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Histological evaluations of organ tissues reveal sublethal effects in a freshwater mussel (*Villosa iris*) exposed to chloride and potassium concentrations below benchmark estimates

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ABSTRACT

Salinization of freshwater ecosystems due to anthropogenic sources will increasingly impact biodiversity. An example of point-source industrial salinization has occurred from historical activities at a U.S. Environmental Protection Agency Superfund Site near Saltville, Virginia USA and its associated chemical waste ponds adjacent to the North Fork Holston River. These point source discharges are documented contributors to mussel declines, partially due to high concentrations of chloride (Cl^-, ≤ 26,000 mg Cl^-/L) and potassium (K^+, ≤ 97 mg K^+/L). During a chronic 61-day laboratory study, Rainbow mussels, *Villosa iris*, were exposed to concentrations of Cl- (0, 416, 831, and 1,663 mg/L) and K+ (0, 4, 8, and 17 mg/L) to determine effects on survival and organ tissues. All test mussels died by day-2 in the 1,663 mg Cl^-/L exposure, and 50% of mussels died by day-13 in the 17 mg K^+/L concentration. Significantly greater abundances of tissue abnormalities were observed in digestive glands and kidneys with exposures to the 4 and 8 mg/L concentrations of K^+ versus the control, and significantly greater abundances of lesions in kidneys were observed in the 416 and 831 mg Cl^-/L concentrations compared to the control. The sublethal effects to digestive glands and kidneys were below reported effect ($EC_{50,\ 20,\ 10}$ and LOEC) concentrations. Significant histological differences between control and baseline (day-0 sample) mussels were observed, suggesting the need for further study on the effects of captivity during longer-term laboratory experiments.

1. Introduction

Salinization of freshwater ecosystems will increasingly become a chronic threat to aquatic biodiversity (Cañedo-Argüelles et al., 2019). For instance, Olson (2019) predicted that salinity may increase by 50% by the end of the century in 50% of USA rivers. Anthropogenic sources of ionic inputs that contribute to increasing salinity include mining, road deicing, urban and agricultural runoff, oil and gas extraction, water treatment, and industrial discharges and seepages (Pandolfo et al., 2012; Gibson et al., 2018; Wang et al., 2018; Salerno et al., 2020; Gillis et al., 2021a). Lethal toxicities of salinity-related ions to freshwater mussel glochidia (larvae) and juveniles have been documented during laboratory studies of water quality benchmarks (Gibson et al., 2018; Wang et al., 2018; Gillis et al., 2021a). However, chronic sublethal effects to vital organs in sub-adult and adult freshwater mussels have been understudied (Rogers et al., 2018). As salinity increases in aquatic environments, sublethal effects to mussels will likely include organ tissue lesions that contribute to impairment of vital somatic functions, including digestion, energy substrate storage, contaminant detoxification, and ion regulation (Rogers et al., 2018).

A historical and continuing source of industrial-related salinity input that is of ongoing concern to mussel conservationists and other interested parties is in the North Fork Holston River (NFHR) of Virginia, USA. The NFHR is located within the Upper Tennessee River Valley System.

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Industrial activities in the NFHR have been present at Saltville, Virginia since the 1800s. After salt was mined for over a century, the Mathieson Chemical Company (which merged with the Olin Corporation in 1954 to become the Olin-Mathieson Chemical Company) opened, and operated soda ash, dry ice, and hydrazine facilities from 1950 to 1980 (Ahlstedt and Rashleigh, 1996; Henley and Neves, 1999). Wastes, consisting of mercury, other heavy metals, ammonia, calcium carbonate, and salt brines from the facility were discharged into settling ponds, and these waste products then seeped into the river over time. The settling ponds and the former chemical plant site were designated by the U.S. Environmental Protection Agency as the Saltville Waste Disposal Ponds Superfund Site on the U.S. National Priorities List in 1983.

Once supporting 42 species of native freshwater mussels, the NFHR mussel fauna has substantially declined over the past 60 years or longer (Ortmann, 1918; Ahlstedt and Rashleigh, 1996). Mussel surveys have shown 9 extant species downstream of Saltville and 13 species upstream (Henley and Neves, 1999; Jones and Neves, 2007), although at least 16 species are known to occur upstream of Saltville [J. Jones (co-author), unpublished data]. The industrial activities at Saltville, as well as seepage of mercury, salts, ammonia, and other ions from the chemical holding ponds are putative causes of mussel declines (Wang and Ingersoll, 2010). Wang and Ingersoll (2010) found maximum levels of K+ and Cl- in seepage waters at the Saltville Superfund Site to be 47 and >18, 200 mg/L, respectively.

Evaluations of digestive gland, gill, and kidney tissues have been used in previous studies to demonstrate effects of contaminants on vital organs of marine and freshwater bivalves (Au, 2004; Rogers et al., 2018). We determined abundances of lesions in vital organs of laboratory Rainbow mussels (*Villosa iris*) to assess condition of organ tissues from exposures to Cl- and K+. Bivalve digestive glands contain diverticula with epithelia consisting of secretory and digestive cells, and exposure of contaminants have resulted in degradation of cytoplasm with effects to intra- and extra-cellular digestion, nutrient absorption, lipid and glycogen storage, and contaminant detoxification (Petrović et al., 2001; Au, 2004; Rogers et al., 2018). Contaminant exposures also have been linked to loss of gill cilia, fusion of gill filaments, inflammation, necrosis, and epithelial cell sloughing with probable effects to respiration, osmoregulation, ion transfer, and food capture and transport (Gómez-Mendikute et al., 2005; Rogers et al., 2018). Kidney cells sequester intra-visceral contaminants by binding them in intracellular lipofuscin granules, which are excreted to the urinary space. Lipofuscin granules that consist of peroxidative by-products and oxidized proteins and carbohydrates result from peroxidation of lysosomal membrane lipids, due to contaminant-related reactive oxygen species (Au, 2004; Moore et al., 2006).

The objective of this study was to quantify abundances of specific lesions in organ tissues and measure survival of sub-adult *V. iris*, during laboratory exposures to Cl^- and K^+. Test concentrations of Cl^- and K^+ were determined from data obtained from measurements taken in the NFHR and other ecological benchmarks that are relevant to a range of environmental settings experiencing increasing salinity.

2. Materials and methods

2.1. Study mussels and test conditions

Sub-adult *V. iris* propagated and cultured at the Freshwater Mollusk Conservation Center (FMCC), Department of Fish and Wildlife Conservation, Virginia Tech, Blacksburg, Virginia were used in this study. At the beginning of the experiment, the mussels were about 12 months old, ranged from 14 mm to 26 mm, and were host-transformed using Rock bass (*Ambloplites rupestris*) in August of 2012. Because of the age of the mussels, they are classified as sub-adults herein; however, all of the mussels exhibited sparse evidence of gametogenesis or production of reproductive acini during the experiment. *Villosa iris* has been designated a species of special concern in Canada, and in the USA, and is listed as endangered in Illinois and Wisconsin, of special concern in Michigan and North Carolina, and proposed for endangered status in Pennsylvania.

Twenty-five closed recirculating down-welling bucket systems were used to hold control and treatment mussels during the exposures. The 5-gallon (18.9 L) buckets contained filtered (200 μm) water from the FMCC pond. Each treatment (low, mid and high Cl^- and K^+) and the control consisted of 5 separate replicate buckets held within a water bath. The water quality data from the controls represents the water quality of the FMCC pond water. At the beginning of the experiment, 9 mussels were randomly selected from all available mussels, and then randomly assigned to each bucket (45 mussels per each treatment and the control). Twenty mussels also were randomly selected to serve as baseline mussels. Histological data from these day-0 baseline mussels were used in subsequent baseline-control and baseline-treatment statistical comparisons. Four mussels were randomly assigned to each of 5 hypothetical baseline replicate buckets. Lengths (mm) of mussels were obtained and recorded before they were placed in their assigned buckets and at the conclusion of the study.

During the 61-day experiment at FMCC from September 11, 2013 to November 11, 2013, the test mussels were fed a commercial algae mixture using Shellfish Diet 1800 (Reed Mariculture, Inc., Campbell, California) at a daily target ration of 10,000 cells/mL using continuous drip-valves.

Stock solutions of ions for high, mid, and low Cl^- and K^+ treatments (concentrations subsequently provided) were created using reagent grades of NaCl or KCl. Solutions of reagent-grade NaCl and KCl were added to the treatment buckets as needed to maintain target concentrations of high, mid, and low Cl^- and K^+ treatments after 100% weekly water changes. Concentrations of Cl-, Na+ and K+ were measured using a Thermo Scientific Orion Cl-, Na+ or K+ electrodes and an Orion EA940 meter (Thermo Fisher Scientific Incorporated, Waltham, Massachusetts).

The test exposure concentrations were determined based on environmentally relevant concentrations. The mean (± standard error, SE) concentration of Cl- observed at 4 contaminated sites within the NFHR Superfund Site (Site) was 3,114 (±1524) mg Cl^-/L, with a range from 200 to 26,000 mg Cl^-/L (Henley et al., 2013). For reference, the acute and chronic United States Environmental Protection Agency (USEPA) National Water Quality Criteria for Cl^- are 860 and 230 mg Cl^-/L, respectively (USEPA, 1988). The mean concentration of K+ found within the NFHR Site was 16.1 (± 5.8) mg K^+/L, with a range from 3.2 to 97.0 mg K^+/L (Henley et al., 2013). Although USEPA acute and chronic aquatic criteria do not exist for K^+, except for the USEPA's general chronic screening value of 53 mg K^+/L (Gillis et al., 2021b; USEPA, 2022), a concentration of 4 mg K^+/L was recommended by Imlay (1973) as the threshold for K+ in rivers that could support freshwater mussels. Our test target concentrations were 705 (low), 1,410 (mid), and 2,820 mg $NaCl^-$/L (high), and 8 (low), 16 (mid), and 32 mg KCl^+/L (high) during this study. Thus, the ionic concentrations used were 415.6, 831.3, and 1,662.5 mg Cl^-/L and 4.2, 8.4, and 16.8 mg K^+/L.

2.1.1. Water quality

Water quality parameters were measured after water changes, except for ammonia. Ammonia was measured as unionized ammonia (mg NH3-N/L) weekly after water changes (Hach method 8155). Nitrate (mg NO_2/L and nitrite (mg NO_3/L) were measured 2 times the first 2 weeks and then weekly [(Hach 8039) and diazotization (Hach 8507) methods, respectfully]. Temperature was maintained at 24°C (± 0.5) usually twice daily and maintained with water heaters. A handheld meter (Oakton Waterproof Double Junction pH Tester 20) determined pH every other week. Percentage oxygen saturation was measured every other week using a YSI Professional Plus Multiparameter Meter, total hardness was measured twice during the 2 months (Hach Method 8213; mg of Ca^+/L as $CaCO_3$), and total alkalinity also was measured twice (Hach Model AL-AP; mg of phenolphthalein alkalinity/L plus mg total

methyl orange alkalinity/L as $CaCO_3$). All meters were calibrated before use with verification standards. Replicate samples were measured from random buckets each week to perform quality assurance/quality control. Water quality parameters were measured from samples on days of sampling.

2.2. Histology

Mussel mortalities in each of the control and treatment buckets were recorded daily during the experiment. We had no way of knowing the levels of mussel mortality that would occur during the study. Therefore, we set an arbitrary 50% mortality level that would trigger collection of live mussel tissues from the treatments to increase the likelihood of tissue collections from live mussels if pronounced unexpected mortalities occurred. The trigger was initiated in 2 cases. An 100% mussel mortality was observed in the 1,662.5 mg Cl^-/L (high Cl^-) treatment on day-2 of the study, and because of the unexpected and sudden complete mortality, no tissues could be collected from the high Cl^- treatment. Also, 50% mortality was observed in the 16.8 mg K^+/L (high K^+) treatment on day-13 of the study, and tissues were collected from the remaining live mussels for subsequent histological evaluations.

At the end of the 61-day experiment, 4 live mussels were randomly selected for tissue collections from each bucket. Organ tissues were dissected and placed in 2 containers (cassettes) per mussel. Because of the small size of the sampled mussels, the complete organs were dissected and placed within the labeled cassettes. One cassette for each mussel contained its gill and the other contained its digestive gland and kidney tissues. The tissue containing cassettes were placed in 10% neutral buffered formalin in labeled containers, and after a week of fixation, the formalin was exchanged with 70% histological grade ethanol. The tissue samples in the cassettes were processed for paraffin embedding, microtoming, and staining using the methods of Rogers et al. (2018). Sections were cut at approximately 50% of the tissue depth and mounted on glass microscope slides. Because the cassettes contained the complete organs of each mussel and all organs were cut with a microtome at 50% of the tissue depths, sections from each mussel were obtained from approximately similar positions within the organs. Tissue sections were placed on one slide and stained with hematoxylin and eosin and another slide was stained using the Long Ziehl-Neelsen carbo fuscin for elaboration of lipofuscin in kidney tissues.

2.3. Microscopical evaluations

Histologically prepared tissues were microscopically evaluated (Olympus BX 41 light microscope, Olympus America, Incorporated, Center Valley, Pennsylvania) using three histologically-based dependent variables. The dependent variables were fractions of digestive gland diverticula cells containing degraded cytoplasm, gill filament termini with cilia, and kidney diverticula cells containing lipofuscin (Rogers et al., 2018).

Quantitative evaluations of organ tissues were performed using a point count method (Chalkey, 1943; Rogers et al., 2018). Six dots were drawn on the ocular eye piece of the microscope, so they appeared over the slides containing organ tissues. Evaluations were only made on target tissues under the dots. Once tissues under the dots were evaluated or determined not to be target tissue, the slide was randomly reoriented to another area of the target organ until data requirements were met. One hundred data per mussel were obtained for the dependent variables associated with the kidney (100X), digestive gland (40X), and gills (40X). 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 were present on the gill filament termini under a dot, then a one was recorded; however, if cilia were absent, then a zero was recorded.

2.4. Data analysis

2.4.1. Water quality

The data analysis toolset in Excel was used to generate the 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 ($p<0.05$) occurred among water quality data among the treatments and control.

2.4.2. Mussel growth and survival

Statistical analyses of growth were not performed. The duration of the study was not long enough to observe mussel growth.

Survival analysis was performed using a global log-rank test in the SAS LIFETEST procedure (SAS Institute, Inc., Cary, NC; SAS code generated by the Statistical Applications and Innovations Group, Department of Statistics, Virginia Tech) to compare survival curves among the different ion treatments and control. The log-rank procedure calculated the observed and expected survival and compared them statistically. If groups had a significantly different survival function ($p<0.05$), a Tukey-Kramer *post hoc* test for multiple comparisons was then performed to determine differences among mussel survival in the treatments and control.

As stated, a 50% mortality trigger was set to initiate collection of tissues from survivors. By day-2 of the experiment, all mussels died in the high Cl^- treatment; therefore, no tissues or data were collected from the dead mussels. By day-13, 50% of mussels in the high K^+ treatment died, and tissues and data from the survivors were collected; histological data from the high K^+ tissues are summarized herein, but were not used in the statistical analyses. However, survival data from the high Cl^- and K^+ treatments were included in the survival analyses.

2.4.2. Histology

Statistical analyses ($p<0.05$) of histological data were performed using a generalized linear mixed model (GLIMMIX) in SAS for binomial data (Henley et al., 2013; Rogers et al., 2018). Within the mixed models, treatment, treatment group buckets, and mussel number were assigned as classes. Overdispersion of models was corrected using a residual term, with bucket as the subject (statistical sampling unit) and treatment as the group. Least-squares means were compared using a Tukey-Kramer *post hoc* test for multiple comparisons to determine statistical differences among the histological data from the baseline, control, and treatments.

3. Results

3.1. Water quality

The measured test exposure concentrations were similar to the study target concentrations during the experiment. The exposure accuracies ranged from 93.8% to 98.1% for KCl and 103.4% to 111.4% for NaCl (Table SI1 in Supplemental Information).

Temperature, pH, dissolved oxygen, hardness, alkalinity, nitrite, nitrate, and ammonia did not differ among buckets over the course of the study ($p>0.05$, Table SI2 in Supplemental Information). Mean temperature of all buckets remained within 0.4 degrees of the target temperature (24°C). Mean ammonia concentrations ranged from 0.03-0.06 mg/L NH3-N, respectively. The USEPA recommended chronic criteria for NH_3-N at pH 8.4 and 24°C is 0.32 mg/L (USEPA, 2013).

3.2. Mussel survival

By day-2 of the study, 100% of mussels in the high Cl- treatment were dead. By day-13, the 50% mortality in the high K+ treatment occurred. Over the 61-day experiment, one mortality occurred in each of the control, mid K+ and mid Cl-, leaving 44 mussels (97.8% survival) in each these groups. No mortality occurred in the low K+ and low Cl-

treatments. Survival was significantly greater among the control, low and mid K+ versus high K+ (p=0.0012), and among control, low, and in mid Cl- versus high Cl- (p=0.0008; Fig. SI1 in Supplemental Information). Survival was not significantly different among control, low, and mid K+ (p=0.9999) or between control, low, and mid Cl- (p>0.9999).

3.3. Histology

Of the 140 total mussels collected for histological sampling, 13 were female, 81 were male, 3 were hermaphrodite, and 43 were of indeterminate sex.

3.3.1. Digestive gland

A significantly lower mean fraction of digestive gland diverticula cells containing degraded cytoplasm occurred in the baseline mussels versus the control (p=0.0204; Fig. 1, Table SI3 in Supplemental Information). The mean fraction was significantly lower in the control than means from the low K+ and mid K+ treatments (p<0.019), and no difference was observed between low and mid K^+ (p=0.1317; Fig. 2, compare Fig. 5A with 5B, 5C, and 5D, Table SI3). No significant differences were observed among means from the control, low, and mid Cl^- treatments (p>0.44) (Table SI3). However, the mean fraction of the baseline mussels was significantly lower than the means of low and mid Cl^- (p<0.004; Fig. 2, Table SI3). Other abnormalities also were noted, including pyknotic and karyolytic nuclei in digestive glands (1 control, 9 low K+, 2 high K+, and 1 low Cl; Fig. 5C) and necrosis in digestive glands (1 control, 1 low K+, 4 mid K+, 2 high K+, 1 low Cl-, and 2 mid Cl-; Fig. 5D).

3.3.2. Gill

The mean fraction of gill filament termini with cilia was significantly lower in the baseline mussels than the mean from the control (p=0.0026; Fig. 1, Table SI3 in Supplemental Information). The mean of fraction from the control was significantly greater than means from the low and mid K^+ treatments (p<0.012), and the means from low and mid K^+ were not significantly different (p=0.9117; Fig. 3, compare Fig. 6A and B, Table SI3). The mean of the control was significantly greater than means from low and mid Cl- (p<0.01), and no differences were observed between low and mid Cl^- (p=0.9274). No differences were observed among the baseline and the low and mid K^+ treatments (p>0.75) and the low and mid Cl^- treatments (p>0.91). During the evaluations, edema of gill filaments was found in gill tissue of 10 mussels (1 baseline, 3 mid K+, 3 low Cl-, and 3 mid Cl-), and fusion of the filaments was observed in one mussel in high K+ and one in low Cl- (Fig. 6B).

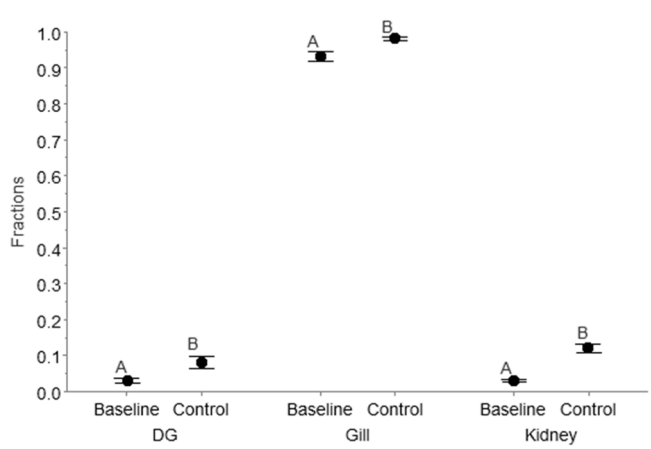

Fig. 1. Interval plot comparing baseline (time-0) and control mussel means and standard error (± SE) bars of fractions of digestive gland cells with lesions, gill filaments with cilia, and kidney cells containing lipofuscin. Dissimilar letters indicate significant differences (p<0.05) among baseline and control within variables only. See Table SI3 in Supplemental Information for means (± SE, n = 5 buckets per baseline and control).

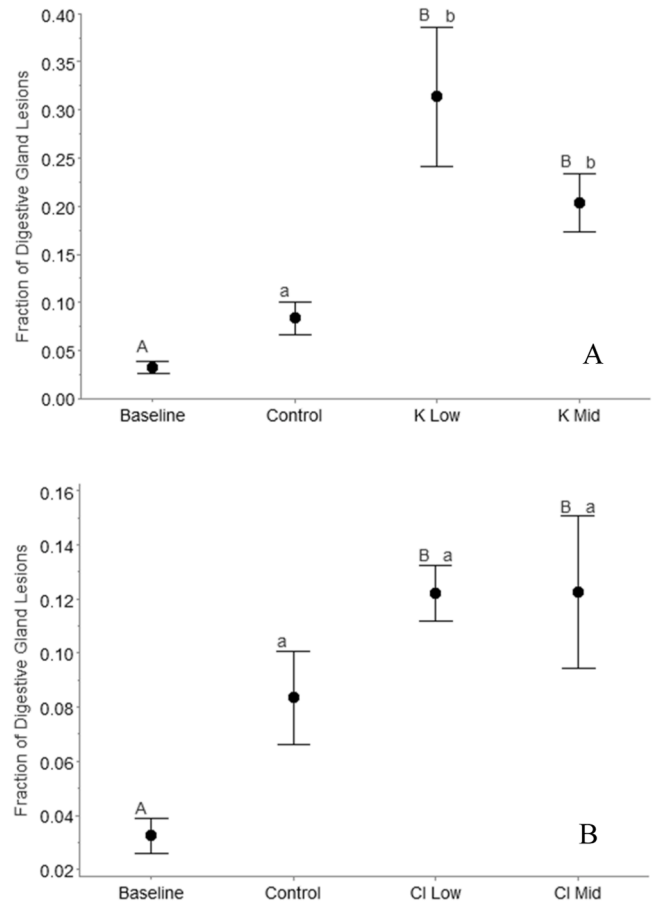

Fig. 2. Interval plots comparing means and standard error bars (± SE) of baseline (time 0), control, and treatments for fractions of digestive gland cells with lesions. Treatments include exposures to 4.2 (K Low) and 8.4 (K Mid) mg K^+/L and 415.6 (Cl Low) and 831.3 (Cl Mid) mg Cl^-/L. Within individual plot panels (A for K^+ and B for Cl^-), dissimilar capital and small letters indicate significant differences (p<0.05) among baseline and treatments and control and treatments, respectively; comparisons of capitol and small letters are not appropriate. See Table SI3 in Supplemental Information for means (± SE, n = 5 buckets per baseline, control, and treatments).

3.3.3. Kidney

A significantly lower mean fraction of kidney diverticula cells containing lipofuscin occurred in baseline mussels compared to the control mussels (p=0.0013; Fig. 1, Table SI3 in Supplemental Information). The mean fraction from both the baseline and controls were significantly lower than the means from low and mid K+ (p<0.014), and no differences were observed between low and mid K^+ (p=0.9053; Figs. 4 and 6C). The mean of both the baseline and control also were lower than means of the low and mid Cl^- treatments (p<0.003), and low and mid Cl^- were not different (p=0.2606).

4. Discussion

Because little research has been conducted on effects of K^+ and Cl^- on sub-adult unionid mussels, the results of this study provide valuable insights concerning lethal and sublethal effects. One hundred percent mortality was observed at day-2 in the 1,662.5 mg Cl^-/L (high Cl^-) treatment, and 50% mortality was observed at day-13 in the 16.8 mg K^+/L (high K^+) treatment. Our results show that sublethal effects to digestive glands and kidneys of sub-adult *V. iris* occurred at much lower concentrations of K^+ than shown to be lethal to mussel glochidia and

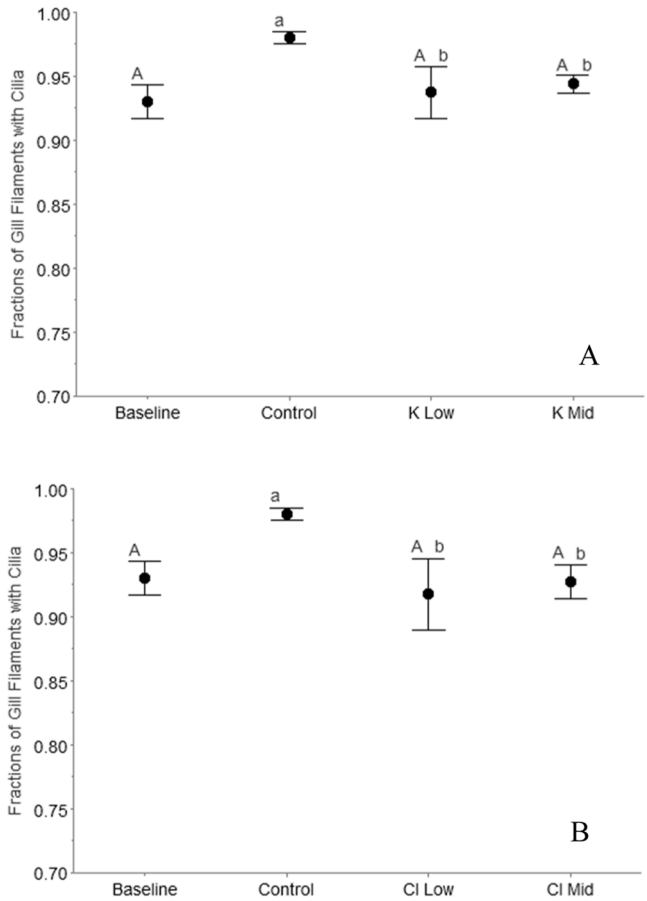

Fig. 3. Interval plots comparing means and standard error bars (± SE) of baseline (time 0), control, and treatments for fractions of gill filaments with cilia. Treatments include exposures to 4.2 (K Low) and 8.4 (K Mid) mg K^+/L and 415.6 (Cl Low) and 831.3 (Cl Mid) mg Cl^-/L. Within individual plot panels (A for K^+ and B for Cl^-), dissimilar capital and small letters indicate significant differences ($p<0.05$) among baseline and treatments and control and treatments, respectively; comparisons of capitol and small letters are not appropriate. See Table SI3 in Supplemental Information for means (± SE, $n = 5$ buckets per baseline, control, and treatments).

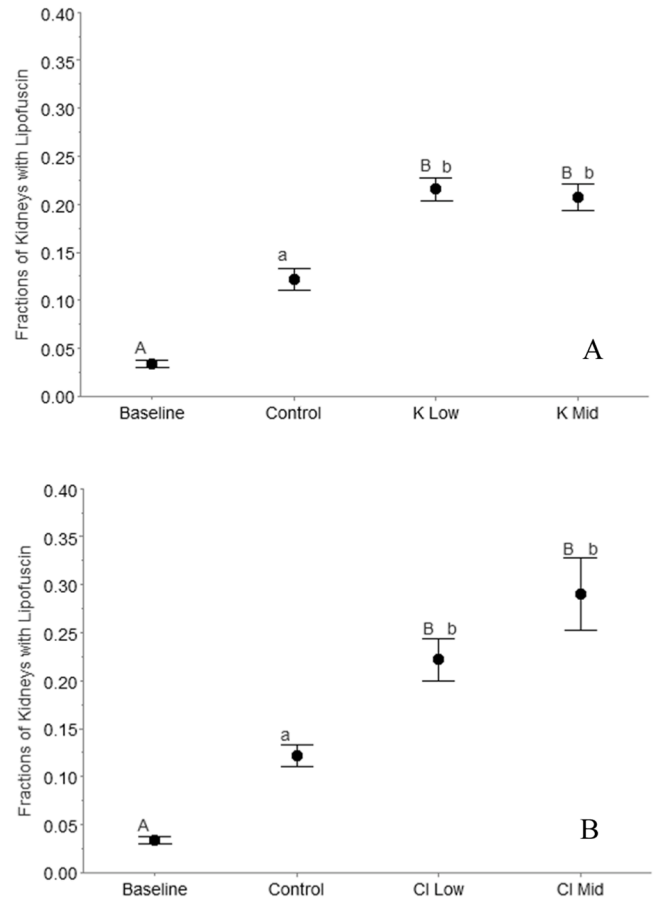

Fig. 4. Interval plots comparing means and standard error bars (± SE) of baseline (time 0), control, and treatments for fractions of kidney cells with lipofuscin. Treatments include exposures to 4.2 (K Low) and 8.4 (K Mid) mg K^+/L and 415.6 (Cl Low) and 831.3 (Cl Mid) mg Cl^-/L. Within individual plot panels (A for K^+ and B for Cl^-), dissimilar capital and small letters indicate significant differences ($p<0.05$) among baseline and treatments and control and treatments, respectively; comparisons of capitol and small letters are not appropriate. See Table SI3 in Supplemental Information for means (± SE, $n = 5$ buckets per baseline, control, and treatments).

juveniles (see literature summary Table SI4 in Supplemental Information). Whereas we observed sublethal effects to our *V. iris* beginning at 4.2 mg K^+/L, benchmark concentrations (commonly reported LC_{50} and EC_{50}) reported in the literature for glochidia and juveniles have ranged from 11.9 to >56 mg K^+/L (Table SI4). Although benchmark concentrations are presented herein, our observations of sublethal effects indicate that LC_{50} and EC_{50} concentrations should not be considered criteria that initiate first concern; therefore, we provide reported LC_{10}, LC_{20}, LOEC concentrations for reference when available. The LC_{50} concentrations of K^+ for glochidia of *V. iris* and the Fatmucket (*Lampsilis siliquoidea*) during 24- and 48-hour tests were 12.8 and 30.0 mg K^+/L, respectively (Wang et al., 2018; Salerno et al., 2020). The EC_{50} (96-h) concentrations for juveniles of multiple mussel species ranged from 11.9 to >56 mg K^+/L (Wang et al., 2017; Gibson et al., 2018, Table SI4). Our sublethal effects to kidneys observed at 4.2 mg K^+/L also was below relative first-concern concentrations; for juvenile *L. siliquoidea*, Wang et al. (2018) and Kunz et al. (2021) estimated an EC_{20} and LOEC of 17 and 87 mg K^+/L, respectively (Table SI4). Imlay (1973) observed 90% mortality of the adult Mucket (*Actinonaias ligamentina*) held in 11 mg K^+/L, and 50% mortality of adult *A. plicata* exposed to 15 mg K^+/L, after 36 days (Table SI4).

Worldwide, there is a lack of K^+ regulations for protection of aquatic life (Gillis et al, 2021b). Currently, no recommended water quality criteria have been established, except for the USEPA's general chronic screening value of 53 mg K^+/L (Gillis et al., 2021b; USEPA, 2022). Based on distributions of unionid mussels in rivers along a gradient of K^+ concentrations, Imlay (1973) recommended 4 mg K^+/L as the maximum safe concentration for adult mussels, and our results substantiate that this recommendation is also protective against sublethal effects. Our results substantiates the hypothesis that the 78 and 97 mg K^+/L concentrations observed by Wang and Ingersol (2010) and Henley et al. (2013) in the NFHR are acutely toxic to freshwater mussels. Our results suggest that the USEPA chronic screening value of 53 mg K^+/L, and even EC_{20} and LOEC estimates (Wang et al., 2018; Kunz et al., 2021), may provide insufficient protection against truly chronic sublethal effects of K^+ to freshwater mussels (Table SI4).

Our results also showed sublethal effects to kidneys at concentrations of Cl^- beginning at 415.6 mg Cl^-/L occurred below reported EC_{50} concentrations for glochidia, juveniles, and adults. Chloride toxicity varies with water hardness (Gillis, 2011; Gibson et al., 2018; Wang et al., 2018). In studies conducted in water with similar hardness to this study, glochidia EC_{50} concentrations for *V. iris* and *L. siliquoidea* ranged from 1, 288 to 1,962 mg Cl^-/L (Gillis, 2011; Pandolfo et al., 2012; Wang et al., 2018) (Table SI4). Salerno et al. (2018) reported sublethal effects of salt exposure below acutely toxic levels (Table SI4). Our observation of sublethal effects that occurred at 415.6 mg Cl^-/L fell within the range of

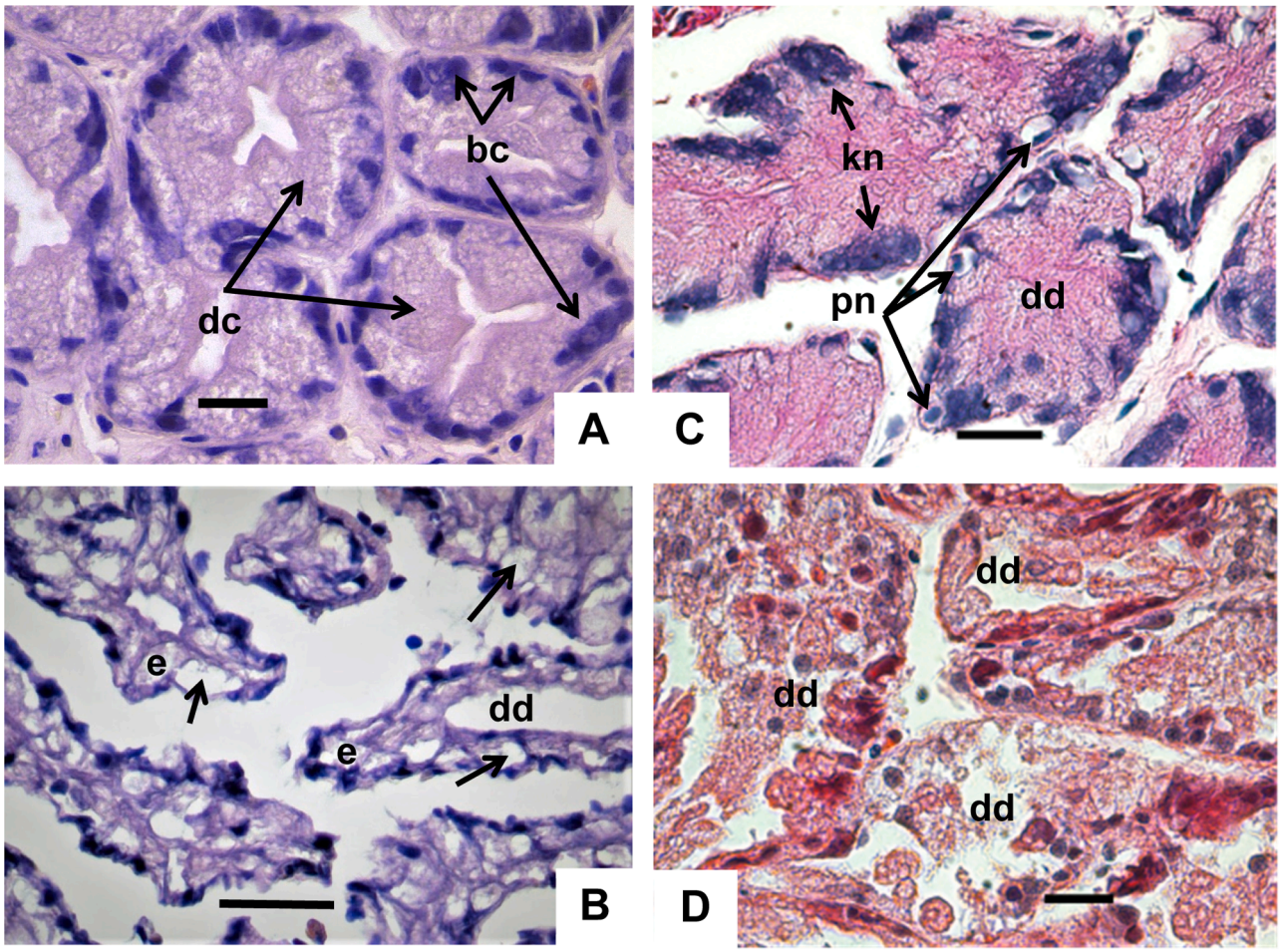

Fig. 5. Digestive gland tissues from *Villosa iris* of this study stained with hematoxylin and eosin. **A**. Normal digestive diverticula containing digestive (dc) and basophilic (bc) cells from low chloride (415.6 mg Cl-/L) treatment. Bar = 4 μm. Panels **B, C**, and **D** contain examples of lesions observed in evaluated digestive gland tissues. **B**. Digestive diverticulum (dd) containing atrophic epithelia (e) with vacuolated (arrows) cytoplasm from low chloride (415.6 mg Cl-/L) treatment. Bar = 4 μm. **C**. Digestive diverticula (dd) with pyknotic (pn) and karyolytic (kn) nuclei from low potassium (4.2 mg K+/L) treatment. Bar = 2 μm. **D**. Necrotic digestive diverticula (dd) from mid potassium (8.4 mg K+/L) treatment. Bar = 2 μm.

LC_{10}, LC_{20}, and LOEC for glochidia (Gillis, 2011; Pandolfo et al., 2012; Prosser et al., 2017; Salerno et al., 2020; Table SI4). The EC_{50} concentrations for juveniles of many mussel ranged from 979 to 3,083 mg Cl^-/L (Bringolf et al., 2007; Pandolfo et al.; 2012; Table SI4). Our sublethal effects of Cl^- also occurred at a concentration that was below LC_{10}, LC_{20}, LOEC estimates (range from >632 to 2,427 mg Cl^-/L) (Pandolfo et al., 2012; Prosser et al., 2017; Wang et al., 2018; Salerno et al., 2020; Wang, N, and 29 co0authors, 2021; Table SI4).

Our mortality result Cl^- concentration (1,662.5 mg Cl^-/L) was in general agreement with results of Blakeslee et al. (2013), who observed 50% mortality of adult *E. complanata* on day-3 and day-4 at exposures of 2,262 and 1,508 mg Cl^-/L, but below the mortality concentration range (1,870 – 4,440 mg Cl^-/L) for *L. siliquoidea* reported by Salerno et al. (2018), and less than reported Cl^- LOEC, LC_{10}, LC_{50}, and EC_{50} concentrations for several mussel species (Bringolf et al., 2007; Pandolfo et al., 2012; Augspurger et al. 2014; Prosser et al, 2017; Salerno et al., 2020; Wang et al. 2021); Table SI4). Even our kidney sublethal concentration (415.6 mg Cl^-/L) was well below the reported presumptive concentrations of first concern (Table SI4). For reference, the USEPA acute Water Quality Criterion is 860 mg Cl^-/L (USEPA, 2022). Our results suggest that this criterion, as well as LC_x concentrations, may not be protective against chronic sublethal effects in sub-adults. Also, recall the maximum concentrations of >18,000 mg Cl^-/L were observed within the NFHR (Wang and Ingersol, 2010; Henley et al., 2013); our results indicate that this maximum is acutely toxic to sub-adults.

We believe that our study is the first to document captivity effects on mussels by statistically comparing baseline versus control using histological data. Before the experiment began, the mussels were held at FMCC in flow-through systems using pond water. The mussels were moved to another FMCC building and placed in closed down-weller buckets using the same pond water, but were additionally fed a commercial algae mix. Thus, moving the mussels from one type of aquaculture system to another led to captivity effects in the control mussels. The baseline fractions of cells containing lesions in digestive glands and lipofuscin in kidneys were significantly lower than those from the control. However, fractions of gill filaments with cilia in control mussels were significantly greater than those in the baseline. We attribute greater cilia in the baseline mussels to exposure to substrate consisting of sediment, sand, and fine gravel in the pre-study aquaculture containers; during this study, the mussels were held on mesh baskets without substrate. Without the comparison of baseline and control, the effects of translocating mussels from one type of aquaculture system to another within one facility would not have been detected.

Although we found no research concerning within-facility translocation during captivity, Roznere et al. (2021) compared RNA transcripts in gill tissues of wild Threeridge, *Amblema plicata*, to those translocated and then captively-held for 11 months. More than 1,200 transcripts were up-regulated in the captive mussels, with 246 of the transcripts assigned to functional annotations, including metabolism (i.e., glycolysis, citric acid cycle, and oxidative phosphorylation) and stress

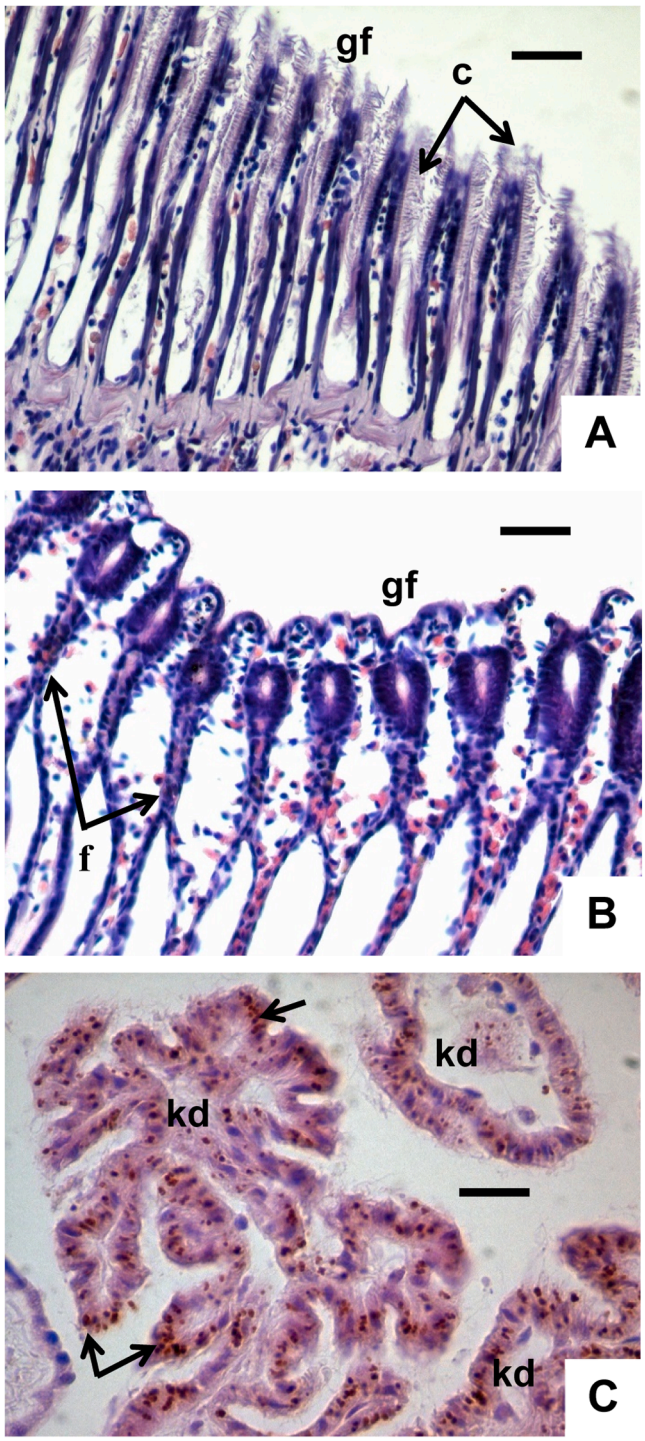

Fig. 6. Gill and kidney tissues from *Villosa iris* stained with hematoxylin and eosin unless otherwise noted. **A**. Normal ciliated (c) gill filaments (gf) from control. Bar = 4 μm. Panels **B** and **C** contain examples of lesions observed in evaluated gill and kidney tissues. **B**. Fusion (f, arrows) and edema of gill filaments (gf) from low chloride (415.6 mg Cl^-/L) treatment. Note absence of cilia on filaments. Bar = 4 μm. **C**. Kidney diverticula (kd) containing high abundance of lipofuscin (arrows, brown inclusions) from high chloride (1,662.5 mg Cl^-/L) treatment. Stained with Long Ziehl-Neelsen Carbo Fuscin. Bar = 2 μm.

response (i.e., heat shock proteins and antioxidants). Concurrently, more than 500 transcripts were down-regulated, with 41 that were assigned to functional annotations, including immune response (i.e., defensin 1 and lysozyme). Although we did not analyze RNA in organ tissues, our abundances of digestive gland and kidney lesions were likely associated with transcript regulations. Determinations of transcript regulations during contaminant exposure studies may offer resolution to document sublethal effects that are not detectable using microscopical evaluation of organ tissues.

Results of statistical comparisons of treatment, baseline, and control data provided some resolution in interpreting results. Although fractions of digestive gland cells containing lesions in the low and mid Cl^- treatments were not significantly different from fractions from the control, these treatments of CL^- were significantly greater than fractions from the baseline mussels (Figs. 1 and 2, Table SI3). However, the same mussels exhibited significantly greater fractions of kidney cells containing lipofuscin compared to both the control and baseline mussels (Figs. 2 and 4, Table SI3), and this indicated that the Cl^- mussels likely endured higher levels of oxidative stress compared to both the baseline and control mussels. Statistical comparisons of treatment, baseline, and control data also provided utility for judging whether results from the gill analyses were biologically meaningful. Fractions of gill filaments with cilia in the low and mid Cl^- and K^+ treatments were significantly lower than those in the control mussels, but fractions from these treatments were not significantly different from the baseline (Fig. 3, Table SI3). Because fractions of cilia in the treatments were comparable to those of the baseline mussels, the differences among them and the control was likely not biologically meaningful (Fig. 3, Table SI3). Additional dependent variables that we did not measure due to funding constraints may provide further resolution to determine sublethal effects of captive and treatments, including metabolomic analyses, quantification of oxidative stress proteins and lipid peroxidation, and measurements of energy stores (Gillis et al., 2014; Roznere et al., 2017).

We did not observe expected statistical increases of all variable fractions from low to mid concentrations of Cl^- and K. In fact, although not significantly different, fractions of lesions in digestive glands from mussels in the low K^+ treatment were greater than fractions in mussels from mid K^+ (Fig. 2, Table SI3). Also, 50% of mussels exposed to the high K^+ concentration died by day-13, but fractions of lesions in digestive glands and lipofuscin in kidney cells in the survivors at day-13 were relatively low compared to similar fractions from the low and mid K^+ concentrations. It is possible that the test mussels initiated a valve closure response as a protective behavioral response to the experimental concentrations of Cl^- and K^+. Valve closure of freshwater mussels has been documented in response to adverse conditions, including emersion, metals, and increased temperature and acidity (Cope et al., 2008).

The observed increase of cellular lesions in digestive glands and kidneys likely indicated negative alterations of physiological functions due to oxidative stress, as indicated by abundance of lipofuscin granules. Lipofuscin granules, consisting of peroxidative by-products and oxidized proteins and carbohydrates, have been related to a wide array of contaminants, and result from peroxidation of lysosomal membrane lipids, due to contaminant-related reactive oxygen species in lysosomes (Au, 2004; Moore et al., 2006). Gillis et al. (2014) documented increased lipid peroxidation and decreased antioxidant capacity in gill tissues of the Fluted-shell, *Lasmigona costata*. Effects of oxidative stress on lysosomes may lead to alterations of cell membrane signaling, enzyme and protein production, and organelle and cell turnover (Moore et al., 2006). The link between occurrence of lipofuscin granules in kidneys of freshwater mussels and oxidative stress has not been established, but the association between their occurrence and pervasive digestive gland lesions in our study, stresses the importance for further research (Rogers et al., 2018).

Degeneration of bivalve digestive gland cells have been linked to disease and an array of contaminant exposures (Au, 2004; Henley et al., 2013; Rogers et al., 2018). Significantly greater fractions of lesions in digestive glands of the K^+-exposed mussels suggest pervasive impacts to important physiological functions that occur within cytoplasmic components (organelles), including intra- and extra-cellular digestion, nutrient absorption, contaminant detoxification, and lipid and glycogen storage (Petrović et al., 2001). It is reasonable to hypothesize that the

lesions lead to diminished energy acquisition and storage functions. Ciparis et al. (2019) observed significantly lower condition index in adult *L. fasciola* exposed to simulated laboratory water from the coal-contaminated Powell River in Virginia, USA and interpreted the results as indication of reduction of total energy stores in the test mussels. Kunz et al. (2021) observed a sublethal LOEC of 24 mg K^+/L for reduced biomass in *L. siliquoidea* (Table SI4). The concurrent pervasive digestive gland lesions and increased occurrences of lipofuscin in kidney cells that likely indicated oxidative stress suggest that our study *V. iris* had reduced condition indices with concurrent declines of stored energy to fuel vital somatic functions.

Bayne (1973) and Thompson et al. (1974) explored the relationship among digestive gland lesions, stress response, and energy substrate utilization in the Blue Mussel (*Mytilus edulis*) during starvation. Thompson et al. (1974) observed progressive lesions in the digestive gland similar to those observed in this study, including reduction of cell volume (atrophy), cytoplasmic vacuolation, deterioration of nuclei, and necrosis. Bayne (1973) observed declines in ratios of oxygen consumed to ammonia nitrogen excreted (N/O ratio) that indicated utilization of stored protein relative to utilization of carbohydrate and lipid reserves, but with more prolonged starvation, declines of carbohydrates and lipids occurred. These energy substrates are primarily stored in and utilized from the digestive gland and other somatic tissues. Our pervasive digestive gland lesions suggest alterations to energy substrate storage and utilization, as well as intra- and extra-cellular digestion, nutrient absorption, and contaminant detoxification (Petrović et al., 2001). Therefore, it is logical to hypothesize that the pervasive deterioration of the organ observed during this study negatively affected somatic energy available for other somatic functions, such as tissue repair, osmoregulation, growth, and gametogenesis.

Because of predicted widescale increasing concentrations of major ions in aquatic ecosystems, it is plausible that wild freshwater mussel populations will incur chronic energetic demands in the future, due to sublethal impacts to vital organs. The energy deficit will likely translate to less energy that can be invested to vital somatic functions. This is of critical importance, because the sublethal effects that we observed in this 61-day chronic study were at concentrations of Cl^- and K^- that were well below published water quality adverse effects (LC_{50} and EC_{50}) and even first concern (LOEC, $LC_{<20}$, and $EC_{<20}$), concentrations for the protection of aquatic life. Thus, the use of adverse effects concentrations of Cl^- and K^+ reported herein, and possibly other major ions, may not be protective against sublethal effects to organ tissues in sub-adult mussels.

CRediT authorship contribution statement

Jennifer J. Rogers: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Visualization. **William F. Henley:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Visualization, Supervision. **Amanda G. Weberg:** Methodology. **Jess W. Jones:** Funding acquisition, Conceptualization, Supervision, Writing – review & editing. **W. Gregory Cope:** Visualization, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2023.106476.

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