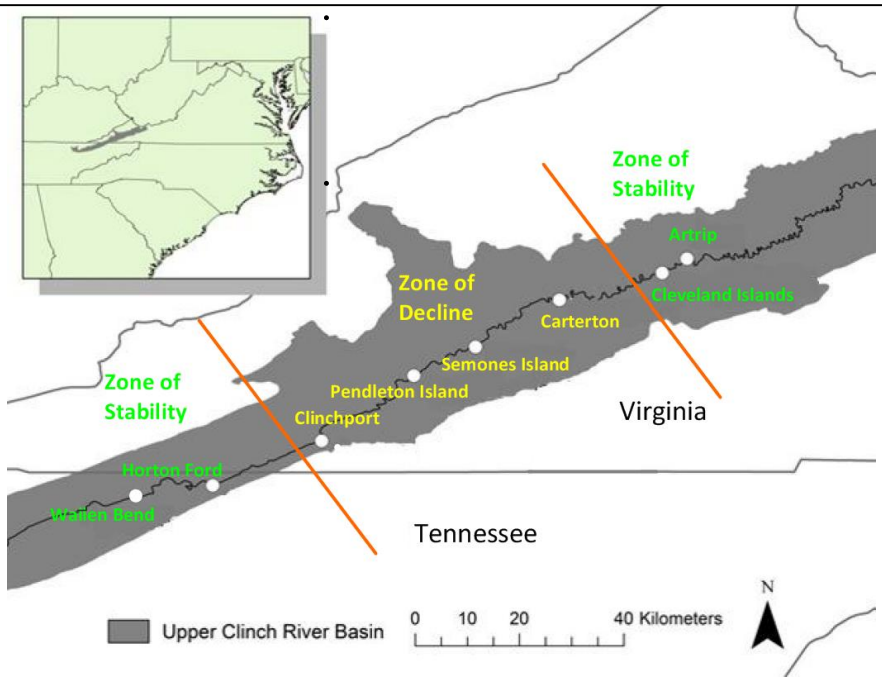


Figure 2. Cages used to hold *Villosa iris* in the Clinch River. Cages were constructed from polyethylene industrial containers with mesh sides and base, and with dimensions of 406.4 mm x 304.8 mm x 114.3 mm.

Figure 3. *In situ c*ages. A. Rebar attached to cage buried in river substrate. B. *Villosa iris* being placed in substrate within cage during June 2012 deployment.

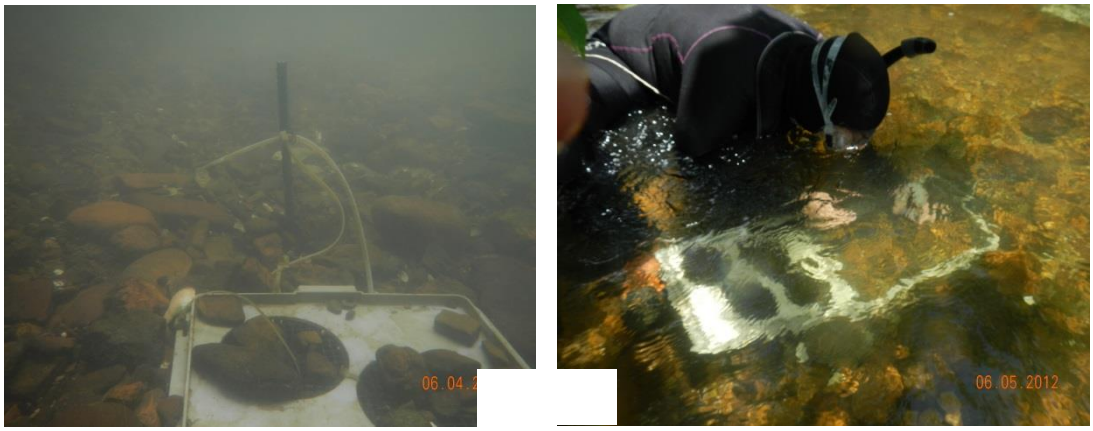


Figure 4. Example of point-count method in gonad to evaluate oogenic and spermatogenic acini. Points B, C, and F would be assigned a 1 for evaluations of fractions of acini containing mature or developing gametes because the points fall directly above acini that contain mature or developing gametes. Points A, D, and E would not be evaluated because they are not on acini.

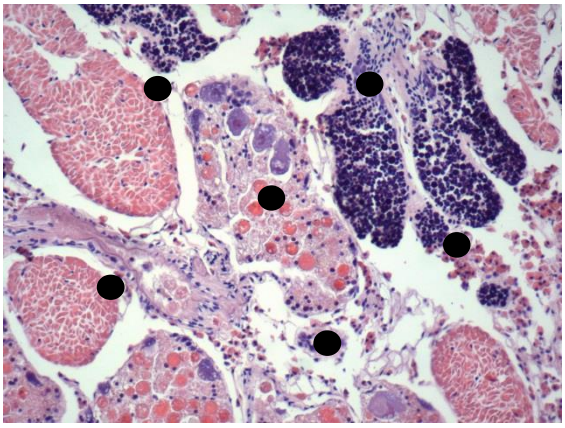


Figure 5. *Villosa iris* growth (by length) from Day 0 to Day 344 at each site in the Zone of Stability (ZS) and Zone of Decline (ZD). Error bars represent 95 percent confidence intervals.

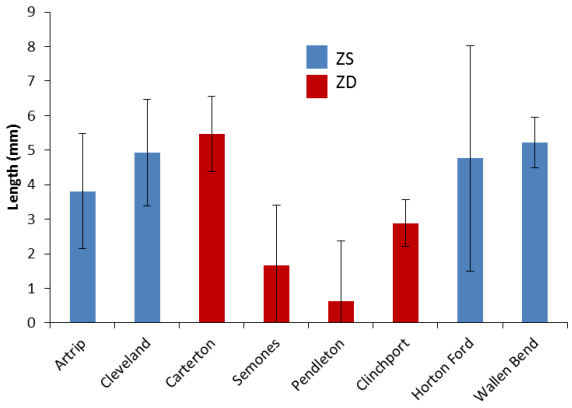


Figure 6. *Villosa iris* percent survival during 344 day deployment at each site in the Zone of Stability (ZS) and Zone of Decline (ZD). Error bars represent 95 percent confidence intervals. LETTERS Indicate significant differences between sites ($p<0.05$).

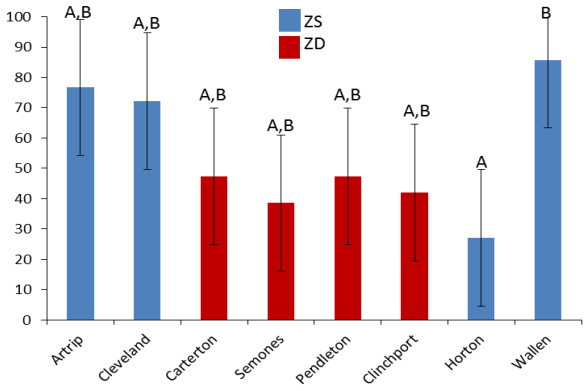


Figure 7. Least squares means (±standard errors) for fractions of digestive gland diverticula cells containing degraded cytoplasm (FDGDC), kidney diverticula cells containing lipofuscin (FKDL), and gill filament termini with cilia (FGFTC) from baseline *Villosa iris* and those held in cages at each site.

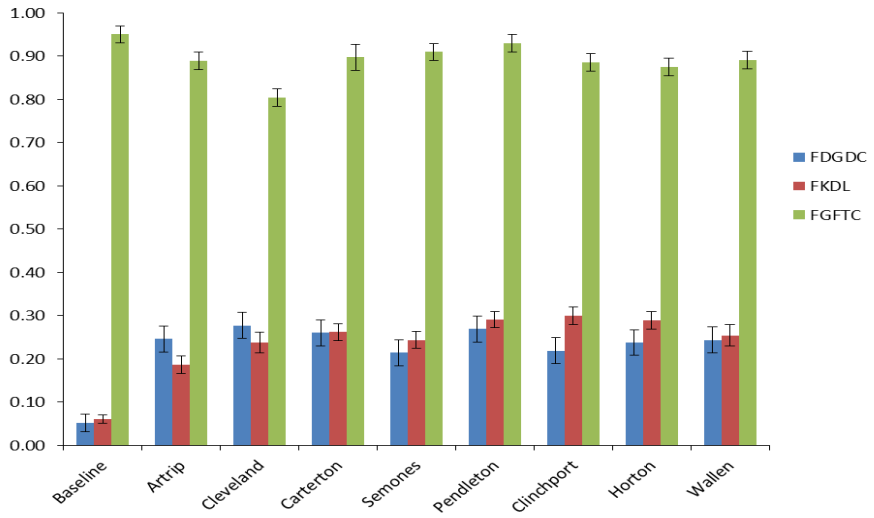


Figure 8. Number of *Villosa iris* of each sex by site. Mussels classified as indeterminate (I) had no tissue for histological evaluations of gonads. Mussels with both oogenic and spermatogenic acini were classified as hermaphrodites (H).

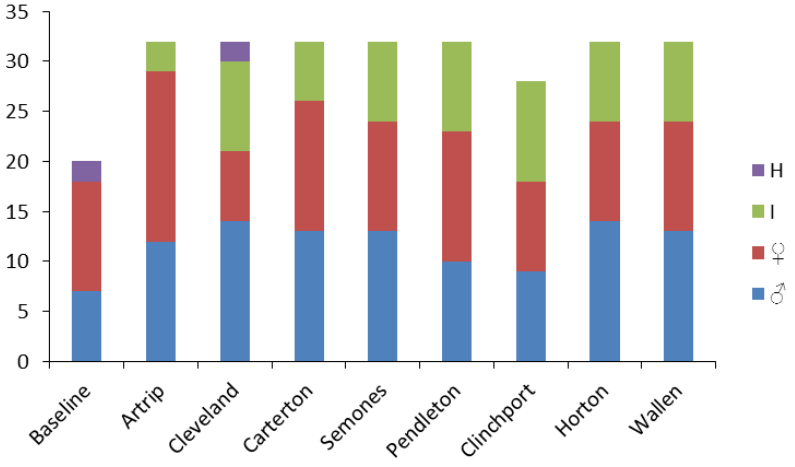


Figure 9. Digestive gland and gill tissues of *Villosa iris* stained with hematoxylin and eosin. A. Digestive diverticulum (dd) containing reduced cytoplasm (rc) from Carterton, sample event 1. Bar = 4 µm. B. Cross section of normal digestive diverticula with lumina (l) and digestive (dc) and basophilic (bc) cells from Wallen Bend, sample event 2. Bar = 2 µm. C. Necrotic gill filaments (gf) with exposed basement membranes (bm) and absent cilia (fac) from Cleveland, sample event 1. Bar = 4 µm. D. Normal ciliated (c) gill filaments (gf) from Wallen Bend, sample event 2. Bar = 2 µm.

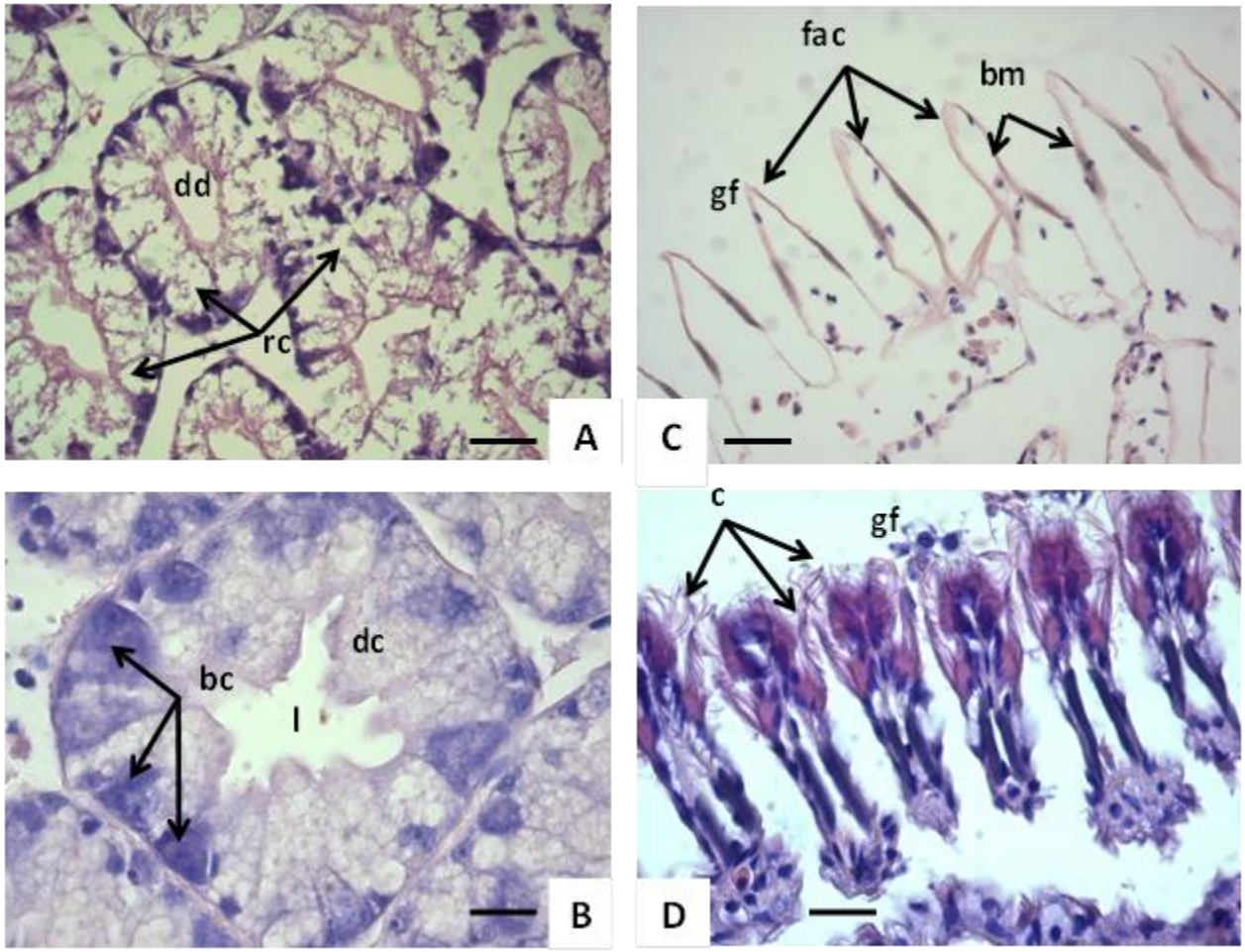


Figure 10. Kidney tissue (stained with Carbol Fuscin) and reproductive tissue (stained with hematoxylin and eosin) of *Villosa iris*. A. Kidney diverticulum (kd) containing high abundance of lipofuscin (lf) (brown inclusions) from Carterton, sample event 2. Bar = 2 µm. B. Spermatogenic acinus (sa) containing spermatozoa (s) from Semones, sample event 1. Bar = 4 µm. C. Oogenic acini (oa) containing resorbing oocytes (r) from Artrip, sample event 1. Bar = 4 µm. D. Hermaphrodite containing oogenic acini (oa) and spermatogenic acini (sa) from a baseline mussel. Bar = 10 µm.

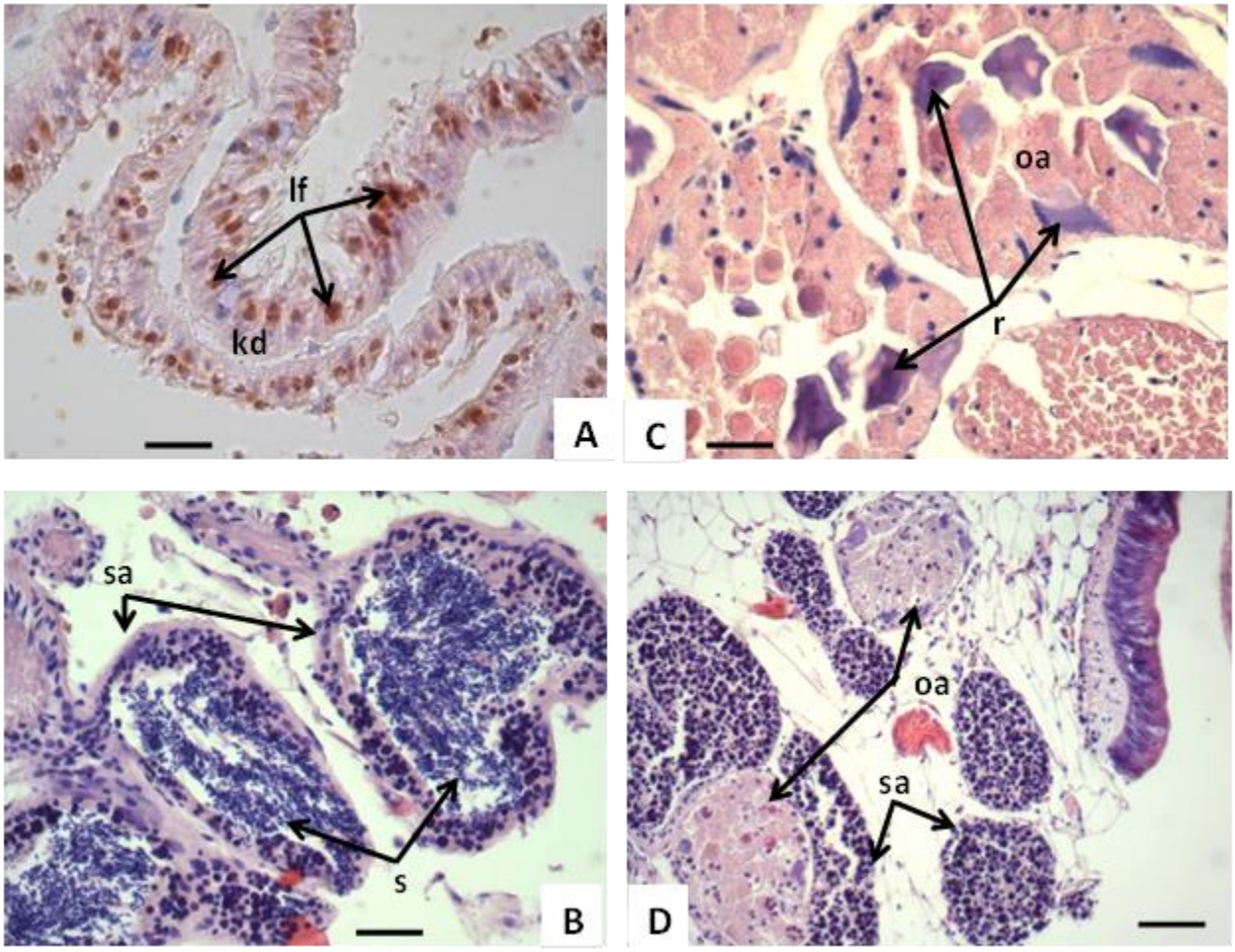
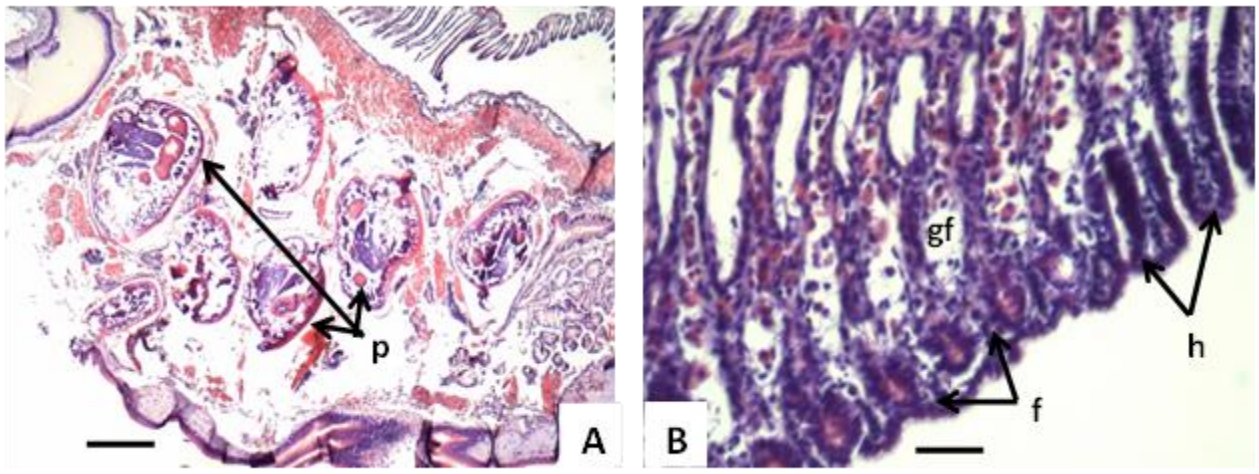


Figure 11. Other histological observations noted in *Villosa iris*; stained with hematoxylin and eosin. A. Parasites in connective tissue from Horton Ford, sample event 2. Bar = 20 µm. B. Fusion (f) and hyperplasia (h) in gill filaments (gf) from Wallen Bend, sample event 2. Bar = 4 µm.



Chapter 2

Effects of Chloride and Potassium on Organ Tissues of Adult *Villosa iris* Using Histological Evaluations

Abstract

Once supporting a diverse assemblage of more than 40 mussel species, the North Fork Holston River (NFHR) has experienced substantial declines in its species diversity and abundance. Historical industrial activities at Saltville, Virginia, as well as continued pollution of the river from chemical waste ponds at this location, are believed to be significant contributors to these declines. Contaminant seepages from the waste ponds that include sodium (Na^+), chloride (Cl^-), and potassium (K^+) have been shown to be toxic to adult and juvenile mussels. A three-month laboratory study was conducted to assess impacts to organ tissues (gills, digestive glands, kidneys, and gonads) of adult *Villosa iris* exposed to environmentally relevant concentrations of Cl^- (230 and 705 mg/L) and K^+ (4 and 8 mg/L) using histological evaluations. No detectable differences were observed among the histological endpoints from mussels held in treatments and control ($p>0.05$). The study design was modified and repeated using increased concentrations of Cl^- (705, 1410, and 2820 mg/L) and K^+ (8, 16, and 32 mg/L) for a two-month exposure period. Survival was significantly higher in control (97.8%), low (100%) and mid (97.8%) K^+ versus high K^+ (50%) ($p<0.001$), and was significantly higher in control (97.8%), low (100%), and mid (97.8%) Cl^- versus high Cl^- (0%) ($p<0.001$). Fractions of gill filaments without cilia and kidney diverticula cells with increased lipofuscin were significantly higher ($p<0.05$) in mussels exposed to increased concentrations of K^+ and Cl^- compared to control mussels. A significantly higher fraction of digestive gland diverticula cells with degraded cytoplasm was found in mussels exposed to increased concentrations of K^+. Significant histological differences between control and baseline mussels occurred in both studies, indicating that captivity influenced mussel tissues. Future studies are needed to more thoroughly address these captivity effects. Both survival and histological data in the second test showed a significant effect of the increased concentrations of Cl^- and K^+, which were representative of those found at some sites (range of 6-18,000 mg/L Cl^- and 1-68 mg/L K^+) in the NFHR downstream of Saltville, Virginia.

Keywords: Freshwater Mussels, Histology, *Villosa iris*, North Fork Holston River, Mussel Declines, Potassium, Chloride

Introduction

An estimated 840 freshwater mussel species are distributed worldwide, but with approximately 300, the greatest diversity is found in North America (Bogan 1993; Graf and Cummings 2007). However, mussels are experiencing declines in North America, with about 10% already extinct and more than 70% in need of protection (Williams et al. 1993; Neves et al. 1997; Strayer et al. 2004; Haag and Williams 2013). These declines are most often attributed to a suite of impacts, including habitat destruction caused by dams, dredging and channelization, which drastically change the riverine and riparian environments, and also eliminate host fish species that are essential to mussel reproduction (Strayer et al. 2004; Haag 2012). Additionally, sedimentation from deforestation, agriculture, and urbanization causes degradation of stream water and benthic substrates. Chemical contamination, primarily derived from agriculture and industry, often introduces heavy metals, pesticides, and mine drainage into the riverine system (Ortmann 1909; Ellis 1931; Cope et al. 2008). Commercial harvesting of freshwater mussels and introduction of exotic species (Asian clam, *Corbicula fluminea*, and zebra mussel, *Dreissena polymorpha*) also have been shown to negatively affect mussel populations (Claassen 1994; Strayer 1999; Strayer et al. 2004).

Freshwater mussels provide important ecosystem services, and with over 70 species federally listed as threatened or endangered. These benthic invertebrates stabilize the lotic substrates and sequester suspended particles, nutrients and some pollutants by filtering stream water (Strayer et al. 1994; Vaughn and Hakenkamp 2001). They also are a source of food to animals such as muskrats, otters, raccoons, sportfish, and ducks (Helfrich et al. 1986).

The North Fork Holston River (NFHR), located within the Upper Tennessee River Valley System, originates in Bland County, Virginia. After flowing for 200 kilometers from its origin, it joins the South Fork Holston River to form the Holston River near Kingsport, Tennessee. The NFHR basin is primarily forested, with agricultural land comprising about 30% of the watershed and urban areas only covering about 1% (Ahlstedt and Rashleigh 1996). The NFHR site of interest for this study is located near the town of Saltville, Virginia.

Industrial activities in the NFHR have been present at Saltville since the 1800s. After salt had been mined for decades, the Mathieson Chemical Company (which merged with the Olin Corporation in 1954 to become the Olin-Mathieson Chemical Company) opened and operated a soda ash (sodium carbonate) facility from 1950 to 1980 (Henley and Neves 1999). The wastes

from this facility were discharged into settling ponds where solids were allowed to settle before the liquid wastes were discharged into the river. This waste consisted mostly of calcium carbonate and chloride brines. The Olin Plant in Saltville also manufactured dry ice and hydrazine, which added more wastes such as heavy metals and ammonia to the holding ponds (Olin 1991; Ahlstedt and Rashleigh 1996).

Once supporting 42 mussel species, the NFHR mussel fauna has substantially declined over the last 60 years or longer (Ortmann 1918; Ahlstedt and Rashleigh 1996). Surveys have shown as few as nine extant species downstream of Saltville and 13 species upstream of Saltville (Henley and Neves 1999; Jones and Neves 2007), although at least 16 species are known to occur upstream (J.W. Jones, USFWS, personal communication). The industrial activities at Saltville, as well as continued pollution of the river from chemical holding ponds, are believed to be significant causes of these declines. Seepage waters from chemical holding ponds add high concentrations of salts and ammonia, as well as various other ions, to the river and are suspected of negatively affecting mussel populations downstream of Saltville (Wang and Ingersoll 2010).

Sodium (Na^+), chloride (Cl^-) and potassium (K^+) are present in the NFHR near Saltville at concentrations that have been shown to be toxic to adult and juvenile mussels (Wang and Ingersoll 2010). Wang and Ingersoll (2010) found maximum levels of Na^+, K^+, and Cl^- in seepage waters from the chemical holding ponds in Saltville to be 6,840, 47, and 22,200 mg/L, respectively.

Histological variables were used in this study to evaluate the condition of mussel tissues exposed to Cl^- and K^+. Evaluations of gonad, digestive gland, gill, and kidney tissues, and have been used in previous studies to demonstrate effects of contaminants on vital organ tissues of marine bivalves (Bayne et al. 1981; Seiler and Morse 1988; Au 2004). Recent studies using histological evaluations of mussels have shown atrophy of digestive gland cells, erosion of gill cilia, and differences in gamete production and resorption of oocytes to be indicators of exposure to contaminants (Henley et al. 2007; 2008a; 2008b). Organ tissues are sites of absorption, sequestration, accumulation and excretion of contaminants, all of which can cause cellular alterations to occur (Seiler and Morse 1988; Domouhtsidou and Dimitruadis 2000).

For example, acini within the gonads are the site of gamete development in mussels, which includes a natural cycle of maturation, spawning and resorption of residual gametes (Kennedy and Battle 1964; Pipe 1987; Dorange and Pennec 1989; Barber 1996; Henley 2010).

Various metal and organic contaminants have been shown to affect this process by suppressing development of gametes in bivalves (Gosling 2003). Resorption of gametes in acini is a natural occurrence after a mussel spawns; however, untimely resorption has been observed as a response to stress and exposure to contaminants (Bayne and Thompson 1970; Bayne et al. 1981; Tay et al. 2003; Henley 2010).

The bivalve digestive gland contains diverticula with epithelial layers made up of secretory and digestive cells. These diverticula cells are the site of intra- and extra-cellular digestion, nutrient absorption, lipid and glycogen storage, and contaminant detoxification (Owen 1970; Lobo-da Cunha 1999; Petrović et al. 2001; Henley 2010). Contaminant exposure can cause cellular alteration in the digestive gland including degradation of cytoplasm in the diverticula cells (Lowe et al. 1981; Au 2004; Usheva et al. 2006; Henley et al. 2013).

Bivalve gills are paired ciliated organs involved in respiration, osmoregulation, and food capture and transport. Because they are the first uptake site in the mussel, gill cells are exposed to many contaminants found in water (Gómez-Mendikute et al. 2005). Loss of cilia along with fusion of gill filaments, inflammation, necrosis, epithelial cell sloughing, and increased mucus production are all histopathological alterations that have been linked to contaminant exposure (Domouhtsidou and Dimitruadis 2000; Lajtner et al. 2003; Gómez-Mendikute et al. 2005; Supanopas et al. 2005; Henley et al. 2013).

The bivalve kidney is responsible for ultrafiltration of hemolymph, ion exchange, and excretion (Dietz et al. 2000; Fahrner and Haszprunar 2002). The kidney also is responsible for sequestering contaminants by excretion of lipofuscin granules. The abundance of lipofuscin can increase as mussels age, but also has been related to contaminant exposure in mussels (Riveros et al. 2002; Kagley et al. 2003).

Thus, the purpose of this study was to assess the condition of vital organ tissues of adult rainbow mussels, *Villosa iris*, exposed to Cl^- and K^+ using microscopical evaluations of histologically prepared gill, digestive gland, kidney, and gonad tissues. Test concentrations of Cl^- and K^+ were determined from data obtained from measurements taken in the NFHR and other ecological benchmarks.

Materials and Methods

The experiment was conducted in the Laboratory for Conservation Aquaculture and Aquatic Ecology, Virginia Tech, Blacksburg, Virginia, and involved a three-month exposure of Cl^- and K^+ running from mid-September through mid-December 2012. After the 2012 results were analyzed; a second study was developed as a follow-up to explore effects of increased concentrations to mussel tissues. Therefore, concentrations were increased and a third level treatment was added to include a low, mid, and high concentration of both Cl^- and K^+ in the 2013 study. To decrease effects of holding mussels in captivity, the duration of the 2013 study was decreased from three months to two months. The follow-up study was conducted from late August through mid-November 2013 in the same facility.

Study Conditions

Twenty-five closed recirculating bucket systems were built for this experiment. Systems were modified downweller buckets designed for culturing juvenile mussels (Barnhart 2006) (Fig. 1), and herein referred to as buckets. Each treatment (low and high Cl^- and K^+ in 2012; low, mid and high Cl^- and K^+ in 2013) consisted of five separate replicate buckets held within a water bath, and herein referred to as treatment banks. There also was one bank of five control buckets held within a water bath, which contained holding water with no additional ions. Eight mussels were placed into each bucket, for a total of 40 mussels per treatment or control bank in 2012. In the 2013 study, nine mussels were placed into each bucket, for a total of 45 mussels per treatment or control bank.

The holding water was pumped from the pond located at The Freshwater Mollusk Conservation Center (FMCC), Department of Fish and Wildlife Conservation, Virginia Tech, Blacksburg. This was the same water used for the propagation and culture of the test mussels. Prior to the study, samples of pond water were sent to the Virginia Tech Soil Testing Laboratory, Blacksburg, Virginia, for analysis of analytes. Additional water quality measurements were conducted at the FMCC to determine alkalinity, hardness, pH, and ammonia concentration (Table 1). The pond water at the start of the study averaged 24°C throughout the day. Due to a lack of chillers in the laboratory to cool the water, and because this was the water temperature in which mussels were currently living, the treatment banks were maintained at 24°C for the remainder of the study.

Adult *V. iris* propagated and cultured at the FMCC were used in this study. These mussels were produced by standard host fish infection techniques and cultured in water recirculating systems using pond water. At the beginning of the 2012 experiment, the mussels were about 21 months old and ranged from 16 mm to 46 mm. At the beginning of the 2013 follow-up experiment, the mussels were about 12 months old and ranged from 14 mm to 26 mm.

Mussels were fed commercial algae using diluted Shellfish Diet® (Reed Mariculture, Inc., Campbell, California). The food was administered continuously using a drip valve delivering 100 mL to each bucket over a 24 hour period. To ensure all treatments and controls received equal amounts of food, samples were randomly collected from buckets from each treatment and control. These 16 mL samples were fixed with three drops of Lugol's iodine solution (5% solution, Fisher Scientific). Algal cells were quantified using a Coulter Counter (Beckman Coulter, Multisizer 3) and measured as cells per mL and mean cell size (µm).

Stock solutions of treatment ions were created using pond water filtered through a 200 µm nylon monofilament bag (Aquatic Ecosystems) and reagent grade NaCl or KCl for treatment banks (high Cl^-, low Cl^-, high K^+, low K^+). Water quality was measured daily for the first weeks to ensure that concentrations of Cl^-, Na^+ and K^+ remained constant. Solutions of NaCl and KCl were added as needed to maintain a constant level of ions. Once it was determined that ion concentrations were remaining constant throughout the week, water quality was measured twice weekly in each replicate. One hundred percent water exchanges (static renewal) were performed weekly, to maintain concentrations of Cl^- or K^+ and to ensure adequate water quality conditions during the exposures.

Concentrations of Cl^-, Na^+ and K^+ were measured using a Thermo Scientific Orion Cl^-, Na^+ or K^+ electrode and Orion EA940 meter (Thermo Fisher Scientific Incorporated, Waltham, Massachusetts). Ammonia was measured as unionized ammonia (NH_3-N) daily during the 2012 study (Thermo Scientific Orion High-Performance Ammonia Electrode) and weekly during the 2013 study (salicylate method, Hach Method 8155). Temperature was maintained at 24 °C (± 0.5) with the use of heaters in the water bath surrounding the buckets and measured twice daily using a digital thermometer. A handheld meter (Oakton Waterproof Double Junction pH Tester 20) was used to measure pH daily during the 2012 study and biweekly during the 2013 study. Bi-weekly measurements were conducted for dissolved oxygen (YSI Professional Plus Multiparameter Meter), total hardness (titration, Hach Method 8213; mg of Ca/L as CaCo3 plus

mg/L of CaCo3), and total alkalinity (Hach Model AL-AP; mg of phenolphthalein alkalinity/L plus mg total methyl orange alkalinity/L as CaCo3). All meters were calibrated before each use with calibration verification standards. Replicate samples were measured from random buckets each week to perform quality assurance/quality control.

2012 Study Concentrations- The test concentrations that the organisms were exposed to in the laboratory study were determined based on previous studies and environmentally relevant concentrations. Solutions were made for high Cl^- exposures to create the concentration equivalent to the mean concentration of Cl^- found at sites in the North Fork Holston River study area (705 mg/L) (Henley et al. 2013). The United States Environmental Protection Agency (USEPA) National Water Quality Chronic Criteria for Cl^- (230 mg/L) was the low Cl^- treatment concentration (USEPA 1988). The high concentration of K^+ was determined from the mean concentration of K^+ found at North Fork Holston study sites (8 mg/L) (Henley et al. 2013). The low concentration of K^+ (4 mg/L) was the recommended threshold for K^+ in rivers supporting freshwater mussels (Imlay 1973).

2013 Study Concentrations- Concentrations for the second round of ion exposures were determined using the previous high concentrations as the new low (705mg/L Cl^- and 8 mg/L K^+). Low level concentrations were doubled to create mid-level concentrations (1410 mg/L Cl^- and 16 mg/L K^+), which were again doubled to create the high concentrations (2820 mg/L Cl^- and 32 mg/L K^+).

Sampling Procedures

Before mussels were removed from the bucket, two histological cassettes per mussel were labeled and organized for treatment and control banks. Each label consisted of a specific code for each mussel (including the bucket and mussel number, and the organ type of the tissue to be contained in the cassette; for example, the code "A1M1 Gill" would represent gill tissue from mussel one in bucket A). Cassette codes were used to track the two cassettes containing tissues from the same mussel throughout histological processing and microscopical evaluations of stained tissues. One cassette held gill tissue, and the other held gonad, digestive gland, and kidney tissues. Bottles of 10% neutral buffered formalin also were prepared and labeled (one bottle per treatment or control) to fix organ tissues at the time of collection (Bancroft and Gamble 2008).

Twenty mussels were randomly selected at the beginning of each experiment, without being exposed to any of the experimental conditions, to be used as baseline specimens for subsequent statistical comparisons. The shell length (mm) of collected mussels was measured and recorded. The entire body mass was first removed from their shell then sliced in half through the dorsal to ventral mid-section of the body using forceps and a scalpel and placed in a labeled histological cassette. Cassettes containing tissues were immediately placed in the formalin bottle labeled as "day 0" mussels.

Sampling events occurred at the end of each study, after three months of exposure in 2012 and two months of exposure in 2013. The studies were set up so that sampling would occur during active gametogenesis. Four mussels were randomly sampled from each bucket, and their tissues were harvested for histological processing. These mussels were processed and preserved using the same procedure as described previously for the baseline mussels. Mussels were fixed in bottles of 10% formalin pre-labeled for each treatment and control bank.

If a bank of buckets had a mortality rate exceeding 50% at any point during either experiment, the sampling event immediately occurred for that treatment bank and it was shut down. This was necessary to ensure data were available from all treatments because histological processing of tissues could only be performed if the mussels were collected while still alive.

Histological Processing

After being fixed in 10% formalin for at least one week, tissues were rinsed with deionized water to remove formalin and placed in a container of 70% ethanol for no more than one week. The gills of each individual were dissected from the visceral mass and placed into separate labeled histology cassettes. The cassettes containing gills, and the cassettes containing the gonad, digestive glands, and kidney, were placed back in the container of 70% ethanol. The tissues underwent a process of dehydration through a progressive series of concentrations of ethyl alcohol and then cleared in 100% xylene in preparation for paraffin embedding. Once embedded in paraffin wax, the tissues were sectioned and mounted on microscope slides labeled with cassette code and tissue type.

Tissue sections were cut from paraffin-embedded tissue blocks using a rotary microtome (Leica RM2125RT, Leica Microsystems Incorporated, Wetzlar, Germany). Sections were cut at approximately 50% of the tissue depth and mounted on glass microscope slides. Tissues from

one mussel were placed on three microscope slides; one slide containing gill tissues, and the other two slides containing gonad, digestive gland, and kidney tissues. Gill tissues and one slide containing visceral organs were stained with hematoxylin and eosin for microscopic evaluations. The duplicate slides containing visceral organs were stained using the Long Ziehl-Neelsen method (Bancroft and Gamble 2008) for elaboration and evaluation of lipofuscin in kidney tissues. Coverslips were mounted over all stained tissues. After staining, the slides were evaluated using light microscopy (Olympus BX 41 light microscope, Olympus America, Incorporated, Center Valley, Pennsylvania).

Histological Evaluations

Gonad, digestive gland, kidney, and gill tissues of *V. iris* sampled during both rounds of this study were microscopically evaluated using five histologically-based dependent variables. The dependent variables were fraction of reproductive acini containing mature and/or developing gametes (FAMD); fraction of acini containing resorbing gametes (FAR); fraction of digestive gland diverticula cells containing degraded cytoplasm (FDGDC); fraction of kidney diverticula cells containing lipofuscin (FKDL); and fraction of gill filament termini with cilia (FGFTC).

Quantitative evaluations of organ tissues were performed by light microscopy using a point count method (Chalkey 1943). Six dots were drawn on the ocular piece of the microscope so they appeared over the slides containing mussel tissues (Fig. 2). Evaluations were only made on target tissues under the dots; thus, up to six evaluations were made at each location of the slide. Once tissue under all six dots was evaluated or determined to not be target tissue, the slide was randomly moved to another area of tissue until all evaluations were conducted for each dependent variable. Fifty evaluations per mussel were conducted for the two dependent variables associated with gonad tissue. One hundred evaluations per mussel were conducted for the dependent variables associated with the kidney, digestive gland, and gills. Data were recorded using a dichotomous dependent variable index using ones (for presence of a particular variable) and zeros (for absence). For example, when evaluating gills, if cilia was present on the gill filament termini then a one was recorded; however, if cilia was absent from the gill filament termini then a zero was recorded (Table 2). Ten percent of all slides were evaluated by a second skilled evaluator (a co-author of this study) for quality assurance and control.

Data Analysis

The majority of statistical analyses were performed in SAS (SAS Institute, Inc., Cary, NC). SAS code for histological variables was modified from a previous histological study (Henley et al. 2013) in consultation with staff at Virginia Tech's Laboratory for Interdisciplinary Statistical Analysis (LISA). SAS code for survival analyses was developed by LISA consultants. Statistical test results in this study were considered significantly different when p-values were <0.05.

Water quality- The data analysis toolset in Excel was used for analysis of all water quality parameters. The descriptive statistics tool was used to determine means and standard errors (SE) for temperature, pH, dissolved oxygen, alkalinity, hardness, K^+, Cl^- and Na^+. The ANOVA: Single Factor tool was used to determine if significant differences occurred among data replicates of each parameter between each high, low, mid treatments and controls.

Algal Count- Mean cell count (cells/mL) and mean cell size (µm) were compared among high and low treatments and control using a one-way ANOVA in SAS.

Growth- Mean growth was compared among high and low treatments and control for the 2012 study using a one-way ANOVA in SAS. The duration of the 2013 study was not long enough to capture mussel growth. Beginning and end shell lengths were recorded; however, growth analyses of mussels were not performed.

Survival- Survival analysis was performed using a global log-rank test in SAS to compare survival curves. The null hypothesis for this test was that the control, low, and high concentrations (or low, mid, and high concentrations in 2013) of either Cl^- or K^+ had the same survival functions. To test this hypothesis, the log-rank procedure calculated the observed and expected survival and compared them statistically. If groups had a significantly different survival function, a Tukey-Kramer post-hoc test for multiple comparisons was then performed to determine differences among each concentration of each treatment and controls.

Histological Evaluations- Statistical analyses of histological data from the gills, digestive glands, and kidneys were performed using a generalized linear mixed model (GLIMMIX) in SAS for binomial data. Within the mixed models for these analyses, treatment and bucket were fixed factors. Overdispersion of models was corrected using a residual term. Bucket was removed from the model if there was not a significant interaction with treatment. If the effect of treatment was significant, least-square means were compared using a Tukey-Kramer post-hoc

test for multiple comparisons. GLIMMIX was also used to analyze histological data from control versus baseline mussel tissues for the gills, digestive glands, and kidneys.

Variables evaluating the gonads were split by sex. Mussels were labeled as indeterminate (I) if the evaluator was unable to determine the sex by the presence of gametes in evaluated tissues. Those mussels with both male and female acini were labeled as hermaphrodites (H) and were removed from the dataset prior to statistical analyses due to the small sample size. Statistical analyses of gonad tissues in the 2012 experiment could not be conducted with SAS GLMMIX because of lack of variation in both the fractions of acini containing mature or developing gametes and fractions of acini containing resorbing gametes. Statistical analyses of gonad tissues in the 2013 experiment were conducted using the same GLMMIX code as described for other tissues above; however, sex also was included with bucket and treatment as a fixed factor in the model. When sex was found to have a significant interaction with treatment, SAS GLMMIX was rerun using a SLICE statement to separately analyze effects of females, males, and indeterminates.

Quality assurance of histological methods was performed using an ANOVA in SAS to compare results of two independent evaluators. Ten percent of slides were randomly chosen for evaluations before evaluations of all slides were conducted.

Results

2012 Study

Water Quality

Potassium, sodium and chloride were measured daily for the first few weeks and then intermittently after it was determined that concentrations were remaining constant. Potassium in the control buckets averaged 2.5 mg/L (range of 1.84-2.75 mg/L). The target concentration for the low level K^+ was 4 mg/L and the actual average concentration measured over the three month study period was 4.10 mg/L (range of 3.28-4.65 mg/L). The target concentration for the high level K^+ was 8 mg/L and the actual average concentration was 7.9 mg/L (range of 6.96-8.78 mg/L). Chloride in the control buckets averaged 16.54 mg/L (range of 11.0-25.3 mg/L). The target concentration for the low level Cl^- was 230 mg/L and the actual average concentration measured over the three month study period was 232.7 mg/L (range of 244-280 mg/L). The target concentration for the high level Cl^- was 705 mg/L and the actual average concentration

was 703.8 mg/L (range of 601-848 mg/L). A target concentration was not set for Na^+ because Cl^- was the ion of interest. The actual concentration of Na^+ measured in control, low Cl^-, and high Cl^- buckets averaged 9.92 mg/L (range of 7.3-16.4 mg/L), 155 mg/L (range of 147-160 mg/L), and 477 mg/L (range of 429-615 mg/L), respectively.

Temperature, dissolved oxygen, hardness, alkalinity, and ammonia did not differ among buckets over the course of the study (p>0.05 for all comparisons) (Tables 3 and 4). Mean pH ranged from 8.52 to 8.58 and did not differ significantly among high and low K^+ treatments and control (p=0.81) or between low Cl^- and control (p=0.06) (Tables 3 and 4); however, mean pH was significantly lower in high Cl^- ($\overline{x}$=8.52) than in control ($\overline{x}$=8.58) (p<0.001) and low Cl^- ($\overline{x}$=8.55) (p=0.02). Mean temperature of all buckets remained within 0.2 degrees of the target temperature (24°C). Mean dissolved oxygen ranged from 87.7 to 88.9 %. Mean range of alkalinity as $CaCo_3$ was 178-195 mg/L and hardness as $CaCo_3$ was 239-262 mg/L. Ammonia was measured for the first five weeks of the study with a mean range of 0.10-0.45 mg/L NH_3-N at pH 8.4 at 24°C; however, these data were discarded due to inaccurate readings by the ammonia probe.

Algal Concentrations

Mean cell count (cells/mL) and cell size (µm) did not differ significantly among control buckets ($\overline{x}$=1808 cells/mL, $\overline{x}$=3.8 µm), high Cl^- ($\overline{x}$=2234 cells/mL, µ=4.0 µm), and low Cl^- ($\overline{x}$=2159 cells/mL, $\overline{x}$=3.8 µm) or among control, high K^+ ($\overline{x}$=1687 cells/mL, $\overline{x}$=3.8 µm), and low K^+ ($\overline{x}$=1774 cells/mL, $\overline{x}$=3.7 µm) (Table 5). P-values were >0.39 for all comparisons.

Growth

Mean mussel growth ranged from 0.02 mm to 0.38 mm in all buckets (Figs. 3 and 4). High K^+ had the highest growth ($\overline{x}$=0.38); however, it was not significantly different than control ($\overline{x}$=0.02) or low K^+ ($\overline{x}$=0.34) (p=0.15). Growth was not significantly different among control ($\overline{x}$=0.02), low Cl^- ($\overline{x}$=0.06), and high Cl^- ($\overline{x}$=0.20) (p=0.23).

Survival

Each treatment and control started with 40 mussels, for a total of 200. Over the three-month study period, the numbers of mussels that survived were 35 (87.5% survival) mussels in

the control bank, 32 (80%) in low K^+, 32 (80%) in high K^+, 37 (92.5%) in low Cl^-, and 26 (65%) in high Cl^- (Figs. 5 and 6). The first mortality occurred on day 46 of the study in the low K^+ treatment. Survival was not significantly different among control, low, and high K^+ (p=0.5808) or between control and low Cl^- (p=0.8413). Survival was significantly higher in control versus high Cl^- (p=0.0218) and in low versus high Cl^- (p=0.0037).

Histology

Tissues from 160 mussels were evaluated for the five dependent histological variables, including 20 mussels from each of 6 treatments, 20 mussels from the control, and 20 baseline mussels.

Gonad- Histological results for the gonads were separated by sex. Out of the 20 baseline mussels, nine were female, eight male, and three hermaphrodites. Of the 20 control mussels, ten were female, one male, and nine indeterminate. Of the 80 mussels in the four treatments, 33 were female (8 High K^+, 8 High Cl^-, 9 Low K^+, and 8 High Cl^-), 18 male (9 High K^+, 3 High Cl^-, 2 Low K^+, and 4 High Cl^-), one hermaphrodite (High K^+), and 28 indeterminate (2 High K^+, 9 High Cl^-, 9 Low K^+, and 8 High Cl^-) (Table 6).

All female mussels collected during the sampling event at the end of the experiment and those used as baseline were in the post-spawning stage of gametogenesis. The majority of acini in all females contained both mature or developing oocytes as well as atretic, resorbing oocytes (Fig. 9A). No statistical analysis was performed on data from either of these variables for females because there was minimal variation among the mussels. The arithmetic means of fractions of acini containing mature or developing oocytes and fractions of acini containing atretic or resorbing gametes are presented in Table 7.

Thirteen males (1 control, 1 low K^+, 8 high K^+, 2 low Cl^-, and 1 high Cl^-) were in the post-spawning stage of gametogenesis and the other fourteen (8 baseline, 1 low K^+, 1 high K^+, 2 low Cl^-, and 2 high Cl^-) were in the ripe or pre-spawn stage of gametogenesis (Fig. 9B). There was seemingly more variation in male reproductive tissue; however, there were no significant differences for either FAMD or FAR among control, low K^+, and high K^+ or among control, low Cl^-, and high Cl^- (p>0.05) (Table 8).

Digestive Gland- The fraction of digestive gland diverticula with degraded cytoplasm (FDGC) was significantly lower in baseline mussels ($\overline{x}$=0.13) than control ($\overline{x}$=0.23) (p=0.0254)

(Table 7) (compare Figs. 9C and 9D). There were no significant differences among high and low K^+ treatments ($\bar{x}$=0.24 and $\bar{x}$=0.27, respectively) and control (p=0.8599) (Table 8) or among high and low Cl^- treatments ($\bar{x}$=0.27 and $\bar{x}$=0.15, respectively) and control (p=0.1181) (Table 9).

Gill- No significant differences were observed for the fractions of gill filaments with cilia among baseline and control (Table 7) or among control and treatments (Tables 8 and 9, Fig. 10A). All p-values were >0.49.

Kidney- Fractions of kidney diverticula cells containing lipofuscin (FKDL) were significantly lower in baseline mussels ($\bar{x}$=0.24) than control mussels ($\bar{x}$=0.36) (p=0.0092) (Table 7, Fig. 10B). There were no significant differences among high K^+ ($\bar{x}$=0.42), low K^+ ($\bar{x}$=0.45) and control (p=0.2450) (Table 8) or among high Cl^- ($\bar{x}$=0.43), low Cl^- ($\bar{x}$=0.38), and control (p=0.3460) for the fraction of kidney diverticula containing lipofuscin (Table 9).

Other Histological Observations- While evaluating tissues for histological variables, other tissue abnormalities also were noted. The most common observation was fusion of gill filaments, which was found in 14 mussels (1 baseline, 2 control, 1 low K^+, 3 high K^+, 2 low Cl^-, and 5 high Cl^-) (compare Figs. 10C and 10A). Parasites were observed in tissues of 13 mussels (2 baseline, 2 control, 4 low K^+, 2 high K^+, 2 low Cl^-, and 1 high Cl^-) (Fig. 10D).

Quality Assurance- Evaluations of ten percent of slides conducted by two independent evaluators showed no significant differences for any variables between evaluators.

2013 Study

Water Quality

Potassium, sodium and chloride were measured daily for the first few weeks and then intermittently after it was determined that concentrations were remaining constant. Potassium in the control buckets averaged 1.34 mg/L (range of 0.22-3.00 mg/L). The target concentration for the low level K^+ was 8 mg/L and the actual average concentration measured over the two month study period was 7.47 mg/L (range of 4.77-10.6 mg/L). The target concentration for the mid-level K^+ was 16 mg/L and the actual average concentration was 15.71 mg/L (range of 12.8-19.5 mg/L). The target concentration for the high level K^+ was 32 mg/L and the actual average concentration was 30.88 mg/L (range of 27.1-34.0 mg/L). Chloride in the control buckets averaged 13.24 mg/L (range of 8.43-23.4 mg/L). The target concentration for the low level Cl^- was 705 mg/L and the actual average concentration measured over the two month study period

was 738.61 mg/L (range of 622-866 mg/L). The target concentration for the mid-level Cl^- was 1410 mg/L and the actual average concentration was 1554.35 mg/L (range of 1250-1810 mg/L). The target concentration for the high level Cl^- was 2820 mg/L and the actual average concentration was 2916.3 mg/L (range of 2760-3130 mg/L). A target concentration was not set for Na^+ because Cl^- was the ion of interest. The actual concentration of Na^+ measured in control, low Cl^-, mid Cl^- and high Cl^- buckets averaged 7.10 mg/L (range of 4.51-14.5 mg/L), 506.03 mg/L (range of 431-635 mg/L), 1018.08 mg/L (range of 886-1360 mg/L), and 2098.00 mg/L (range of 1910-2290 mg/L), respectively.

Temperature, pH, dissolved oxygen, hardness, alkalinity, nitrite, nitrate, and ammonia did not differ among buckets over the course of the study (p>0.05 for all comparisons) (Tables 10 and 11). Mean temperature of all buckets remained within 0.4 degrees of the target temperature (24°C). Mean pH of buckets ranged from 8.23 to 8.33. Mean dissolved oxygen ranged from 85.3 to 86.2 %. Mean range of alkalinity as $CaCo_3$ was 233-245 mg/L and hardness as $CaCo_3$ was 205-215 mg/L. Mean nitrite, nitrate, and ammonia ranged from 0.01 to 0.04 mg/L NO_2, 0.27-0.86 mg/L NO_3, and 0.03-0.06 mg/L NH_3-N, respectively. The NH_3-N was higher than the draft USEPA chronic criteria of 0.014 mg/L at pH 8.4 and 24°C (USEPA 2009).

Survival

Each treatment and control started with 45 mussels, for a total of 315. By day two of the study, 100% of mussels in the high Cl^- treatment were dead. By day thirteen, the 50% mortality threshold in the High K^+ treatment occurred, so tissues from surviving mussels were collected for histological evaluations and that treatment was shut down. Over the remaining 2-month study period, one mortality occurred in the control, mid K^+ and mid Cl^-, leaving 44 mussels (97.8% survival) in each bank. All 45 mussels survived in both the low K^+ and low Cl^- treatments. Survival was not significantly different among control, low, and mid K^+ (p=0.9999) or between control, low, and mid Cl^- (p>0.9999). Survival was significantly higher in control, low and mid K^+ versus high K^+ (p=0.0012) and control, low, and in mid Cl^- versus high Cl^- (p=0.0008) (Figs. 7 and 8).

Histology

Tissues from 140 mussels were evaluated for the five dependent histological variables. This included 20 mussels from each of 5 treatments, 20 mussels from the control, and 20 baseline mussels. All mussels in the high Cl^- treatment died within two days, so no histology was performed on those mussels. All histological results presented excluded the High Cl^- treatment.

Gonad- Histological results for the gonads were separated by sex. Out of the 20 baseline mussels, one was female, seventeen male, and two indeterminate. Of the 20 control mussels, two were female, five male, and seventeen indeterminate. Of the 100 mussels in the five treatments, 10 were female (3 High K^+, 3 Mid K^+, 2 Low K^+, and 2 Low Cl^-), 59 male (13 High K^+, 8 Mid K^+, 13 Low K^+, 12 Mid Cl^-, and 13 Low Cl^-), three hermaphrodite (2 High K^+ and 1 Mid Cl^-), and 28 indeterminate (2 High K^+, 9 Mid K^+, 5 Low K^+, 7 Mid Cl^-, and 5 Low Cl^-) (Table 12).

All female mussels collected during the sampling event at the end of the experiment and those used as baseline were either in the pre-spawning stage of gametogenesis (5 total) or their reproductive stage was indeterminate (8 total). Of the male mussels, 43 were in the pre-spawning stage, seven were in post-spawning stage, 12 were in the resorbing stage, and the reproductive stage of 19 were indeterminate. One hermaphrodite was in the pre-spawning stage of gametogenesis and the reproductive stage of the other two was indeterminate.

There was not a significant interaction between sex and treatment for fractions of reproductive acini containing mature and/or developing gametes (FAMD) in any comparisons from the 2013 study ($p>0.05$); therefore, sex was removed from the model. Once sex was removed, FAMD was still not significantly different between baseline ($\overline{x}=0.85$) and control mussels ($\overline{x}=0.54$) ($p=0.1352$). The FAMD also was not significantly different among low K^+ ($\overline{x}=0.66$), mid K^+ ($\overline{x}=0.98$), high K^+ ($\overline{x}=0.99$), and control ($p=0.9401$) or among low Cl^- ($\overline{x}=0.77$), mid Cl^- ($\overline{x}=0.72$), and control ($p=0.5280$). There was a significant interaction with sex and treatment for fractions of acini containing resorbing gametes (FAR) between control and baseline mussels ($p=0.0055$), among control and K^+ treatments ($p=0.0001$), and among control and Cl^- treatments ($p=0.0031$); therefore, analyses of FAR were separated by sex. The mean fractions of acini containing resorbing gametes (FAR) did not differ significantly between baseline ($\overline{x}=0.01$) and control males ($\overline{x}=0.22$) ($p=0.28$). There was only one female mussel and two assigned as indeterminate in the baseline; therefore, comparisons were not made between

baseline and control female or indeterminate mussels. The FAR in males did not differ significantly among control ($\overline{x}$=0.22), low ($\overline{x}$=0.40), mid ($\overline{x}$=0.24), and high K^+ ($\overline{x}$=0.11) (p=0.4404) or among control, low ($\overline{x}$=0.13), and mid Cl^- ($\overline{x}$=0.18) (p=.9994). The FAR in females did not differ significantly among control ($\overline{x}$=0.81), low ($\overline{x}$=0.77), mid ($\overline{x}$=0.62), and high K^+ ($\overline{x}$=0.84) (p=0.8847). The FAR in mussels assigned as indeterminate also did not differ significantly among control ($\overline{x}$=0.25), low ($\overline{x}$=0.69), mid ($\overline{x}$=0.44), and high K^+ ($\overline{x}$=0.50) (p=0.3051) or among control, low ($\overline{x}$=0.03), and mid Cl^- ($\overline{x}$=0.08) (p>0.9997) (Table 12).

Digestive Gland- A significantly lower mean fraction of digestive gland diverticula cells containing degraded cytoplasm (FDGDC) occurred in baseline mussels ($\overline{x}$=0.03) versus control mussels ($\overline{x}$=0.08) (p=0.0275) (Table 13) (compare Figs. 9C and 9D). FDGDC was significantly lower in the control versus low K^+ ($\overline{x}$=0.30) (p<.0001) and mid K^+ ($\overline{x}$=0.20) (p=0.0158). There were no significant differences between control and high K^+ ($\overline{x}$=0.16) (p=0.1654), Low Cl^- ($\overline{x}$=0.12) (p=0.4243), or Mid Cl^- ($\overline{x}$=0.10) (p=0.7356) treatments or between low and mid K^+ (p=0.148), low and high K^+ (p=0.0662), mid and high K^+ (p=0.8768), and mid and low Cl^- (p=0.9076) (Tables 14 and 15).

Gill- The mean fractions of gill filament termini with cilia (FGFTC) was significantly lower in the baseline mussels ($\overline{x}$=0.93) versus control ($\overline{x}$=0.98) (p=0.0054) (Table 13) (Fig. 10A). FGFTC was significantly higher in the control versus high K^+ ($\overline{x}$=0.88) (p=0.0009) and mid Cl^- ($\overline{x}$=0.93) (p=0.0353). Mussels in the mid K^+ treatment ($\overline{x}$=0.95) had significantly higher FGFTC versus mussels exposed to high K^+ (p=0.0429). There were no significant differences between low K^+ ($\overline{x}$=0.95), mid K^+, or Low Cl^- ($\overline{x}$=0.94) and control (p=0.2933, p=0.1581, and p=0.0559, respectively) or between mid and low K^+ (p=0.9954), low and high K^+ (p=0.0726), and mid and low Cl^- (p=0.9853) (Tables 14 and 15).

Kidney- A significantly lower mean fraction of kidney diverticula cells containing lipofuscin (FKDL) occurred in baseline mussels ($\overline{x}$=0.03) compared to the control mussels ($\overline{x}$=0.12) (p=0.0008) (Table 13) (Fig. 10B). FKDL was significantly lower in control mussels versus low K^+ ($\overline{x}$=0.21) (p=0.0020), mid K^+ ($\overline{x}$=0.21) (p=0.0081), low Cl^- ($\overline{x}$=0.22) (p=0.0010), and mid Cl^- ($\overline{x}$=0.28) (p<0.0001). FKDL was significantly lower in high K^+ ($\overline{x}$=0.06) versus low K^+ (p=0.0224) and mid K^+ (p=0.0312). There were no significant differences between control and high K^+ (p=0.4148), low and mid K^+ (p=0.9879), or mid and low Cl^- (p=0.0876) (Tables 14 and 15).

Other Histological Observations- While evaluating tissues for the histological variables, other abnormalities also were noted. The most common observation was pyknotic nuclei found in tissues digestive glands, which was found in 13 mussels (1 control, 9 low K^+, 2 high K^+, and 1 low Cl^-) (compare Figs. 11A and 11C). Atrophy of digestive gland tissue was noted in four mussels (1 low K^+, 1 mid K^+, and 2 mid Cl^-) (Fig. 11C). Necrosis in the digestive gland was found in 10 mussels (1 control, 1 low K^+, 4 mid K^+, 2 high K^+, 1 low Cl^-, and 2 mid Cl^-) (Fig. 11B compared to 9C), as well as edema, found in gill tissue of 10 mussels (1 baseline, 3 mid K^+, 3 low Cl^-, and 3 mid Cl^-) (Fig. 11D compared to 10A). Fusion of gill filaments was found in one mussel in high K^+ and one in low Cl^- (Fig. 11D compared to 9C).

Discussion

Results from the 2012 study indicated that the test concentrations of Cl^- or K^+ used in the study had no significant adverse effects on gill, digestive gland, kidney or gonad tissues of *V. iris*, although the high concentration of Cl^- caused significantly higher mortality than the control or low Cl^-. The kidney and digestive gland tissues of control mussels had significantly higher abundance of lipofuscin and degraded cytoplasm, respectively, than the baseline mussels, demonstrating that captivity effects, such as inadequate food or lack of burrowing substrate, were potentially affecting mussel tissues in all buckets.

The shortened duration of the 2013 study did not sufficiently address impacts of captivity; however, both survival and histological results indicated a significant effect of the increased concentrations of Cl^- and K^+. All mussels within the high Cl^- treatment were dead (100% mortality) by day two, leaving no mussels from this treatment bank for histological evaluations. While the purpose of this study was to examine sub-lethal chronic exposures of Cl^- and K^+, it is clear that exposure to Cl^- levels near 2820 mg/L are acutely toxic to *V. iris*. Mussels exposed to the high level (32 mg/L) of K^+ also demonstrated acute toxicity, with 50% of the mussels dead by day 13. Other studies have demonstrated chronic lethal toxicity with exposures to lower concentrations of K^+. For example, in separate 52 day exposures, Imlay (1973) reported that 11 mg/L K^+ was lethal to 90% of three species of freshwater mussels and 20 mg/L K^+ was reported to be lethal to 73% of *D. polymorpha* (unpublished report, Aquatic Sciences, Inc.; Wildridge et al. 1998). Water samples collected from the North Fork Holtson

River near Saltville, Virginia have exceeded these concentrations with some measurements as high as 18,000 mg/L Cl^- and 68 mg/L K^+, demonstrating that these areas could not support freshwater mussels (Henley et al. 2013).

The gills of baseline mussels had significantly less cilia than control mussels; however, the actual percentages were 93 versus 98. Both of these represent a high percentage of healthy gill tissue remaining. Gills of control mussels had more cilia than high K^+ and mid Cl^-; and the fraction of gill filament with cilia in mid K^+ was higher than mussels in high K^+, showing that the higher level of K^+, even at a short exposure time of 13 d decreased cilia. The exact mode of action of K^+ is unclear; however, loss of ciliary action and compromised integrity of the gill tissues, ultimately causing asphyxiation and death has been seen in mussels exposed to K^+ (Fisher et al. 1991), and my results support these findings. A lack of significant difference in histological evaluations of gill tissues of control versus mid and low K^+ or low Cl^- does not necessarily mean that the gill tissues were not adversely impacted. Fisher et al. (1991) examined the effects of K^+ on gill tissue of *Dreissena polymorphia* and were unable to detect histological differences between treatment and controls when using similar light microscopy methods to those used in this study. However, when electron microscopy was used for histological evaluations, lesions and vacuolation were evident in gill tissue of mussels exposed to K^+ (Fisher et al. 1991).

The digestive glands of control mussels again showed significantly more degraded cytoplasm and kidneys showed a higher abundance of lipofuscin than baseline mussels in the 2013 study. Despite reduced exposure time, degraded tissues in control mussels indicated that test conditions were still inadequate for holding mussels in captivity for the duration of the 2013 study. However, mussels exposed to some treatments showed significantly more degraded tissues than control mussels. Mussels exposed to both the low and mid concentrations of potassium had significantly more degraded cytoplasm in the digestive glands than the control mussels. The digestive glands of mussels in the high K^+ treatment were not significantly different than control mussels, but were only exposed for 13 d, opposed to the full 61 d exposure of other treatments, due to the high mortality rate. The digestive gland of mussels exposed to both low and mid concentrations of Cl^- were not significantly different than control, indicating that chronic exposures to concentrations as high as 1410 mg/L Cl^- do not cause degraded cytoplasm in epithelia of digestive gland diverticulum.

The significantly higher abundances of lipofuscin in kidney epithelial cells of mussels exposed to low and mid K^+ and low and mid Cl^- concentrations indicate a significant impact of both K^+ and Cl^-. Again, the high K^+ treatment did not show significant differences, but this can be attributed to the shorter exposure time of mussels in this treatment. The abundance of lipofuscin has been related to contaminant exposure in mussels (Riveros et al. 2002; Kagley et al. 2003), and the results from this study support these previous findings.

While captivity effects seemed to decrease in the 2013 study, they were still present. In the future several changes should be made to help decrease captivity effects. Juvenile *V. iris* and *Epioblasma capsaeformis* cultured in a sediment substrate showed significantly greater growth and survival compared to juveniles in no substrate (Jones et al. 2005). Gatenby (2000) hypothesized that a lack of burrowing substratum contributed to a low survival rate of freshwater mussels suspended in cages in hatchery ponds. Future studies could use redesigned holding systems which include substrate for mussels to burrow. Fine sediment has the ability to sorb ambient K^+; therefore, caution should be used if holding mussels in substrate to insure the mussels are actually being exposed to the intended concentration of the target contaminant (Fisher et al. 1991).

The recommended concentration of food used for this study was 10,000 cells/mL for each bucket over 24h, which was based on feeding protocols used at the FMCC (D. Hua, FMCC, personal communication). Other studies have recommended feeding adult mussels held in captivity at a concentration as high as 100,000 cells/mL at least twice daily (Gatenby 2000). Mussels starved in captivity showed degraded cytoplasm in the digestive gland after four weeks (Thompson et al. 1974). Mussels were fed daily during both the 2012 and 2013 studies; however, if available food was still not adequate, degraded cytoplasm in digestive glands of mussels held eight or twelve weeks in captivity is possible. It is clear that adequate food is essential for the health of adult mussels held in captivity; however, the exact amount needed to support the number of adult mussels in each bucket still needs to be determined. An increase in food can be accomplished by increasing the algal concentrations fed directly to each bucket or by performing water exchanges of fresh pond water twice weekly as opposed to just weekly.

Conclusion and Recommendations

It is evident from both the survival and histological results, especially during the 2013 study, that mussel tissues become degraded when exposed to increased concentrations similar to those found near Saltville, Virginia in the North Fork Holston River. Populations of *Villosa iris* and other mussel species will continue to decline and be excluded from this area until water quality is improved.

Future laboratory studies examining the health and function of mussel tissues exposed to contaminants will need to be improved to further reduce effects of captivity. Different systems could be used that allow the mussels to burrow in substrate. Also, food requirements for *V. iris* should be further examined to determine the optimal algal concentrations for feeding.

This study was designed to examine effects of salt concentrations found in the NFHR; however, the results have broader management implications. Increases in salinity in freshwater have been found in other systems due to runoff from road deicers, urban lands, and agriculture (Godwin et al. 2003; Anning and Flynn 2014). It is important that these salinity increases, including elevated concentrations of K^+ and Cl^-, are monitored and regulated so that mussels and other freshwater fauna do not continue to decline.

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Table 1. Water quality measurements of pond water sampled at The Freshwater Mollusk Conservation Center, Department of Fish and Wildlife Conservation, Virginia Tech, Blacksburg. The pond water was used as holding water for both the 2012 and 2013 studies. "<" indicates concentrations less than the instrument detection limit.

Parameter	mg/L	Parameter	mg/L	Parameter	mg/L
Ag	<0.006	Fe	0.01	Se	<0.022
As	<0.021	Hg	<0.025	Tl	<0.021
B	0.019	K	2.54	Zn	<0.006
Be	<0.001	Mg	29.874	NH_3-N	0.029
Ca	27.088	Na	8.864	NO_3	0.1
Cd	<0.005	Ni	<0.006	NO_2	0
Cl	20.2	P	<0.027	pH	8.45
Cr	<0.007	Pb	<0.016	Alkalinity	143
Cu	<0.003	S	<0.023	Hardness-$CaCo_3$	225

Table 2. Dependent variables used to assess the condition of vital organ tissues from adult *Villosa iris.* Fifty observations were acquired from gonadal tissues, and 100 observations were obtained from digestive gland, kidney, and gill tissues of each evaluated mussel. Each datum was recorded as either a one or zero, as described in this table.

Dependent Variable	Recorded as One	Recorded as Zero
Fractions of acini containing mature or developing gametes	acini contains mature or developing gametes	acini does not contain mature or developing gametes
Fractions of acini containing resorbing gametes	acini contains resorbing gametes	acini does not contain resorbing gametes
Fractions of digestive gland diverticula cells containing degraded cytoplasm	diverticula contains degraded cytoplasm	diverticula does not contain degraded cytoplasm
Fractions of gill filaments with cilia	cilia present on gill filament	cilia not present on gill filament
Fractions of kidney cells containing lipofuscin	kidney cell contains lipofuscin	kidney cell does not contain lipofuscin

Table 3. Arithmetic means (± standard errors) of water quality measurements for control and low and high K^+ during the 2012 study. No significant differences were measured for any water quality parameters (p>0.05).

Parameter	Unit	Control	Low K^+	High K^+
Temperature	°C	23.9 (0.04)	23.9 (0.03)	23.8 (0.04)
Dissolved Oxygen	%	88.9 (0.56)	88.1 (0.82)	88.1 (0.52)
pH	log [H^+]	8.58 (0.01)	8.58 (0.01)	8.57 (0.01)
Hardness	mg/L	244.7 (18.2)	239.2 (9.4)	261.3 (16.2)
Alkalinity	mg/L	194.8 (11.7)	191.7 (19.3)	186.3 (14.4)
Ammonia	mg/L	0.33 (0.11)	0.17 (0.06)	0.10 (0.01)

Table 4. Arithmetic means (± standard errors) of water quality measurements for control and low and high Cl^- during the 2012 study. No significant differences were measured for any water quality parameters besides pH (p>0.05). LETTERS indicate significant differences in water pH (p<0.05). Comparisons were made across rows.

Parameter	Unit	Control	Low Cl^-	High Cl^-
Temperature	°C	23.9 (0.04)	23.8 (0.03)	23.9 (0.04)
Dissolved Oxygen	%	88.9 (0.56)	87.8 (0.58)	87.7 (0.82)
pH	log [H^+]	8.58 (0.01)^A	8.55 (0.01)^A	8.52 (0.01)^B
Hardness	mg/L	244.7 (18.2)	249.0 (21.9)	243.1 (12.6)
Alkalinity	mg/L	194.8 (11.7)	178.5 (16.5)	190.9 (14.4)
Ammonia	mg/L	0.33 (0.11)	0.28 (0.07)	0.45 (0.15)

Table 5. Algal count (cells/mL) and cell size (µm) observed in treatment and control buckets in the 2012 study. Samples were taken from one randomly chosen bucket in each treatment and control during 2 sample events. Algal counts and size were measured using a hemocytometer.

	11/20/2012		12/14/2012	
Control/Treatment	Cell Count (cells/mL)	Mean Cell Size (µm)	Cell Count (cells/mL)	Mean Cell Size (µm)
Control	981	3.652	2636	3.983
Low K^+	1963	3.792	1585	4.010
High K^+	2443	3.787	931	3.706
Low Cl^-	3404	3.832	914	3.734
High Cl^-	2535	3.952	1933	4.051

Table 6. Arithmetic mean (± standard errors) of fractions of reproductive acini containing mature and/or developing gametes (FAMD) and acini containing atretic or resorbing gametes (FAR) observed in female (♀), male (♂), and hermaphroditic (H) *Villosa iris* from day zero baseline, control, and treatments in the 2012 study. No significant differences were measured for any comparisons of FAMD or FAR (p>0.05).

Treatment	Sex	n	FAMD	FAR
Baseline	♀	9	0.94 (0.06)	0.94 (0.06)
	♂	8	1.00 (0.00)	0.00 (0.00)
	H	3	1.00 (0.00)	1.00 (0.00)
Control	♀	10	0.99 (0.01)	0.99 (0.01)
	♂	1	0.98 (0.00)	0.96 (0.00)
Low K^+	♀	9	0.99 (0.01)	0.99 (0.01)
	♂	2	0.76 (0.01)	0.06 (0.06)
High K^+	♀	8	0.97 (0.02)	0.97 (0.02)
	♂	9	0.85 (0.04)	0.68 (0.09)
	H	1	1.00 (0.00)	1.00 (0.00)
Low Cl^-	♀	8	0.99 (0.01)	0.99 (0.00)
	♂	4	0.91 (0.06)	0.36 (0.21)
High Cl^-	♀	8	1.00 (0.01)	1.00 (0.00)
	♂	3	0.93 (0.07)	0.26 (0.26)

Table 7. Arithmetic mean (± standard errors) of fractions of digestive gland cells with abnormal cytoplasm, fractions of gill filaments with cilia, and fractions of kidney cells containing lipofuscin observed in baseline and control *Villosa iris* in the 2012 study. LETTERS indicate significant differences in histological variables (p<0.05). Comparisons were made within columns.

	Histological Variables		
Treatment	**Digestive Gland**	**Gill**	**Kidney**
Baseline	0.13 (0.02)^{A}	0.84 (0.03)^{A}	0.24 (0.02)^{A}
Control	0.23 (0.04)^{B}	0.86 (0.02)^{A}	0.36 (0.04)^{B}

Table 8. Arithmetic mean (± standard errors) of fractions of digestive gland cells with abnormal cytoplasm, fractions of gill filaments with cilia, and fractions of kidney cells containing lipofuscin observed in *Villosa iris* from Control, and Low and High K^+ treatments in the 2012 study. LETTERS indicate significant differences in histological variables between treatments (p<0.05). Comparisons were made within columns.

	Histological Variables		
Treatment	**Digestive Gland**	**Gill**	**Kidney**
Control	0.23 (0.04) A	0.86 (0.02) A	0.36 (0.04) A
Low K^+	0.27 (0.03) A	0.82 (0.03) A	0.45 (0.04) A
High K^+	0.24 (0.05) A	0.83 (0.04) A	0.42 (0.03) A

Table 9. Arithmetic mean (± standard errors) of fractions of digestive gland cells with abnormal cytoplasm, fractions of gill filaments with cilia, and fractions of kidney cells containing lipofuscin observed in *Villosa iris* from Control, and Low and High Cl^- treatments in the 2012 study. LETTERS indicate significant differences in histological variables between treatments (p<0.05). Comparisons were made within columns.

	Histological Variables		
Treatment	**Digestive Gland**	**Gill**	**Kidney**
Control	0.23 (0.04) A	0.86 (0.02) A	0.36 (0.04) A
Low Cl^-	0.27 (0.05) A	0.88 (0.02) A	0.38 (0.03) A
High Cl^-	0.15 (0.02) A	0.87 (0.02) A	0.43 (0.03) A

Table 10. Arithmetic means (± standard errors) of water quality measurements for control and low and high K^+ during the 2013 study. No significant differences were observed for any water quality parameters (p>0.05). [1]Measurements only taken on two occasions for control, mid and low; no measurements taken for high K^+. [2]Measurements only taken on two occasions for control, mid and low; measurements only taken once on high K^+.

Parameter	**Unit**	**Control**	**Low K^+**	**Mid K^+**	**High K^+**
Temperature	°C	23.66 (0.07)	23.73 (0.06)	23.61 (0.07)	23.73 (0.32)
Dissolved	%	85.31 (0.44)	86.00 (0.34)	86.62 (0.39)	86.82 (0.39)
pH	log	8.23 (0.04)	8.31 (0.04)	8.30 (0.05)	8.30 (0.06)
Hardness[1]	ppt	211 (15)	205 (9.0)	214.5 (8.5)	-
Alkalinity[1]	mg/L	245 (55.0)	233.0 (57.0)	238.0 (62.0)	-
Ammonia	mg/L	0.03 (0.01)	0.04 (0.01)	0.04 (0.02)	0.05 (0.02)
Nitrate[2]	mg/L	0.27 (0.09)	0.61 (0.27)	0.38 (0.15)	0.86 (0.27)
Nitrite[2]	mg/L	0.04 (0.02)	0.01 (0.01)	0.01 (0.002)	0.02 (0.01)

Table 11. Arithmetic means (± standard errors) of water quality measurements for control and low and high Cl^- during the 2013 study. No significant differences were observed for any water quality parameters (p>0.05). [1]Measurements only taken on two occasions for control, mid and low; no measurements taken for high Cl^-. [2]Measurements only taken on two occasions for control, mid and low; no measurements taken for high Cl^-.

Parameter	**Unit**	**Control**	**Low Cl^-**	**Mid Cl^-**	**High Cl^-**
Temperature	°C	23.66 (0.07)	23.66 (0.07)	23.91 (0.07)	24.34 (0.17)
Dissolved	%	85.31 (0.44)	85.90 (0.38)	85.20 (0.23)	86.30 (0.21)
pH	log	8.23 (0.04)	8.32 (0.04)	8.33 (0.04)	8.27 (0.26)
Hardness[1]	ppt	211 (15)	208.0 (7.0)	206.0 (4.0)	-
Alkalinity[1]	mg/L	245 (55.0)	245.0 (55.0)	240.0 (30.0)	-
Ammonia	mg/L	0.03 (0.01)	0.05 (0.01)	0.06 (0.02)	0.06 (0.03)
Nitrate[2]	mg/L	0.27 (0.09)	0.48 (0.13)	0.68 (0.19)	-
Nitrite[2]	mg/L	0.04 (0.02)	0.01 (0.002)	0.01 (0.002)	-

Table 12. Arithmetic mean (± standard errors) of fractions of reproductive acini containing mature and/or developing gametes (FAMD) and acini containing atretic or resorbing gametes (FAR) observed in female (♀), male (♂), indeterminate (I) and hermaphroditic (H) mussels from day zero baseline, control, and treatments in the 2013 study. No significant differences were observed for any comparisons of FAMD or FAR (p>0.05).

Treatment	Sex	n	FAMD	FAR
Baseline	♀	1	0.48 (0.00)	0.00 (0.00)
	♂	17	0.92 (0.04)	0.01 (0.01)
	I	2	0.00 (0.00)	0.00 (0.00)
Control	♀	2	0.82 (0.16)	0.81 (0.19)
	♂	5	0.90 (0.06)	0.22 (0.05)
	I	13	0.40 (0.18)	0.25 (0.07)
Low Cl^-	♀	2	0.94 (0.06)	0.96 (0.04)
	♂	13	0.90 (0.06)	0.13 (0.07)
	I	5	0.11 (0.11)	0.03 (0.03)
Mid Cl^-	♀	0	-	-
	♂	12	0.96 (0.03)	0.18 (0.06)
	H	1	1.00 (0.00)	0.54 (0.00)
	I	7	0.14 (0.50)	0.08 (0.03)
Low K^+	♀	2	0.95 (0.05)	0.77 (0.13)
	♂	13	0.88 (0.07)	0.40 (0.08)
	I	5	0.20 (0.50)	0.69 (0.79)
Mid K^+	♀	3	0.34 (0.10)	0.62 (0.18)
	♂	8	0.96 (0.03)	0.24 (0.06)
	I	9	0.67 (0.14)	0.44 (0.09)
High K^+	♀	3	0.84 (0.16)	0.84 (0.16)
	♂	13	0.95 (0.03)	0.11 (0.04)
	H	2	0.85 (0.13)	0.57 (0.22)
	I	2	0.50 (0.50)	0.50 (0.04)

Table 13. Least square mean (± standard errors) of fractions of digestive gland cells with abnormal cytoplasm, fractions of gill filaments with cilia, and fractions of kidney cells containing lipofuscin observed in *Villosa iris* from baseline and control in the 2013 study. LETTERS indicate significant differences in survival between treatments ($p<0.05$). Comparisons were made within columns.

	Histological Variables		
Treatment	**Digestive Gland**	**Gill**	**Kidney**
Baseline	0.03 (0.01) ^{A}	0.93 (0.01) ^{A}	0.03 (0.01) ^{A}
Control	0.08 (0.02) ^{B}	0.98 (0.01) ^{B}	0.12 (0.02) ^{B}

Table 14. Least square mean (± standard errors) of fractions of digestive gland cells with abnormal cytoplasm, fractions of gill filaments with cilia, and fractions of kidney cells containing lipofuscin observed in *Villosa iris* from Control, and Low, Mid and High K^+ treatments in the 2013 study. LETTERS indicate significant differences in survival between treatments ($p<0.05$). Comparisons were made within columns.

	Histological Variables		
Treatment	**Digestive Gland**	**Gill**	**Kidney**
Control	0.08 (0.02) ^{A}	0.98 (0.01) ^{A}	0.12 (0.02) ^{A}
Low K^+	0.30 (0.03) ^{B}	0.95 (0.02) ^{AB}	0.21 (0.02) ^{B}
Mid K^+	0.20 (0.03) ^{B}	0.95 (0.01) ^{A}	0.21 (0.02) ^{B}
High K^+	0.16 (0.04) ^{AB}	0.88 (0.02) ^{B}	0.06 (0.03) ^{A}

Table 15. Least square mean (± standard errors) of fractions of digestive gland cells with abnormal cytoplasm, fractions of gill filaments with cilia, and fractions of kidney cells containing lipofuscin observed in *Villosa iris* from Control, and High and Low Cl^- treatments in the 2013 study. LETTERS indicate significant differences in survival between treatments ($p<0.05$). Comparisons were made within columns.

	Histological Variables		
Treatment	**Digestive Gland**	**Gill**	**Kidney**
Control	0.08 (0.02) ^{A}	0.98 (0.01) ^{A}	0.12 (0.02) ^{A}
Low Cl^-	0.12 (0.03) ^{A}	0.94 (0.02) ^{AB}	0.22 (0.02) ^{B}
Mid Cl^-	0.10 (0.03) ^{A}	0.93 (0.02) ^{B}	0.28 (0.02) ^{B}

Figure 1. Schematic of downweller bucket system used for Cl^- and K^+ studies. A. Upper bucket. B. Water level. C. Lower bucket. D. Pump attached to bulkhead. E. View from above showing bottom of upper bucket with F. Mesh covered holes (modified from Barnhart 2006).

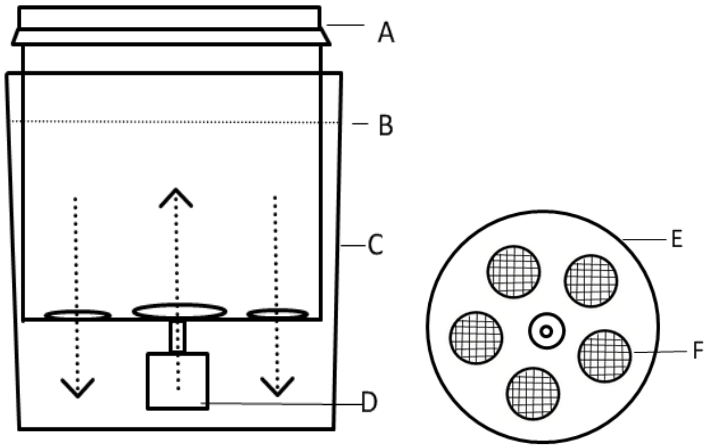


Figure 2. Example of point-count method in gonad to evaluate oogenic and spermatogenic acini. Points B, C, and F would be assigned a 1 for evaluations of fraction of acini containing mature or developing gametes, because the points fall directly above acini that contain mature or developing gametes. Points A, D, and E would not be evaluated because they are not on acini.

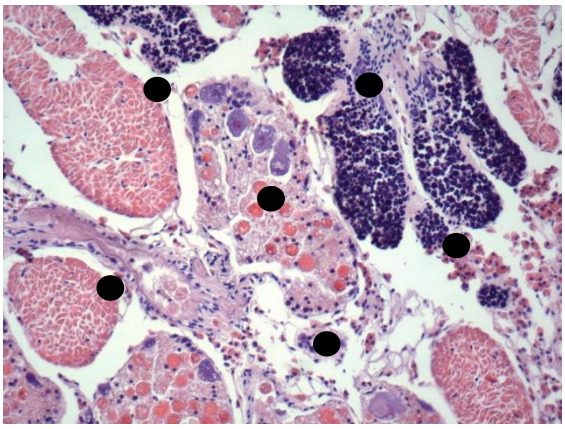


Figure 3. Mean growth (by length) of *Villosa iris* held in control, low and high K^+ treatments during the 2012 study. Error bars represent 95 percent confidence intervals. LETTERS indicate significant differences in growth between treatments ($p<0.05$).

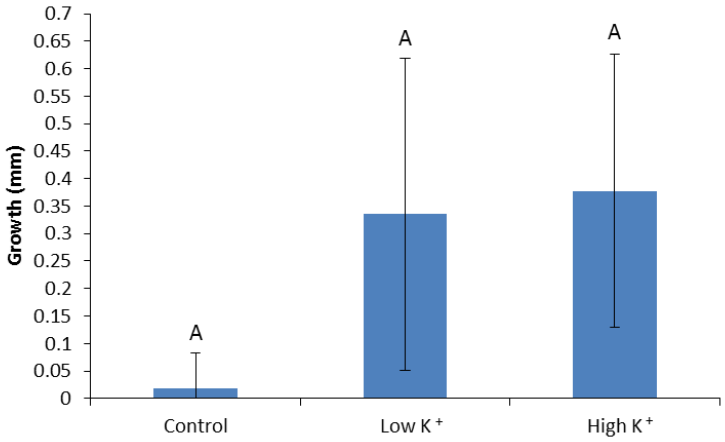


Figure 4. Mean growth (by length) of *Villosa iris* held in control, low and high Cl^- treatments during the 2012 study. Error bars represent 95 percent confidence intervals. LETTERS indicate significant differences in growth between treatments ($p<0.05$).

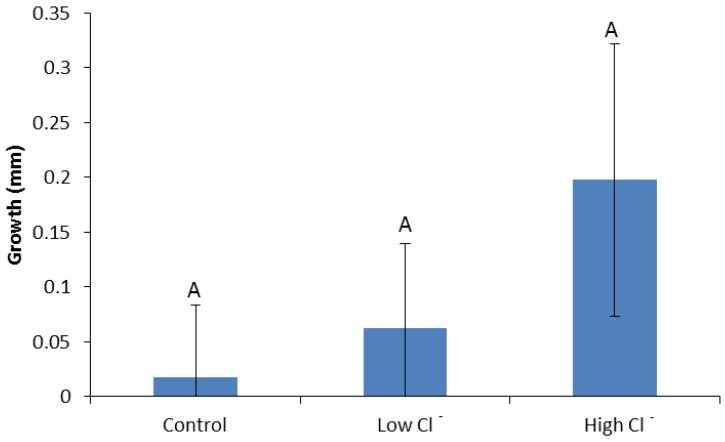


Figure 5. Percent survival of *Villosa iris* per day held in control, low and high K^+ treatments during the 2012 study. LETTERS indicate significant differences in survival between treatments (p<0.05).

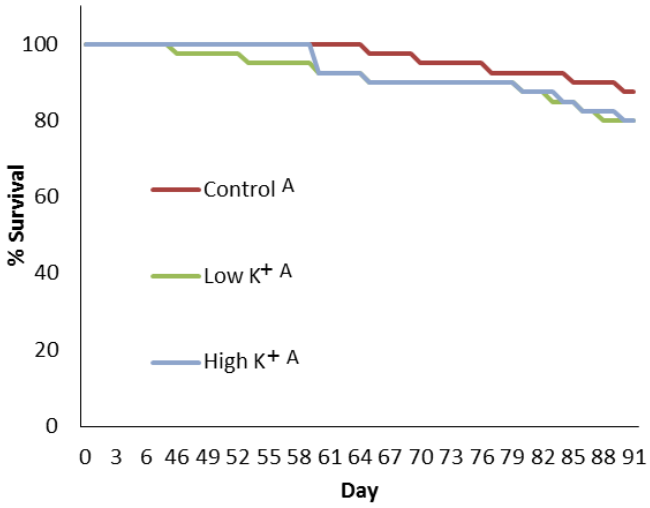


Figure 6. Percent survival of *Villosa iris* per day held in control, low and high K^+ treatments during the 2012 study. LETTERS indicate significant differences in survival between treatments (p<0.05).

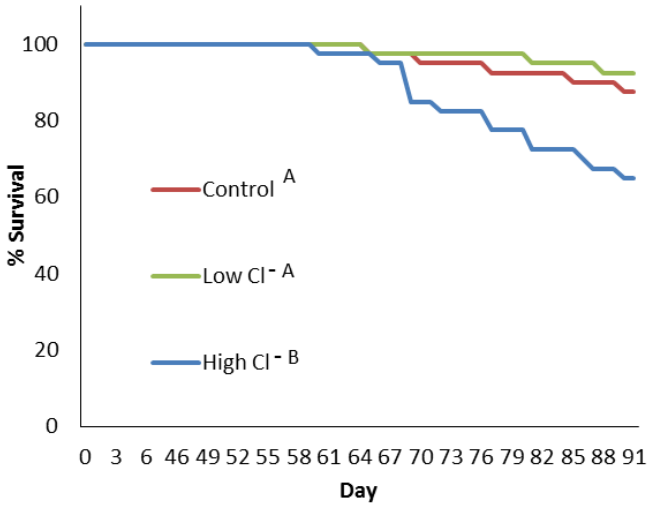


Figure 7. Percent survival of *Villosa iris* per day held in control, low, mid and high K^+ treatments during the 2013 study. LETTERS indicate significant differences in survival between treatments (p<0.05). *Mortality threshold of 50% occurred, so remaining surviving mussels were sampled on Day 13 and the high K^+ treatment was shut down.

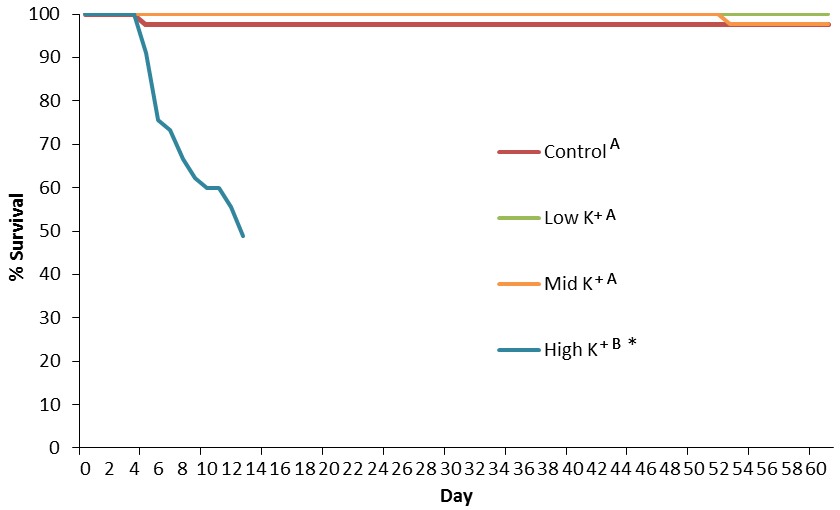


Figure 8. Percent survival of *Villosa iris* per day held in control, low, mid and high Cl^- treatments during the 2013 study. LETTERS indicate significant differences in survival between treatments (p<0.05).

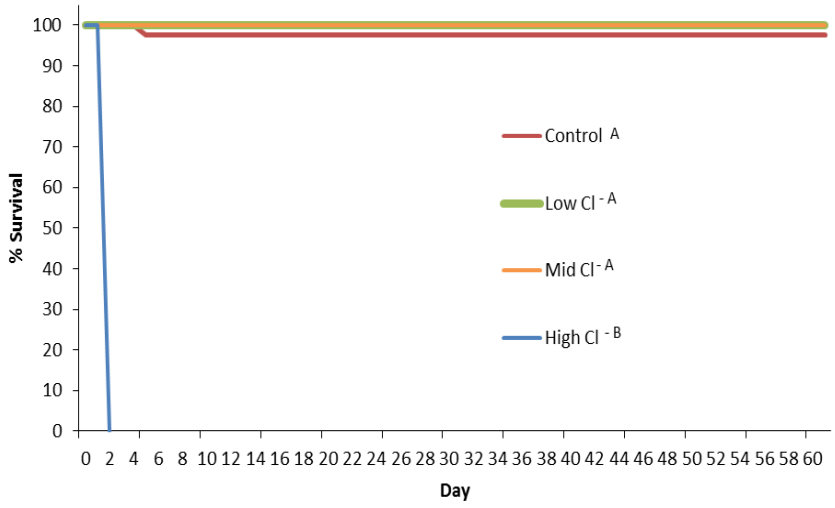


Figure 9. Reproductive and digestive gland tissues of *Villosa iris* stained with hematoxylin and eosin. A. Oogenic acini (oa) containing resorbing oocytes (r) from low NaCl, 2013 study. Bar = 2 µm. B. Spermatogenic acinus (sa) containing spermatozoa (s) in ripe stage of gametogenesis, from baseline, 2012 study. Bar = 4 µm. C. Cross section of normal digestive diverticula with lumina (l) and digestive (dc) and basophilic (bc) cells from high NaCl, 2012 study. Bar = 4 µm. D. Digestive diverticulum (dd) containing reduced cytoplasm (rc) from low NaCl, 2012 study. Bar = 4 µm

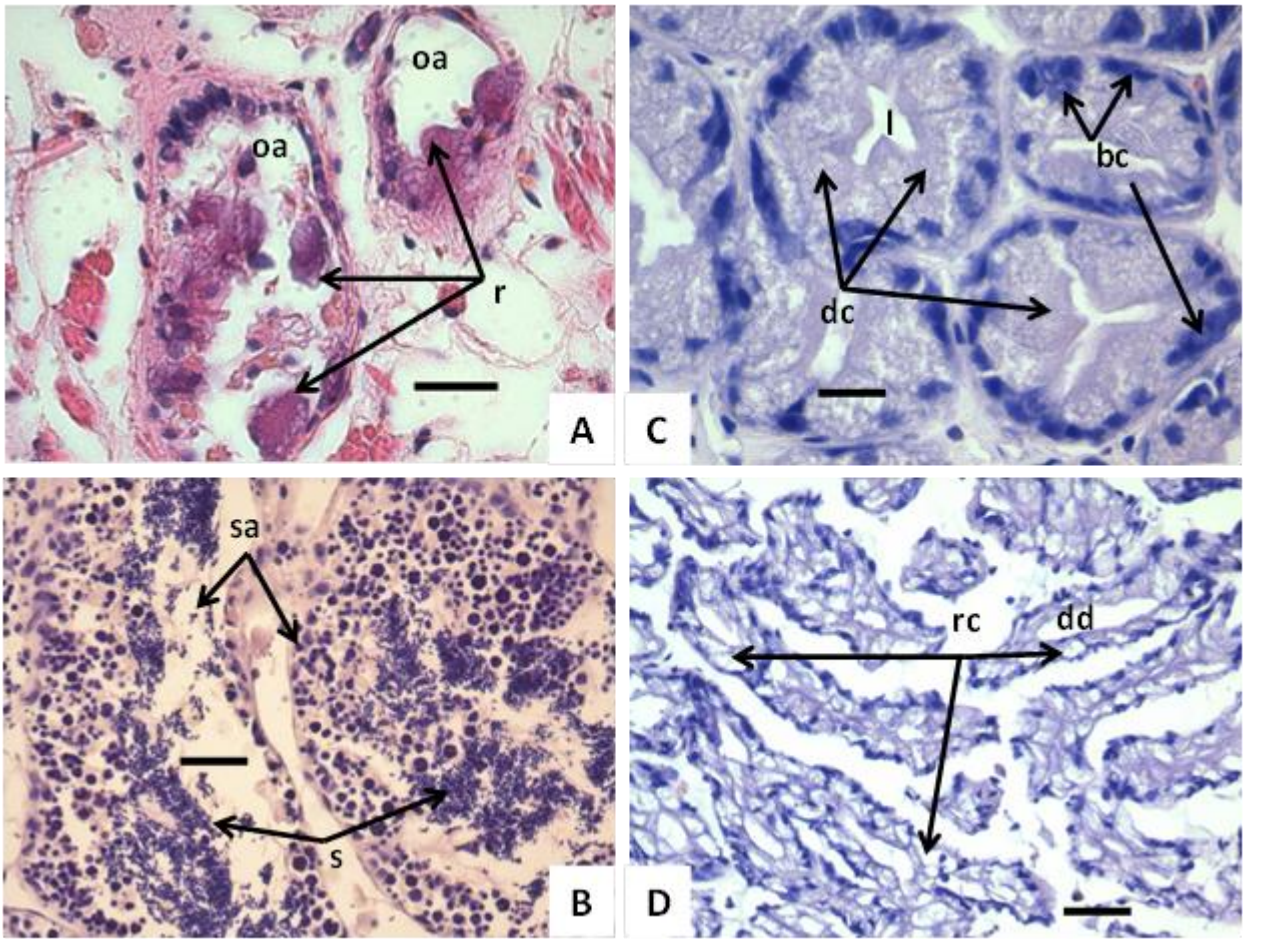


Figure 10. Gill and intestinal tissue (stained with hematoxylin and eosin) and kidney tissue (stained with Carbol Fuscin) of *Villosa iris*. A. Normal ciliated (c) gill filaments (gf) control, 2012 study. Bar = 4 µm. B. Kidney diverticulum (kd) containing high abundance of lipofuscin (lf) (brown inclusions) from high NaCl, 2012 study. Bar = 2 µm. C. Fusion (f) in gill filaments (gf) from low NaCl, 2012 study. Bar = 4 µm. D. Parasite (p) in intestine (i) from low KCl, 2012 study. Bar = 10 µm.

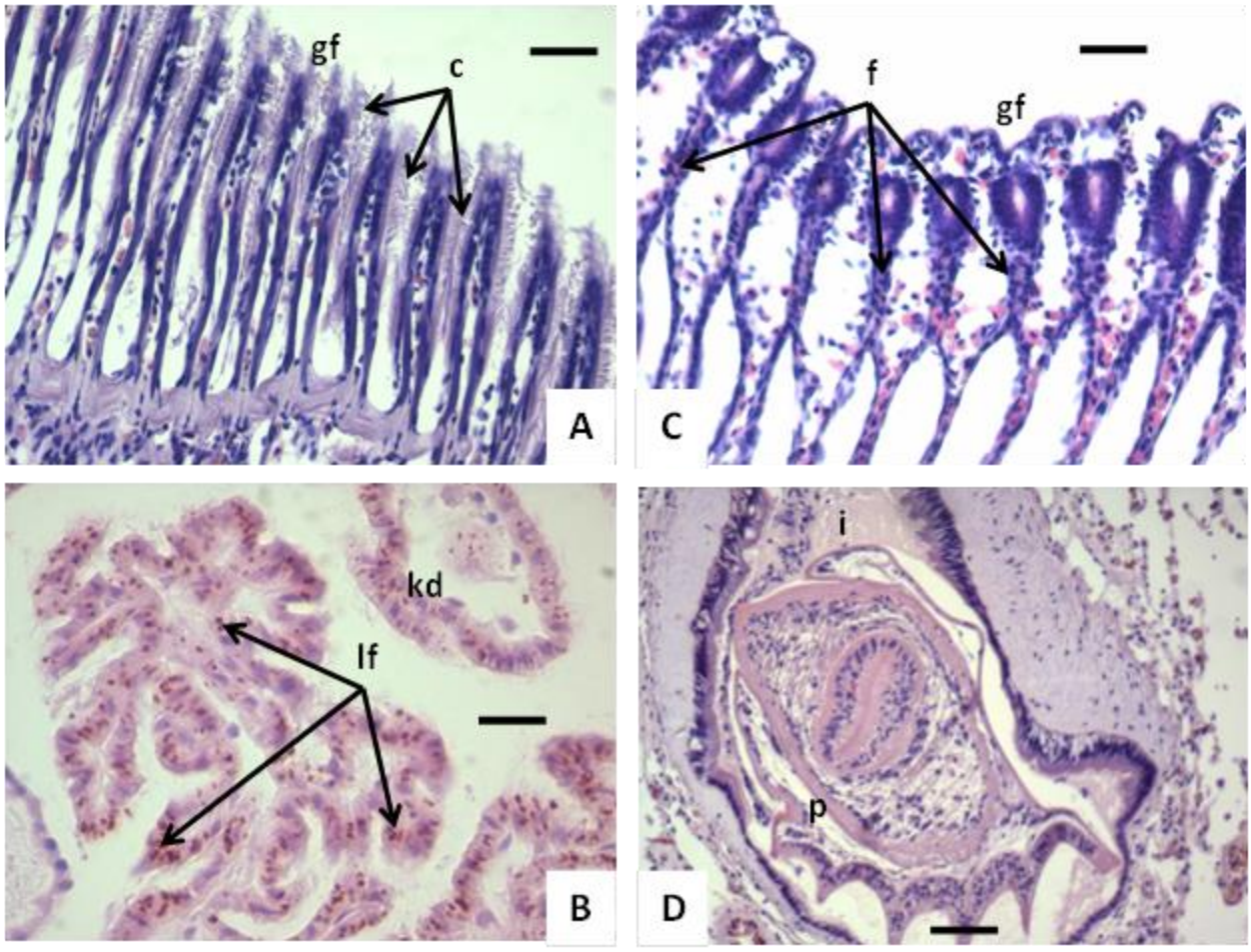
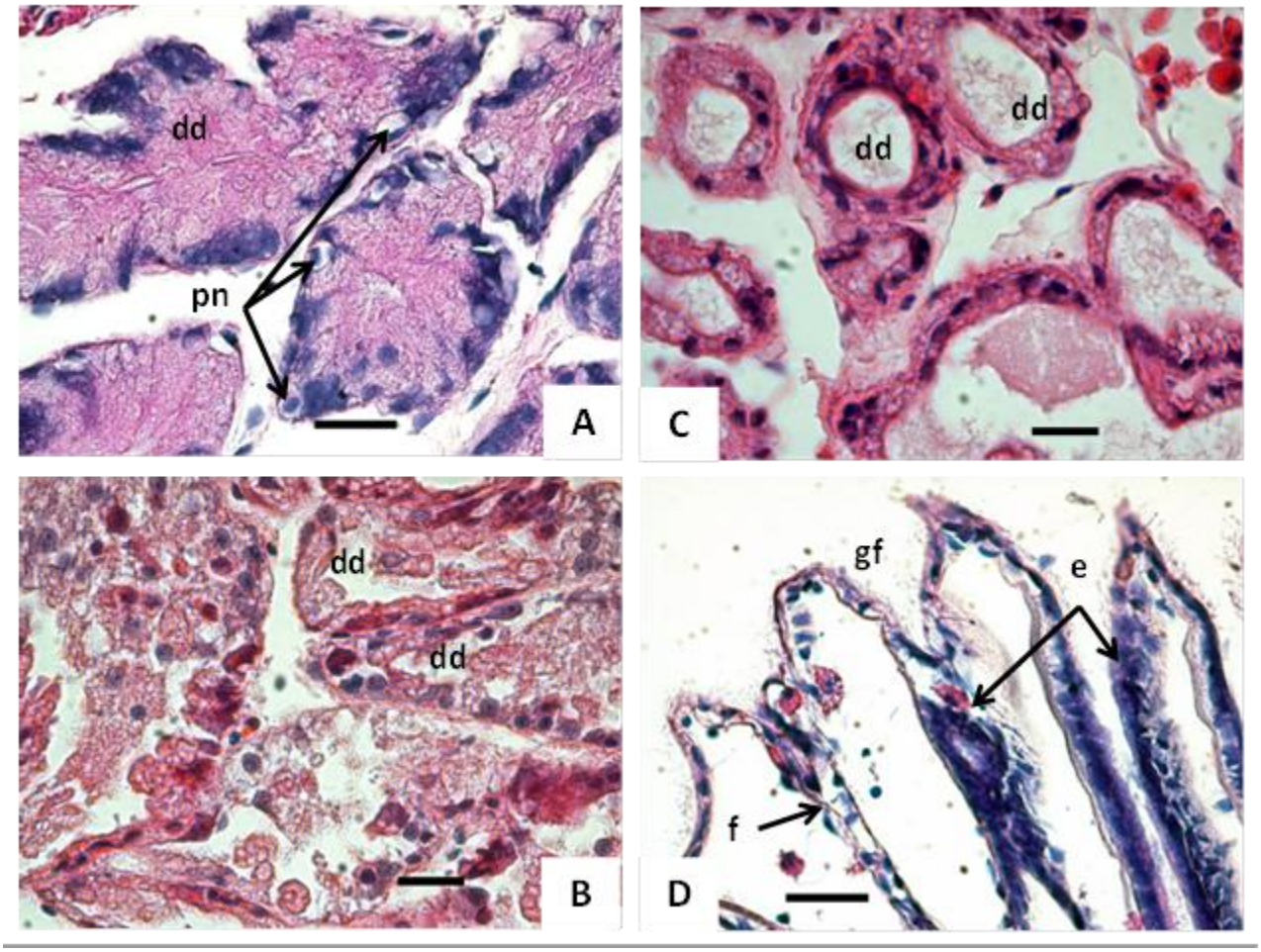


Figure 11. Other histological observations noted in *Villosa iris*; stained with hematoxylin and eosin. A. Pyknotic nuclei (pn) found in digestive gland diverticulum (dd), from low KCl, 2013 study. Bar = 2 µm. B. Necrosis in digestive gland diverticulum (dd), from mid KCl, 2013 study. Bar = 2 µm. µm. C. Atrophy in digestive gland diverticulum (dd), from mid NaCl, 2013 study. Note diminution of epithlial layers of diverticula compared to those in Fig. 3C. Bar = 2 µm. D. Edema (e) and fusion (f) in gill filaments (gf) from mid NaCl, 2013 study. Bar = 2 µm.



Chapter 3

Effects of Un-Ionized Ammonia on Organ Tissues of Adult *Villosa iris* Using Histological Evaluations

Abstract

Once supporting a diverse assemblage of more than 40 mussel species, the North Fork Holston River (NFHR) has experienced substantial declines in its species diversity and abundance. Historical industrial activities at Saltville, Virginia, as well as continued pollution of the river from chemical waste ponds at this location, are believed to be significant contributors of these declines. Contaminant seepages from the waste ponds containing ammonia have been shown to be toxic to mussel populations downstream of Saltville. Freshwater mussels have been shown to be very sensitive to ammonia. A three-month laboratory study was conducted to assess impacts to organ tissues (gills, digestive glands, kidneys, and gonads) of adult *Villosa iris* exposed to environmentally relevant concentrations of un-ionized ammonia (0.014 and 0.15 mg/L NH_3-N) using histological evaluations. Results of evaluations for all variables indicated no detectable differences among the histological endpoints from mussels held in treatments and control ($p>0.05$). Survival and growth also were not significantly different among treatments and control ($p>0.05$). Significant histological differences between control and baseline mussels occurred ($p<0.05$), indicating that captivity influenced condition of mussel tissues. Additionally, maintaining consistent levels of and measuring ammonia concentrations was difficult, which resulted in unreliable data. Future studies need to more thoroughly address both the captivity effects and how best to conduct long-term (>60 days) ammonia exposures to adult mussels. Potential improvements for captivity include increasing available algal food, providing substrate for mussels to burrow, and decreasing time in captivity. The use of higher quality electrodes and reconstituted water instead of pond water could improve ammonia studies.

Keywords: Freshwater Mussels, Histology, *Villosa iris*, North Fork Holston River, Mussel Declines, Ammonia

Introduction

An estimated 840 freshwater mussel species are distributed worldwide, but with approximately 300, the greatest diversity is found in North America (Bogan 1993; Graf and Cummings 2007). However, mussels are experiencing declines in North America, with about 10% already extinct and more than 70% in need of protection (Williams et al. 1993; Neves et al. 1997; Strayer et al. 2004; Haag and Williams 2013). These declines are most often attributed to a suite of impacts, including habitat destruction caused by dams, dredging and channelization, which drastically change the riverine and riparian environments, and also eliminate host fish species that are essential to mussel reproduction (Strayer et al. 2004; Haag 2012). Additionally, sedimentation from deforestation, agriculture, and urbanization causes degradation of stream water and benthic substrates. Chemical contamination, primarily derived from agriculture and industry, often introduces heavy metals, pesticides, and mine drainage into the riverine system (Ortmann 1909; Ellis 1931; Cope et al. 2008). Commercial harvesting of freshwater mussels and introduction of exotic species (Asian clam, *Corbicula fluminea*, and zebra mussel, *Dreissena polymorpha*) also have been shown to negatively affect mussel populations (Claassen 1994; Strayer 1999; Strayer et al. 2004).

Freshwater mussels provide important ecosystem services, and with over 70 species federally listed as threatened or endangered, the United States Endangered Species Act of 1973 drives conservation actions to monitor and protect remaining populations and the ecosystem services that they provide. These benthic invertebrates stabilize the lotic substrates and sequester suspended particles, nutrients and some pollutants by filtering stream water (Strayer et al. 1994; Vaughn and Hakenkamp 2001). They also are a source of food to animals such as muskrats, otters, raccoons, sportfish, and ducks (Helfrich et al. 1986).

The North Fork Holston River (NFHR), located within the Upper Tennessee River Valley System, originates in Bland County, Virginia. After flowing for 200 kilometers from its origin, it joins the South Fork Holston River to form the Holston River near Kingsport, Tennessee. The NFHR basin is primarily forested, with agricultural land comprising about 30% of the watershed and urban areas only covering about 1% (Ahlstedt and Rashleigh 1996). The NFHR site of interest for this study is located near the town of Saltville, Virginia.

Industrial activities in the NFHR have been present at Saltville since the 1800s. After salt had been mined for decades, the Mathieson Chemical Company (which merged with the Olin Corporation in 1954 to become the Olin-Mathieson Chemical Company) opened and operated a soda ash (sodium carbonate) facility from 1950 to 1980 (Henley and Neves 1999). The wastes from this facility were discharged into settling ponds where solids were allowed to settle before the liquid wastes were discharged into the river. This waste consisted mostly of calcium carbonate and chloride brines. The Olin Plant in Saltville also manufactured dry ice and hydrazine, which added more wastes such as heavy metals and ammonia to the holding ponds (Olin 1991; Ahlstedt and Rashleigh 1996). Additionally, ammonia can enter the river directly from municipal effluent discharges and animal excretion and indirectly from agricultural runoff, nitrogen fixation, and air deposition (USEPA 2013).

Once supporting 42 mussel species, the NFHR mussel fauna has substantially declined over the last 60 years or longer (Ortmann 1918; Ahlstedt and Rashleigh 1996). Surveys have shown as few as nine extant species downstream of Saltville and 13 species upstream of Saltville (Henley and Neves 1999; Jones and Neves 2007), although at least 16 species are known to occur upstream (J. W. Jones, USFWS, personal communication). The industrial activities at Saltville, as well as continued pollution of the river from chemical holding ponds, are believed to be significant causes of these declines. Seepage waters from chemical holding ponds add high concentrations of salts and ammonia, as well as various other ions, to the river and are suspected of negatively affecting mussel populations downstream of Saltville (Wang and Ingersoll 2010).

Un-ionized ammonia (NH_3-N) is present in the NFHR at a seepage point from a chemical waste holding pond near Saltville at concentrations that have been shown to be toxic to adult and juvenile mussels (Wang and Ingersoll 2010). Wang and Ingersoll (2010) found levels of total ammonia in these seepage waters up to 10 mg N/L at pH about 7.6. More recently, NH_3-N was measured near Saltville at concentrations that exceed both the accepted Virginia and EPA acute and chronic water quality criteria for the protection of aquatic life (USEPA 2009; Henley et al. 2013). Henley et al. (2013) found maximum levels of NH_3-N to be as high as 5.7 mg/L.

The histological variables used in this study to evaluate the condition of mussel tissues exposed to ammonia included evaluations of gonad, digestive gland, gill, and kidney tissues, and have been used in previous studies to demonstrate effects of contaminants on vital organ tissues of marine bivalves (Bayne et al. 1981; Seiler and Morse 1988; Au 2004). Recent studies using

histological evaluations of mussels have shown atrophy of digestive gland cells, erosion of gill cilia, and differences in gamete production and resorption of oocytes to be indicators of exposure to contaminants (Henley et al. 2007; 2008a; 2008b). Organ tissues are sites of absorption, sequestration, accumulation and excretion of contaminants, all of which can cause cellular alterations to occur (Seiler and Morse 1988; Domouhtsidou and Dimitruadis 2000).

For example, acini within the gonads are the site of gamete development in mussels, which includes a natural cycle of maturation, spawning and resorption of residual gametes (Kennedy and Battle 1964; Pipe 1987; Dorange and Pennec 1989; Barber 1996; Henley 2010). Various metal and organic contaminants have been shown to affect this process by suppressing development of gametes in bivalves (Gosling 2003). Resorption of gametes in acini is a natural occurrence after a mussel spawns; however, untimely resorption has been observed as a response to stress and exposure to contaminants (Bayne and Thompson 1970; Bayne et al. 1981; Tay et al. 2003; Henley 2010).

The bivalve digestive gland contains diverticula with epithelial layers made up of secretory and digestive cells. These diverticula cells are the site of intra- and extra-cellular digestion, nutrient absorption, lipid and glycogen storage, and contaminant detoxification (Owen 1970; Lobo-da Cunha 1999; Petrović et al. 2001; Henley 2010). Contaminant exposure can cause cellular alteration in the digestive gland including degradation of cytoplasm in the diverticula cells (Lowe et al. 1981; Au 2004; Usheva et al. 2006; Henley et al. 2013).

Bivalve gills are paired ciliated organs involved in respiration, osmoregulation, and food capture and transport. Because they are the first uptake site in the mussel, gill cells are exposed to many contaminants found in water (Gómez-Mendikute et al. 2005). Loss of cilia along with fusion of gill filaments, inflammation, necrosis, epithelial cell sloughing, and increased mucus production are all histopathological alterations that have been linked to contaminant exposure (Domouhtsidou and Dimitruadis 2000; Lajtner et al. 2003; Gómez-Mendikute et al. 2005; Supanopas et al. 2005; Henley et al. 2013).

The bivalve kidney is responsible for ultrafiltration of hemolymph, ion exchange, and excretion (Dietz et al. 2000; Fahrner and Haszprunar 2002). The kidney also is responsible for sequestering contaminants by excretion of lipofuscin granules. The abundance of lipofuscin can increase with age, but also has been related to contaminant exposure in mussels (Riveros et al. 2002; Kagley et al. 2003).

Thus, the purpose of this study was to assess the condition of vital organ tissues of adult rainbow mussels, *Villosa iris*, exposed to NH_3-N using microscopical evaluations of histologically prepared gill, digestive gland, kidney, and gonad tissues. Test concentrations of NH_3-N were determined from data obtained from measurements taken in the NFHR and other ecological benchmarks.

Material and Methods

Study Conditions

The experiment was conducted at the Laboratory for Conservation Aquaculture and Aquatic Ecology, Virginia Tech, Blacksburg, Virginia, and involved a three-month exposure of NH_3-N running from mid-September through mid-December 2012. Fifteen closed recirculating systems were built for this experiment. Systems were modified downweller buckets designed for culturing juvenile mussels (Barnhart 2006) (Fig. 1), and herein referred to as buckets. Each treatment consisted of five separate replicate buckets held within a water bath, and herein referred to as treatment banks. There also was one bank of five control buckets held within a water bath, which contained holding water with no additional ammonia. Eight mussels were placed into each bucket, for a total of 40 mussels per treatment or control bank.

The holding water was pumped from the pond located at The Freshwater Mollusk Conservation Center (FMCC), Department of Fish and Wildlife Conservation, Virginia Tech, Blacksburg. This was the same water used for the propagation and culture of the test mussels. Prior to the study, samples of pond water were sent to the Virginia Tech Soil Testing Laboratory, Blacksburg, Virginia, for analysis of analytes. Additional water quality measurements were conducted at the FMCC to determine alkalinity, hardness, pH, and ammonia concentration (Table 1). The pond water at the start of the study averaged 24°C throughout the day. Due to a lack of chillers in the laboratory to cool the water, and because this was the water temperature in which mussels were currently living, the treatment banks were maintained at 24°C for the remainder of the study.

Adult *V. iris* propagated and cultured at the FMCC were used in this study. These mussels were produced by standard host fish infection techniques and cultured in water recirculating systems using pond water. At the beginning of the experiment, the mussels were about 21 months old and ranged from 14 mm to 39 mm.

Mussels were fed commercial algae using diluted Shellfish Diet® (Reed Mariculture, Inc., Campbell, California). The food was administered continuously using a drip valve delivering 100 mL to each bucket over a 24 hour period. To ensure all treatments and controls received equal amounts of food, samples were randomly collected from buckets from each treatment and control. These 16 mL samples were fixed with three drops of Lugol's iodine solution (5% solution, Fisher Scientific). Algal cells were quantified using a Coulter Counter (Beckman Coulter, Multisizer 3) and measured as cells per mL and mean cell size (µm).

Stock solutions were created using pond water filtered through a 200 µm nylon monofilament bag (Aquatic Ecosystems) and reagent grade ammonium chloride (NH_4Cl, 99.9% purity, Fisher Scientific). As opposed to trying to maintain a constant ammonia concentration, the treatment buckets were spiked with either the high or low level concentration three times a week. Temperature and pH affect the percentage of NH_3-N in total ammonia nitrogen (TAN) (Emerson et al. 1975). Therefore, water temperature was maintained at a temperature of 24^{o}C (± 0.5) using aquaculture heaters set in the water bath surrounding the buckets and measured twice daily using a digital thermometer and pH was measured throughout the study. One hundred percent water exchanges were performed weekly.

The test concentrations that the buckets were spiked with in the laboratory study were determined based on environmentally relevant concentrations. The high concentration of NH_3-N was determined from the mean concentration of NH_3-N found at North Fork Holston River study sites (0.15 mg/L at pH 8.4 and 24^{o}C) (Henley et al. 2013). The draft EPA Aquatic Life Water Quality Chronic Criterion for NH_3-N when mussels are present (0.014 mg/L at pH 8.4 and 24^{o}C) was the concentration used in the low NH_3-N treatment (USEPA 2009).

Ammonia was measured daily using an Orion High-Performance Ammonia Electrode and Orion EA940 meter (Thermo Fisher Scientific Incorporated, Waltham, Massachusetts). A handheld meter (Oakton Waterproof Double Junction pHTester 20) was used to measure pH daily. Bi-weekly measurements were conducted for dissolved oxygen (YSI Professional Plus Multiparameter Meter), total hardness (titration, Hach Method 8213; mg of Ca/L as CaCo3 plus mg/L of CaCo3) and total alkalinity (Hach Model AL-AP; mg of phenolphthalein alkalinity/L plus mg total methyl orange alkalinity/L as CaCo3). All meters were calibrated before each use with calibration verification standards. Replicate samples were measured from random systems each week to perform quality assurance/quality control.

Sampling Procedures

Before mussels were removed from the bucket, two histological cassettes per mussel were labeled and organized for treatment and control banks. Each label consisted of a specific code for each mussel (including the bucket and mussel number, and the organ type of the tissue to be contained in the cassette; for example, the code "A1M1 Gill" would represent gill tissue from mussel one in bucket A). Cassette codes were used to track the two cassettes containing tissues from the same mussel throughout histological processing and microscopical evaluations of stained tissues. One cassette held gill tissue, and the other held gonad, digestive gland, and kidney tissues. Bottles of 10% neutral buffered formalin also were prepared and labeled (one bottle per treatment or control) to fix organ tissues at the time of collection (Bancroft and Gamble 2008).

Twenty mussels were randomly selected at the beginning of the experiment, without being exposed to any of the experimental conditions, to be used as baseline specimens for subsequent statistical comparisons. The shell length (mm) of collected mussels was measured and recorded. The entire body mass was first removed from their shell and then sliced in half through the dorsal to ventral mid-section of the body using forceps and a scalpel and placed in a labeled histological cassette. Cassettes containing tissues were immediately placed in the formalin bottle labeled as "day 0" mussels.

The only sampling event occurred at the end of the study, after three months of exposure. The study was set up so that sampling would occur during probable active gametogenesis. Four mussels were randomly sampled from each bucket, and their tissues were harvested for histological processing. These mussels were processed and preserved using the same procedure as described previously for the baseline mussels. Mussels were fixed in bottles of 10% formalin pre-labeled for each treatment and control bank.

If a bank of buckets had a mortality rate exceeding 50% at any point during either experiment, the sampling event immediately occurred for that bank and that treatment bank was shut down. This was necessary to ensure data were available from all treatments because histological processing of tissues could only be performed if the mussels were collected while still alive.

Histological Processing

After being fixed in 10% formalin for at least one week, tissues were rinsed with deionized water to remove formalin and placed in a container of 70% ethanol for no more than one week. The gills of each individual were dissected from the visceral mass and placed into separate labeled histology cassettes. The cassettes containing gills, and the cassettes containing the gonad, digestive glands, and kidney, were placed back in the container of 70% ethanol. The tissues underwent a process of dehydration through a progressive series of concentrations of ethyl alcohol and then cleared in 100% xylene in preparation for paraffin embedding. Once embedded in paraffin wax, the tissues were sectioned and mounted on microscope slides labeled with cassette code and tissue type.

Tissue sections were cut from paraffin-embedded tissue blocks using a rotary microtome (Leica RM2125RT, Leica Microsystems Incorporated, Wetzlar, Germany). Sections were cut at approximately 50% of the tissue depth and mounted on glass microscope slides. Tissues from one mussel were placed on three microscope slides; one slide containing gill tissues, and the other two slides containing gonad, digestive gland, and kidney tissues. Gill tissues and one slide containing visceral organs were stained with hematoxylin and eosin for microscopic evaluations. The duplicate slides containing visceral organs were stained using the Long Ziehl-Neelsen method (Bancroft and Gamble 2008) for elaboration and evaluation of lipofuscin in kidney tissues. Coverslips were mounted over all stained tissues. After staining, the slides were evaluated using light microscopy (Olympus BX 41 light microscope, Olympus America, Incorporated, Center Valley, Pennsylvania).

Histological Evaluations

Gonad, digestive gland, kidney, and gill tissues of *V. iris* sampled during both rounds of this study were microscopically evaluated using five histologically-based dependent variables. The dependent variables were fraction of reproductive acini containing mature and/or developing gametes (FAMD); fraction of acini containing resorbing gametes (FAR); fraction of digestive gland diverticula cells containing degraded cytoplasm (FDGDC); fraction of kidney diverticula cells containing lipofuscin (FKDL); and fraction of gill filament termini with cilia (FGFTC).

Quantitative evaluations of organ tissues were performed by light microscopy using a point count method (Chalkey 1943). Six dots were drawn on the ocular piece of the microscope

so they appeared over the slides containing mussel tissues (Fig. 2). Evaluations were only made on target tissues under the dots; thus, up to six evaluations were made at each location of the slide. Once tissue under all six dots was evaluated or determined to not be target tissue, the slide was randomly moved to another area of tissue until all evaluations were conducted for each dependent variable. Fifty observations per mussel were conducted for the two dependent variables associated with gonad tissue. One hundred observations per mussel were conducted for the dependent variables associated with the kidney, digestive gland, and gills. Data were recorded using a dichotomous dependent variable index using ones (for presence of a particular variable) and zeros (for absence). For example, when evaluating gills, if cilia was present on the gill filament termini then a one was recorded; however, if cilia was absent from the gill filament termini then a zero was recorded (Table 2).

Data Analysis

The majority of statistical analyses were performed in SAS (SAS Institute, Inc., Cary, NC). The SAS code for histological variables was modified from a previous histological study (Henley et al. 2013) in consultation with staff at Virginia Tech's Laboratory for Interdisciplinary Statistical Analysis (LISA). The SAS code for survival analyses was developed by LISA consultants. Statistical test results in this study were considered significantly different when *p*-values were <0.05.

Water quality- The data analysis toolset in Excel was used for analysis of all water quality parameters. The descriptive statistics tool was used to determine means and standard errors (SE) for temperature, pH, dissolved oxygen, alkalinity, and hardness. The ANOVA: Single Factor tool was used to determine if significant differences occurred among data replicates of each parameter between each low and high NH_3-N and controls.

Algal Count- Mean cell count (cells/mL) and mean cell size (µm) were compared among low and high NH_3-N and control using a one-way ANOVA in SAS.

Growth- Differences in growth among treatment and control banks were determined using an ANOVA in SAS. If banks had a significantly different growth function, a Tukey-Kramer post-hoc test for multiple comparisons was then performed to determine differences among each concentration of NH_3-N and controls.

Survival- Survival analysis was performed using a global log rank test in SAS to compare survival curves. The null hypothesis for this test was that the low and high concentrations of NH_3-N and control had the same survival functions. To test this hypothesis, the log rank procedure calculated the observed and expected survival and compared them. If groups had a significantly different survival function, a Tukey-Kramer post-hoc test for multiple comparisons was then performed to determine differences among each concentration of NH_3-N and controls.

Histological Evaluations- Statistical analyses of histological data from the gills, digestive glands, and kidneys were performed using a generalized linear mixed model (GLIMMIX) in SAS for binomial data. Within the mixed models for these analyses, treatment and bucket were fixed factors. Overdispersion of models was corrected using a residual term. Bucket was removed from the model if there was not a significant interaction with treatment. If the effect of treatment was significant, least-square means were compared using a Tukey-Kramer post-hoc test for multiple comparisons. GLIMMIX was also used to analyze histological data from control versus baseline mussel tissues for the gills, digestive glands, and kidneys.

Variables evaluating the gonads were split by sex. Mussels were labeled as indeterminate (I) if the evaluator was unable to determine the sex by the presence of gametes in evaluated tissues. Those mussels with both male and female acini were labeled as hermaphrodites (H) and were removed from the dataset due to the small sample size. Statistical analyses of gonad tissue could not be conducted with SAS GLMMIX because of lack of variation in both the fractions of acini containing mature or developing gametes and fractions of acini containing resorbing gametes.

Quality assurance of histological methods was performed using an ANOVA in SAS to compare results of two independent evaluators (co-authors of this study). Ten percent of slides were randomly chosen for evaluations before evaluations of all slides were conducted.

Results

Water Quality

Ammonia was measured daily for the first two months and then every other day for the final month. Only data from the first five weeks are presented in the results, due to equipment failure which caused inaccurate readings of NH_3-N throughout the third month of the study. Mean NH_3-N in the control banks was 0.33 mg/L (range of 0.0 to 3.9 mg/L). The low NH_3-N

treatment banks were spiked with 0.014 mg/L NH_3-N three days a week, and the actual average concentration measured over the study period was 0.08 mg/L (range of 0.0 to 0.56 mg/L). The high NH_3-N treatment banks were spiked with 0.15 mg/L NH_3-N three days a week, and the mean concentration measured over the study period was 0.22 mg/L (range of 0.0 to 1.31 mg/L). The low NH_3-N treatment banks had significantly lower NH_3-N than the control and high treatment (p<0.001) buckets. Ammonia in the high NH_3-N treatment was not significantly different than the control (p=0.0707).

Temperature, pH, dissolved oxygen, hardness, alkalinity, and ammonia did not differ among treatment and control banks over the course of the study (p>0.05 for all comparisons) (Table 3). Mean pH was 8.58 for all banks (Table 3). Mean temperature of all buckets remained within 0.1 degrees of the target temperature (24°C). Mean dissolved oxygen ranged from 88.2 to 88.9 %. Mean range of alkalinity as $CaCo_3$ was 180-195 mg/L and hardness as $CaCo_3$ was 244-257 mg/L.

Algal Concentrations

Mean cell count (cells/mL) did not differ significantly among control systems ($\overline{x}$=1808 cells/mL), low NH_3-N ($\overline{x}$=5210 cells/mL), and high NH_3-N ($\overline{x}$=6131 cells/mL) (p=0.5873). Mean cell size (µm) did not differ significantly among control systems ($\overline{x}$=3.8 µm), low NH_3-N ($\overline{x}$=3.7 µm), and high NH_3-N ($\overline{x}$=3.7 µm) (p=0.7129) (Table 4).

Growth

Mean growth ranged from 0.02 mm to 0.14 mm in all systems (Fig. 3). Low NH_3-N had the highest growth ($\overline{x}$=0.14); however it was not significantly different than control ($\overline{x}$=0.02) or high NH_3-N ($\overline{x}$=0.13) (p=0.66).

Survival

Both treatments and control started with 40 mussels, for a total of 120. Over the 3-month study period, 35 (87.5%) mussels in the control bank, 31 (77.5%) in low NH_3-N, and 31 (77.5%) in high NH_3-N survived (Fig. 4). The first mortality occurred on day 64 of the study in the low NH_3-N treatment. Survival was not significantly different among control, low, and high NH_3-N (p=0.4488).

Histology

Tissues from 80 mussels were evaluated for the five dependent histological variables. Four mussels were collected from each bucket (sampling unit); for a total of 20 mussels from both treatments, 20 mussels from the control, and 20 baseline mussels.

Gonad- Histological results for the gonads were separated by sex. Out of the 20 baseline mussels, nine were female, eight male, and three hermaphrodites. Of the 20 control mussels, ten were female, one male, and nine indeterminate. Of the 40 mussels in the two treatments, 20 were female (11 Low NH_3-N, 9 High NH_3-N), 5 male (2 Low NH_3-N, 3 High NH_3-N), one hermaphrodite (1 Low NH_3-N), and 14 indeterminate (6 Low NH_3-N, 8 High NH_3-N) (Table 5).

All female mussels collected during the sampling event and those used as baseline were in the post-spawning stage of gametogenesis. The majority of acini in all female mussels contained both mature or developing oocytes as well as atretic, resorbing oocytes (Fig. 5A). No statistical analysis was performed on either of these variables for females because there was minimal variation among the mussels. Thirteen males (8 baseline, 2 low NH_3-N, and 3 high NH_3-N) were in the ripe or pre-spawn stage of gametogenesis (Fig. 5B), and one male from control was in the post-spawning stage of gametogenesis. With only one male in the control and five males in the treatments, there were not enough male mussels for statistical analysis. The arithmetic means of fractions of acini containing mature or developing gametes (FAMD) and fractions of acini containing atretic or resorbing gametes (FAR) for females and males are presented in Table 5.

Digestive Gland- The fraction of digestive gland diverticula with degraded cytoplasm (FDGC) was significantly lower in baseline mussels ($\overline{x}$=0.13) than control ($\overline{x}$=0.23) (p=0.0254) (Table 7) (compare Figs. 5C and 5D). There were no significant differences among low and high NH_3-N treatments ($\overline{x}$=0.19 and $\overline{x}$=0.28, respectively) and control (p=0.1673) (Table 7).

Gill- No significant differences were observed for the fractions of gill filaments with cilia among baseline ($\overline{x}$=0.84) and control ($\overline{x}$=0.86) (p=0.6211) (Table 6) or among low and high NH_3-N treatments ($\overline{x}$=0.89 and $\overline{x}$=0.87, respectively) and control (p=0.4849) (Table 7) (Fig. 6A).

Kidney- Fractions of kidney diverticula cells containing lipofuscin (FKDL) were significantly lower in baseline mussels ($\overline{x}$=0.24) than control mussels ($\overline{x}$=0.36) (p=0.0092) (Table 6, Fig. 6B). There were no significant differences among low and high NH_3-N treatments ($\overline{x}$=0.37 and $\overline{x}$=0.42, respectively) and control (p=0.3227) (Table 7).

Other Histological Observations- While evaluating tissues for histological variables, other abnormalities also were noted. The most common observation was fusion of gill filaments, which was found in 7 mussels (1 baseline, 2 control, 1 low NH_3-N, and 3 high NH_3-N) (Fig. 6C). Parasites were observed in 6 mussels (2 baseline, 2 control, 1 low NH_3-N, and 1 high NH_3-N) (Fig. 6D).

Discussion

Results from the 2012 study indicated that the test concentrations of NH_3-N used in the study had no significant adverse effects on gill, digestive gland, kidney, or gonad tissues of *V. iris*. In addition, the kidney and digestive gland tissues of control mussels had significantly higher abundance of lipofuscin and degraded cytoplasm, respectively, than the baseline mussels, demonstrating that issues with captivity, such as inadequate food or lack of burrowing substrate, were potentially affecting mussel tissues in all buckets.

Performing an experiment using the methods and materials used in this study was problematic. Working with ammonia in a laboratory setting was known to be difficult before the study began. Ammonia is a volatile chemical, so maintaining consistent concentrations is challenging, especially when live specimens are used. To minimize these difficulties, this study aimed to examine effects of pulses of ammonia being added to a system as opposed to maintaining a constant concentration. Temperature and pH were maintained as constants so that they would not change the percentage TAN measured as NH_3-N in the control or treatment banks. The major issue arose when trying to measure NH_3-N.

Methods to measure low-level concentrations of NH_3-N were used but response times of the electrode were known to be longer with concentrations below 1.0 ppm NH_3-N and ammonia absorption from the air may have become a source of error (Thermo Scientific 2009). Samples were covered to prevent this; however, because concentrations were generally below 1.0 ppm NH_3-N, the longer response times potentially introduced error. Another issue was that the deionized water used to create calibration standards had ammonia present, likely introducing additional error into the calibration used for the electrode.

This study used filtered pond water as the holding water for the mussels to improve captive conditions by providing natural communities of algae which was needed as supplemental

food. The complex communities of algae and bacteria already present in this water likely changed the concentration of ammonia found in the systems. Algae and denitrifying bacteria can uptake ammonia and therefore decrease concentrations (Durborrow et al 1997). To minimize this effect, we switched out the buckets every week and let them dry until the next water exchange to minimize the algae and bacteria growing in the buckets. We were not able to measure the amount of algae or bacteria in the buckets, but determining a method to monitor and remove these algae and bacterial communities would be beneficial in future studies. Additionally, pond water could be replaced with reconstituted hard water in future ammonia studies; however, additional supplemental feeding would be necessary. This method was used in previous studies of ammonia exposures where maintaining the ammonia levels did not seem as problematic as in this study (Wang et al. 2007, 2011).

Temperature and feeding also can affect denitrifying bacteria in aquaculture systems. Lower water temperatures decrease aerobic bacterial activity, which can slow down the denitrification process (Durborrow et al. 1997). Systems were maintained at a temperature of 24°C during this study, which was warm enough to allow for the growth of bacteria. Decreasing the temperature of the systems for future studies could also help eliminate denitrifying bacteria. Increased ammonia beyond the added concentrations can occur from bacterial decomposition of organic matter from the pond water or from the algal food that was added to all systems as well as from excretion (Hargreaves and Tucker 2004). Over feeding mussels can result in excess ammonia, while under feeding can affect mussel health. It is therefore important to determine the optimal food concentrations for mussels prior to conducting laboratory experiments.

Captivity effects were apparent in histological results in this study. Degraded tissues in control mussels indicated that test conditions were inadequate for holding mussels in captivity, while there were no significant differences between control and treatments. The digestive glands of control mussels showed significantly more degraded cytoplasm and kidneys showed a higher abundance of lipofuscin than baseline mussels. The gills of baseline mussels had significantly less cilia than control mussels; however, the actual percentages were 93 versus 98. Both of these represent a high percentage of healthy gill tissue remaining.

In the future, several changes should be made to help decrease captivity effects. Juvenile *V. iris* and *Epioblasma capsaeformis* cultured in a sediment substrate showed significantly greater growth and survival compared to juveniles in no substrate (Jones et al. 2005). Gatenby

(2000) hypothesized that a lack of burrowing substratum contributed to a low survival rate of freshwater mussels suspended in cages in hatchery ponds. Substrate was not used in this study. Future studies could use redesigned holding systems which include using inorganic decontaminated sand for substrate for mussels to burrow. Suspended and bed sediment and has the ability to sorb ammonia; therefore, caution should be used if holding mussels in substrate to insure the mussels are actually being exposed to the intended concentration of the target contaminant (Canadian Council of Ministers of the Environment 2010).

The recommended concentration of food used for this study was 10,000 cells/mL for each bucket over 24h, which was based on feeding protocols used at the FMCC (D. Hua, FMCC, personal communication). Other studies have recommended feeding adult mussels held in captivity at a concentration as high as 100,000 cells/mL at least twice daily (Gatenby 2000). Mussels starved in captivity showed degraded cytoplasm in the digestive gland after four weeks (Thompson et al. 1974). Mussels were fed daily; however, if available food was still not adequate, degraded cytoplasm in digestive glands of mussels held eight or twelve weeks in captivity is possible. It is clear that adequate food is essential for the health of adult mussels held in captivity; however, the exact amount needed to support the number of adult mussels in each bucket still needs to be determined. An increase in food can be accomplished by increasing the algal concentrations fed directly to each bucket or by performing water exchanges of fresh pond water twice weekly as opposed to just weekly.

Conclusion and Recommendations

Future laboratory studies examining the health and function of mussel tissues exposed to contaminants will need to be improved to include better methodology and equipment for controlling and measuring ammonia and to reduce effects of captivity. The bucket systems designed for this study could be slightly modified to allow the mussels to burrow in substrate. Future studies should use reconstituted hard water as opposed to pond water. Also, food requirements for *V. iris* should be further examined to determine the optimal algal concentrations for feeding.

This study was designed to examine effects of ammonia concentrations found in the NFHR; however, ammonia issues are present in many river systems. Increases in ammonia in freshwater have been found in other aquatic systems due to municipal effluent discharges,

industrial processes, and agricultural runoff (USEPA 2013). It is important that these ammonia increases are properly monitored and if necessary, regulated so that mussels and other freshwater fauna do not continue to decline.

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Table 1. Water quality measurements of pond water sampled at The Freshwater Mollusk Conservation Center, Department of Fish and Wildlife Conservation, Virginia Tech, Blacksburg. The pond water was used as holding water for both the 2012 and 2013 studies. "<" indicates concentrations less than the instrument detection limit.

Parameter	mg/L	Parameter	mg/L	Parameter	mg/L
Ag	<0.006	Fe	0.01	Se	<0.022
As	<0.021	Hg	<0.025	Tl	<0.021
B	0.019	K	2.54	Zn	<0.006
Be	<0.001	Mg	29.874	NH_3-N	0.029
Ca	27.088	Na	8.864	NO_3	0.1
Cd	<0.005	Ni	<0.006	NO_2	0
Cl	20.2	P	<0.027	pH	8.45
Cr	<0.007	Pb	<0.016	Alkalinity	143
Cu	<0.003	S	<0.023	Hardness-$CaCo_3$	225

Table 2. Dependent variables used to assess the condition of vital organ tissues from adult *Villosa iris.* Fifty observations were acquired from gonadal tissues, and 100 observations were obtained from digestive gland, kidney, and gill tissues of each evaluated mussel. Each datum was recorded as either a one or zero, as described in this table.

Dependent Variable	Recorded as One	Recorded as Zero
Fractions of acini containing mature or developing gametes	acini contains mature or developing gametes	acini does not contain mature or developing gametes
Fractions of acini containing resorbing gametes	acini contains resorbing gametes	acini does not contain resorbing gametes
Fractions of digestive gland diverticula cells containing degraded cytoplasm	diverticula contains degraded cytoplasm	diverticula does not contain degraded cytoplasm
Fractions of gill filaments with cilia	cilia present on gill filament	cilia not present on gill filament
Fractions of kidney cells containing lipofuscin	kidney cell contains lipofuscin	kidney cell does not contain lipofuscin

Table 3. Arithmetic means and (± standard errors) for water quality measurements for control and low and high NH_3-N. LETTERS indicate significant differences in measured ammonia concentrations ($p<0.05$). No significant differences were measured for any other water quality parameters ($p>0.05$).

Parameter	Unit	Control	Low NH_3	High NH_3
Temperature	°C	23.9 (0.04)	23.9 (0.5)	23.9 (0.04)
Dissolved Oxygen	%	88.94 (0.56)	88.23 (0.85)	88.37 (0.59)
pH	log $[H^+]$	8.58 (0.01)	8.58 (0.01)	8.58 (0.01)
Hardness	mg/L	244.74 (18.19)	249.75 (7.44)	256.5 (12.14)
Alkalinity	mg/L	194.8 (11.67)	180.1 (6.81)	184.67 (4.06)
Ammonia	mg/L	0.33 (0.06)^A	0.08 (0.01)^B	0.22 (0.03)^A

Table 4. Algal count (cells/mL) and cell size (µm) observed in treatment and control buckets in the 2012 study. Samples were taken from one randomly chosen bucket in each treatment and control during 2 sample events. Algal counts and size were measured using a hemocytometer.

	11/20/2012		12/14/2012	
System	Cell Count (cells/mL)	Mean Cell Size (µm)	Cell Count (cells/mL)	Mean Cell Size (µm)
Control	981	3.652	2636	3.983
Low NH_3-N	1837	3.734	8584	3.627
High NH_3-N	2627	3.825	9636	3.658

Table 5. Arithmetic mean (± standard errors) of fractions of reproductive acini containing mature and/or developing gametes (FAMD) and acini containing atretic or resorbing gametes (FAR) observed in female (♀), male (♂), and hermaphroditic (H) *Villosa iris* from day zero baseline, control, and treatment systems. No significant differences were measured for any comparisons of FAMD or FAR (p>0.05).

Treatment	Sex	n	FAMD	FAR
Baseline	♀	9	0.94 (0.06)	0.94 (0.06)
	♂	8	1.00 (0.00)	0.00 (0.00)
	H	3	1.00 (0.00)	1.00 (0.00)
Control	♀	10	0.99 (0.01)	0.99 (0.01)
	♂	1	0.98 (0.00)	0.96 (0.00)
Low NH_3-N	♀	11	0.99 (0.01)	0.99 (0.12)
	♂	2	1.00 (0.00)	0.00 (0.00)
	H	1	1.00 (0.00)	0.20 (0.00)
High NH_3-N	♀	9	0.99 (0.00)	0.99 (0.00)
	♂	3	1.00 (0.00)	0.00 (0.00)

Table 6. Arithmetic mean (± standard errors) of fractions of digestive gland cells with abnormal cytoplasm, fractions of gill filaments with cilia, and fractions of kidney cells containing lipofuscin observed in *Villosa iris* from baseline and control systems. LETTERS indicate significant differences in survival between treatments (p<0.05). Comparisons were made within columns.

	Histological Variables		
Treatment	Digestive Gland	Gill	Kidney
Baseline	0.13 (0.02) A	0.84 (0.03) A	0.24 (0.02) A
Control	0.23 (0.04) B	0.86 (0.02) A	0.36 (0.04) B

Table 7. Arithmetic mean (± standard errors) of fractions of digestive gland cells with abnormal cytoplasm, fractions of gill filaments with cilia, and fractions of kidney cells containing lipofuscin observed in *Villosa iris* from Control, and Low and High NH_3-N treatments. LETTERS indicate significant differences in survival between treatments ($p<0.05$). Comparisons were made within columns.

	Histological Variables		
Treatment	**Digestive Gland**	**Gill**	**Kidney**
Control	0.23 (0.04) A	0.86 (0.02) A	0.36 (0.04) A
Low NH_3-N	0.19 (0.03) A	0.89 (0.01) A	0.37 (0.03) A
High NH_3-N	0.28 (0.04) A	0.87 (0.02) A	0.42 (0.03) A

Figure 1. Schematic of downweller bucket system used for NH_3-N studies. A. Upper bucket. B. Water level. C. Lower bucket. D. Pump attached to bulkhead. E. View from above showing bottom of upper bucket with F. Mesh covered holes (modified from Barnhart 2006).

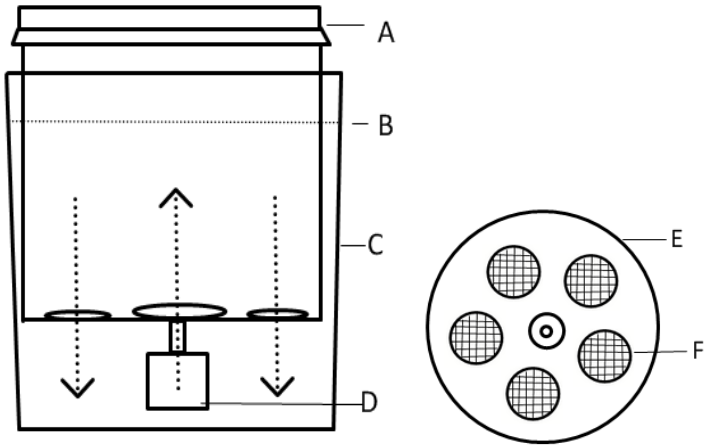

Figure 2. Example of point-count method in gonad to evaluate oogenic and spermatogenic acini. Points B, C, and F would be assigned a 1 for evaluations of fraction of acini containing mature or developing gametes because the points fall directly above acini that contain mature or developing gametes. Points A, D, and E would not be evaluated because they are not on acini.

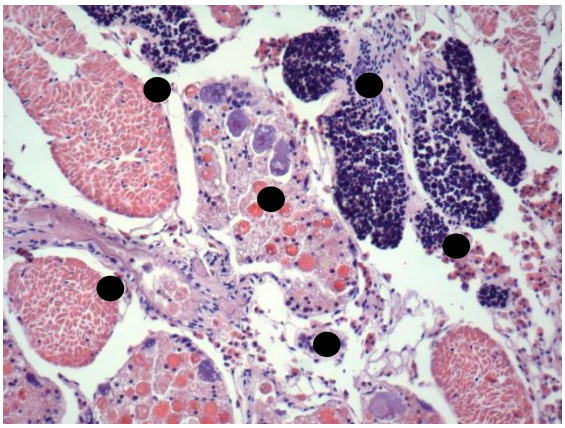

Figure 3. Mean growth (by length) of *Villosa iris* held in control, low and high NH_3-N treatments during the study. Error bars represent 95 percent confidence intervals. LETTERS indicate significant differences in growth between treatments (p<0.05).

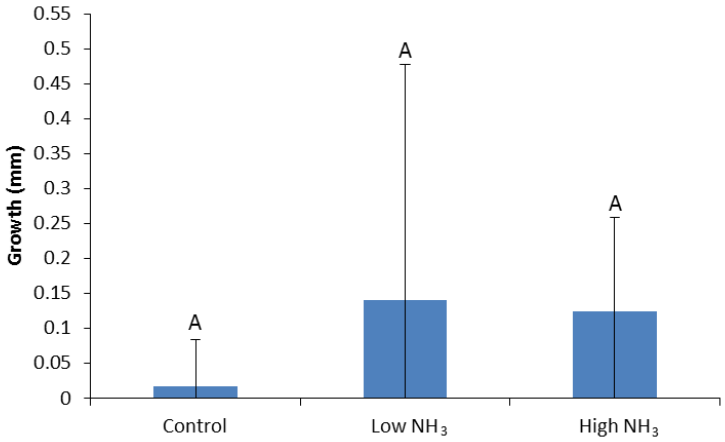

Figure 4. Percent survival of *Villosa iris* per day held in control, low and high NH_3-N treatments during the study. LETTERS indicate significant differences in survival between treatments (p<0.05).

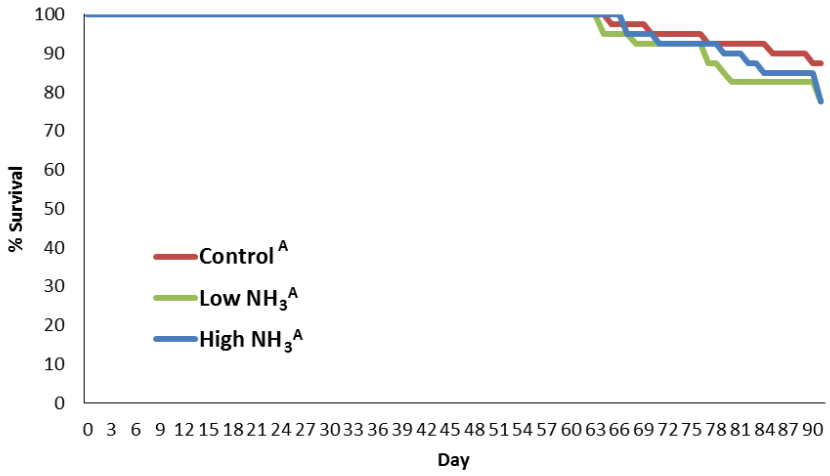

Figure 5. Reproductive and digestive gland tissues of *Villosa iris* stained with hematoxylin and eosin. A. Oogenic acini (oa) containing resorbing oocytes (r). Bar = 2 µm. B. Spermatogenic acinus (sa) containing spermatozoa (s) in ripe stage of gametogenesis, study. Bar = 4 µm. C. Cross section of normal digestive diverticula with lumina (l) and digestive (dc) and basophilic (bc) cells. Bar = 4 µm. D. Digestive diverticulum (dd) containing reduced cytoplasm (rc). Bar = 4 µm

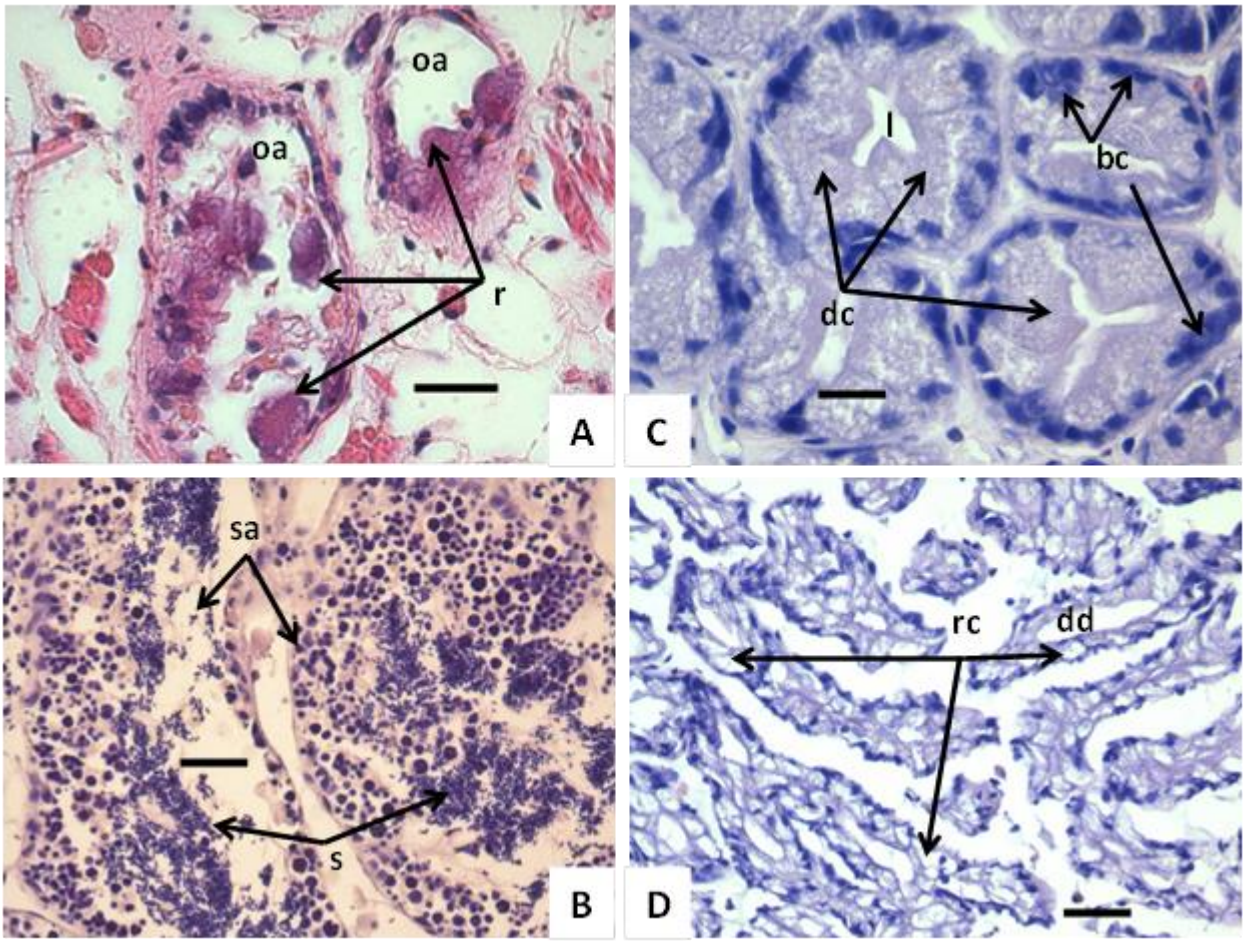

Figure 6. Gill and intestinal tissue (stained with hematoxylin and eosin) and kidney tissue (stained with Carbol Fuscin) of *Villosa iris*. A. Normal ciliated (c) gill filaments (gf). Bar = 4 µm. B. Kidney diverticulum (kd) containing high abundance of lipofuscin (lf) (brown inclusions). Bar = 2 µm. C. Fusion (f) in gill filaments (gf). Bar = 4 µm. A. Parasite (p) in intestine (i). Bar = 10 µm.