GROWTH AND SURVIVAL OF FRESHWATER MUSSELS IN RESPONSE TO CONTAMINANTS IN THE UPPER CONASAUGA RIVER, GA

by

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(Under the Direction of Brian Irwin and Peter Hazelton)

ABSTRACT

Freshwater mussels are one of the most imperiled and diverse faunal groups in North America, facing widespread decline due to habitat degradation, invasive species, and pollution. This study aimed to assess the effects of sediment- and water-borne contaminants on juvenile mussel growth and survival in the Conasauga River watershed. Two complementary approaches were used: (1) a 28-day sediment toxicity trial evaluated the effects of field-collected sediments on mussel growth and survival in a controlled laboratory setting, and (2) an *in situ* silo deployment assessed juvenile mussel responses to ambient water conditions across multiple tributaries. Mussel growth varied significantly across sites in both experiments, and multivariate models identified sediment and water chemistry, nutrient concentrations, and land use variables as predictors. Data from this research contribute to ongoing conservation efforts in the Conasauga River by identifying potential sediment and water contaminants and ambient stream conditions that are harmful to freshwater mussels.

INDEX WORDS: Unionida, ecotoxicology, conservation, *in situ* exposures, sediment contamination

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DEDICATION

I would like to dedicate this thesis to my family. First, to all the Martins on the farm — my parents, siblings, grandparents, aunt and uncle, and nieces and nephew. Coming home to Kentucky and being with you all is what kept me going. And to my found family here in Athens, you have been and continue to be the highlight of my daily life. I am indebted and incredibly grateful to you all.

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CHAPTER 1

LITERATURE REVIEW AND INTRODUCTION

Background on Freshwater Mussels

Unionida, or freshwater mussels, are an incredibly diverse order of bivalves in the phylum Mollusca with over 950 total species globally (Graf & Cummings, 2021). Taxa from this order are found on 6 continents, but diversity is concentrated in Asia and North America (Bogan, 2008; Graf & Cummings, 2021). The order has six families, but the vast majority of diversity and the greatest geographical range is from Unionidae with 78% of the total species (Bogan & Roe, 2008; Graf and Cummings, 2021). North America has only two (Unionidae and Margaritiferidae) of the six families of Unionida, and there are approximately 302 species of freshwater mussels found there (Graf and Cummings 2021). The distribution of freshwater mussels is influenced by factors such as historic glaciation and climate stability but is heavily influenced by their reliance on host fish for dispersal of their larvae (Haag, 2012; Lopes-Lima et al., 2017; Schwalb et al., 2013).

The distribution of freshwater taxa in the U.S. is concentrated in the Southeast, specifically Alabama, Tennessee, and Georgia (Elkins et al., 2019). There are nearly 240 species of freshwater mussel and over 810 species of fishes and crayfish found in the region (Elkins et al. 2019). Georgia is home to 126 species of freshwater mussels, or 40% of total North American freshwater mussel diversity (Georgia Department of Natural Resources, 2024). Paired with diversity are high rates of both endemism and imperilment (Elkins et al., 2019). Freshwater ecosystems are experiencing disproportionately greater rates of species decline compared to

terrestrial systems (Collen et al., 2014). Unionid mussels prove to be a quintessential example of this phenomenon. In the Southeast, approximately 32% of freshwater mussel species are listed as vulnerable, endangered, critically endangered, or extinct under current IUCN red list (2024). The sources of these declines is thought to stem from habitat degradation, sedimentation, pollution, poor land use practices, dams, and channelization, although many studies fail to evaluate causal mechanisms (Downing et al., 2010; Haag, 2019; Strayer et al., 2004).

Unionids are thought to be long lived, but longevity can vary both between and within species with ranges spanning 4 to 190 years (Haag & Rypel, 2011). The life cycle of mussels begins when male mussels release gametes (sperm aggregates called spermatozeugmata) into the water column. Female mussels siphon the sperm and release eggs from the gonads into the specialized gill chambers, or marsupial, where the eggs are fertilized and brooded. The eggs remain in the marsupia and mature into the larval, glochidia stage (Haag, 2012).

Perhaps the most fascinating of freshwater mussel life stages is the obligate parasitic larval phase. Glochidia are expelled from female mussels and attach to a host animal. This is typically a fish species although there are instances of this host relationship occurring on amphibians like salamanders (Barnhart et al., 2008; Howard, 1951). To infect fishes with glochidia, mussels develop elaborate adaptations to attract hosts and release glochidia. For example, mussels may use broadcast spawning where glochidia are released freely into the water with no further adaptation to attract a host (Barnhart et al., 2008). Additionally, some species create aggregates of glochidia that serve to protect glochidia and attract fishes. These conglutinates and superconglutinates mimic the color and form of fish prey sources like worms (*Cyprogenia aberti*) and fly pupae (*Ptychobranchus subtentum*) to further entice suitable hosts (Barnhart et al., 2008). Mussels primarily from the tribe *Lampsilini*, have adapted intricate

mantle lures which attract fish, who then rupture the protruding marsupium (Barnhart et al. 2008).

Once mussels have attached to the fins, scales, or gills of a host, they are encapsulated by the host tissues (Rogers-Lowery & Dimock, 2006). Regardless of host infection strategy, research shows that mussels derive nutrients from the host fish during infection (Fritts et al., 2013). Finally, juvenile freshwater mussels are sloughed off their host and settle into the sediment on the stream bed where they stay for the first 0-4 years of life as they mature into adults (Barnhart et al. 2008).

Freshwater mussels provide essential ecosystem services because of their unique biology and ecology. To begin, freshwater mussel excretions can provide benthic communities with nutrients such as nitrogen (Atkinson et al., 2014). Additionally, mussels can remove suspended solids from the water column and deposit organic material into the sediment (Vaughn et al., 2004). The burrowing behavior of mussels can increase water and aerate the sediment, while also releasing nutrients from the sediment into the water column and stabilizing loose sediment (Vaughn & Hakenkamp, 2001). These services support the diversity of other aquatic taxa. Vaughn and Spooner (2006) showed that densities of unionids and macroinvertebrates were positively correlated, and this could be due to mussel shells serving as structures for aquatic insect larvae to attach (Lawfield et al., 2014). Finally, because of their sensitivity, unionids can be used as bioindicators and their presence is considered evidence of ecosystem health (Vaughn, 2018).

Toxicology & Environmental Stressors

Aquatic ecotoxicology is a broad and rapidly evolving field. While much of the research has traditionally focused on fish and zooplankton, studies on freshwater mussels are increasingly gaining attention (Connors et al., 2019). Due to the marked decline in unionid populations, research has sought to identify potential causes of their imperilment, with environmental contaminants being a significant factor (Downing et al., 2010). In addition to illuminating causes for population decline, several traits add to the suitability of freshwater mussels as model organisms for ecotoxicology research: they are highly sensitive to certain pollutants, their complex life cycle and feeding behaviors expose them to multiple contamination routes, and they are nearly sessile and some are long-lived, so chronic exposure to contaminants can be representative of a river system over time (Farris & Van Hassel, 2007).

Freshwater mussels can be more sensitive than commonly used test species, like fishes and zooplanktons, to environmental contaminants like major ions, nitrate and sulfate, metals, pesticides, and ammonia (Bringolf et al., 2007; Kunz et al., 2021; March et al., 2007; Wang et al., 2020; Wang et al., 2008; Wang, Ivey, et al., 2017; Zipper et al., 2014). These contaminants are usually related to altered land use in the watershed. Pesticides, nitrate, sulfate, and ammonia contamination are associated with agriculture and wastewater effluents, while metals like lead, zinc, cadmium, manganese, and copper are often associated with mining, and major ions can come from runoff in urban settings (Singh et al., 2024). Water quality parameters can influence mussel sensitivity to contaminants. For example, the toxicity of potassium chloride decreased in chronic exposures when water hardness increased from soft (25 mg/L) to very hard (300 mg/L) (Wang et al., 2023). Ammonia toxicity has also been shown to increase with rising pH levels

(Wang et al., 2008). Similarly, elevated temperatures have been associated with increased chromium toxicity (Wang, Kunz, et al., 2017). Confounding with this sensitivity, the complex life cycle of freshwater mussels makes them vulnerable to multiple exposure routes (Cope et al., 2008).

All life stages of mussels can be particularly sensitive to environmental contaminants, but early life stages like larvae and juveniles are thought to be the most sensitive (Augspurger et al., 2007; Wang et al., 2007). However, the decreased sensitivity of adult mussels could be due to their contaminant avoidance behaviors, where they close their shells in response to high concentrations of contaminants (Cope et al., 2008). Adult mussels are exposed to contaminants primarily through surface water as they are siphoning and feeding, but they are partially buried in sediment which is an additional exposure route. Glochidia can be exposed to contaminants while in the marsupial gills and while attached to fish hosts (Cope et al. 2008). Juveniles, which spend the first 0 to 4 years buried in the sediment pedal feeding, are susceptible to contaminants found in sediment and pore water (Cope et al. 2008; Haag 2012). Controlled laboratory assays have been developed specifically to examine the toxicity of pollutants via these life-stage-specific exposure routes of freshwater mussels (ASTM International, 2020, 2022; Hazelton et al., 2012).

Methods for toxicology studies with freshwater mussels can be found in resources like the American Society for Testing and Materials (ASTM). These assays vary by duration, exposure, age of organism, and most notably the contaminant being tested. For glochidia and juveniles, acute toxicity tests last between 24 hours and 96 hours, respectively. The short test duration is meant to mimic the exposure time glochidia might face in the wild once they have been released from marsupium and before they encyst on a host fish (ASTM International, 2022; Fritts et al., 2014). Toxicity tests using glochidia and juveniles are typically dosed water only

exposure, or may have some lab control sediment, because of the short duration and the small size of the organism (ASTM 2022). Additionally, for these studies, the contaminant being tested is usually applied at differing concentrations across treatment replicates but kept constant for the treatment it is assigned and monitored for the duration of the trial (ASTM 2022). Water quality parameters like dissolved oxygen, temperature, pH, conductivity, and hardness are kept constant for these trials and monitored regularly throughout. The endpoints for acute water-only exposures are typically only survival used to calculate a median lethal concentration (LC50).

Recent guidelines have been developed for toxicity trials incorporating sediments. These guidelines are adapted from 28-day water only exposures with juveniles and sediment exposures using *Hyalella azteca* (Amphipods) and *Chironomus dilutus* (Midges). Trials are typically chronic, rather than acute, and utilize juveniles exclusively. Chronic exposures are typically 28 days long, which is meant to represent a substantial portion of a freshwater mussel's lifespan, even though this can vary dramatically among species (ASTM 2020; Haag and Rypel 2011). Sediment toxicity trials have much of the same experimental design as acute water only exposures, where water chemistry is monitored and kept constant, but the endpoints include growth (change in length and biomass) as well as survival (ASTM 2020). Additionally, mussels are fed during the trial and the overlaying water in the test chambers is renewed daily. Sediment toxicity tests are uniquely useful for the assessment of toxicity of freshwater mussels because juveniles live buried in the sediment and because of mussels' sensitivity to contaminants that are found in sediment (Cope et al. 2008).

While laboratory experiments are valuable for assessing the toxicity of contaminants and establishing protective water quality criteria, they often lack the environmental realism needed to fully capture the complexity of natural ecosystems. Laboratory studies typically do not account

for the interactive effects of multiple chemical stressors or the cumulative impacts of long-term exposure (Chapman, 2002). In essence, they represent a simplified abstraction of the challenges mussels face in the wild. Combining laboratory experiments with *in situ* methods provides a more comprehensive understanding of the impacts of contaminants on mussel populations. This integrated approach allows researchers to better evaluate how contaminants influence mussel growth, survival, and overall population health in an actual watershed of concern.

In situ mussel exposures can assess toxicity of waterborne contaminants in specific watersheds while experiencing the variability of natural systems. These studies are often exploratory but are useful to evaluate growth and survival in streams and relate them to faunal decline, water quality, underlying geology, food availability, and contamination or nutrients found in water and sediment (Archambault et al., 2017; Cope et al., 2021; Haag et al., 2019; Skorupa et al., 2024).

There are a few methods for *in situ* exposures, like Whitlock-Vibert (W-V) boxes used in Haag et al. (2019), but the most used method is mussel silos. Silos are made of concrete, dome shaped, and possess an inner chamber with a grate where mussels are contained during deployments. They are positioned on the stream bed so that the dome is faced upright, allowing for water flowing over top the silo to pull water from underneath and through the inner chamber, delivering food and oxygenated water, while removing waste (Bernoulli Principle) (Haag et al., 2019; Patterson et al., 2018). Silo deployments typically occur over the summer months (April - September) using juvenile mussels. For silos to function properly and be retrieved at the end of the trial, they are placed in water with moderate velocity that is at least 0.5 m deep, and on stable substrate. During this time growth end points like length and biomass are measured periodically

throughout the deployment, and survival is measured at the end. Additionally, water and sediment are sampled and analyzed for chemistry and contaminants.

In situ studies are often in areas where populations of mussels are declining or to assess the viability of streams for reintroduction. Haag and others(2019) found that defaunated streams had similar characteristics including low total organic carbon, low water temperature, and a similar underlying geomorphology of karst, and ultimately this led to a reduction in growth of mussels during silo deployments. Skorupa et al. (2024) demonstrated that high mussel growth was related to high concentration of Chlorophyll-*a* and temperature, and that in cool and unproductive streams, increases levels of sodium, magnesium, calcium, and potassium may reduce growth. Other studies found that contaminants such as Polycyclic Aromatic Hydrocarbons (PAHs) and Mn are stressors to mussels and are negatively correlated with mussel densities (Cope et al. 2021).

Challenges in the Conasauga River

The Conasauga River in northwest Georgia and southern Tennessee is a free-flowing portion of the headwaters of the Coosa River basin and is prioritized for the protection of globally rare species like native fishes and freshwater mussels (Albanese et al., 2015). Data from natural history collections have shown that since the 1960's mussel species richness has decreased by 72% in Conasauga with a 97% decline in the total number of individuals (Haag 2019). Alteration of land use temporally coincides with these changes in species composition. While the headwaters of the Conasauga River are protected by the Chattahoochee National Forest, much of the land in the watershed is influenced by agriculture like poultry production and row crops, forestry, urban development, and industrial effluent (Freeman et al., 2007).

Agricultural contaminants like nitrogen, phosphorus, steroid hormones, and glyphosates degradation product (aminomethyl phosphonic acid (AMPA)) have been detected in surface waters and sediments in the Conasauga mainstem and its tributaries at levels that are harmful to aquatic taxa (Lasier et al., 2016). Maximum AMPA in surface water was detected at a concentration of 5.7 mg/L, but was not found to be toxic to aquatic invertebrates, similar to previous studies on fathead minnow (*Pimephales promelas*) and Daphnia (Lasier et al., 2016; Levine et al., 2015). However, glyphosate formulations typically contain 0.25-1.0 percent surfactant which can be toxic to juvenile mussels at levels as low as 0.5 mg/L (Bringolf et al., 2007). Further, Lasier et al. (2016) detected seasonal differences in the concentration of contaminants, with the highest concentrations of sediment glyphosate and AMPA occurring in May 2012 contrasting with low concentrations found in November 2013. The high concentration of contaminants coincides with the phenology of glochidia released in the spring and summer before juveniles of many species drop off fish hosts and burrow into sediment in the Summer and Fall (Haag 2012).

In addition to agriculture land use, there is also urbanization in the watershed. The average percentage developed land use in the study is approximately 13% but is as high as 37% in the Mill Creek watershed that include Dalton, GA. This land use is characterized by impervious surfaces, which significantly affect water quality due to runoff. Water runoff from impervious surfaces can alter water hardness, pH, alkalinity, and total suspended solids (Muller et al., 2020). Additionally, this runoff can introduce contaminants, including metals (e.g., lead, zinc, and copper) and nutrients (e.g., nitrogen and phosphorus) (Muller et al. 2020). Emerging contaminants like perfluoro octane sulfonate (PFOS) have been detected in the Conasauga (Konwick et al., 2008). PFOS concentrations were detected beyond the recommended aquatic

chronic water guidelines at sites downstream of wastewater land application system in Dalton, Georgia where carpet manufacturing and other industry is present (Beach et al., 2006; Konwick et al., 2008).

Research Needs and Objectives

Given the importance of the Conasauga River's biodiversity, further information is needed on the potential drivers of species decline—particularly contaminants associated with agricultural and developed land use. Research such as this supports more informed and targeted conservation efforts. To combat declines, resource managers may implement actions such as population reintroduction, augmentation, or habitat restoration, often in the absence of clearly defined causes. Studies that evaluate local environmental stressors help identify system-specific characteristics contributing to species loss and are essential for prioritizing sites for effective restoration and long-term recovery.

To better understand mussel declines in the Conasauga River, we evaluate the lethal and sublethal effects of agricultural and municipal contaminants in the watershed on freshwater mussels. This project combines the use of *in situ* water exposures, as well as laboratory sediment toxicity tests with field collected sediment across a gradient of contaminated sites within the watershed. Our trial targeted the vulnerable life stage of juveniles exposed to contaminants in the sediment at environmentally relevant concentrations and mixtures. Lab work is important to understand foundational mussel toxicological profile, but there is an added benefit to pairing these experiments with field exposure to get the most holistic yet thorough look at the conditions in the system. Besser et al. (2015) and Wang et al. (2013) have shown that the toxicity of contaminants from mining activities observed in the laboratory, were consistent with declines in mussel populations observed in the field.

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CHAPTER 2

NUTRIENT AND CONTAMINANT EFFECTS ON JUVENILE FRESHWATER MUSSEL GROWTH IN CONTROLLED LABORATORY TRIALS[1]

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Abstract

Freshwater mussels are some of the most imperiled taxa in the world. Threats to mussel populations have been attributed to numerous causes, including habitat degradation or loss from dams, pollution, and invasive species. We conducted laboratory exposure trials to assess the effects of multiple contaminant stressors in sediment on freshwater mussel survival and growth. We conducted substrate-exposure studies in the laboratory using sediment collected from throughout the Conasauga River, GA, watershed following established sediment toxicity test conditions. During the summer of 2024, sediments were collected at 13 study sites representing a gradient of expected municipal and agricultural contamination and varying land use practices. Juvenile mussels (2024 average start length ~1.5 mm) were exposed to these sediments at the University of Georgia's Aquatic Biotechnology and Environmental Laboratory. In 2024, the average survival across all sites and the control was 96% for a 28-day duration. Average percent change in length was 72%. Average percent change in weight was 435%. Multiple linear regression revealed that magnesium, the sum of toxic units from multiple pesticides, and National Discharge Pollution Discharge Elimination System permit density had a significant negative effect on growth, while carbon, potassium, and phosphorus had a significant positive effect on growth. Data collected could further our understanding of the role sediment contaminants and nutrients play in the decline of freshwater mussels.

Introduction

Freshwater mussel populations in the U.S. have experienced dramatic declines throughout their ranges since the mid-20th century (Haag, 2019). The causes of declines are thought to stem from habitat alteration, poor water quality, pollution, poor land use practices,

dams or impoundments, and alteration of hydrology (Downing et al., 2010; Haag, 2019; Strayer et al., 2004). The Southeastern U.S. contains a multitude of aquatic biodiversity including 589 fish species, 234 mussel species, and 221 crayfish species, despite incompatible land use and habitat alteration found in the region (Elkins et al., 2019). Much of the freshwater taxa in the Southeast is imperiled with nearly a third of the unionid species being vulnerable, endangered, critically endangered, or extinct under the current IUCN list. Previous research has highlighted correlative relationships between population declines and potential explanatory variables (Downing et al., 2010). Nonetheless, conservation strategies such as population reintroductions, augmentations, habitat restoration, and investigations into emerging stressors remain valuable tools for protecting freshwater mussels (Freshwater Mollusk Conservation Society, 2016; Haag & Williams, 2014). However, more targeted research is essential to uncover the unique characteristics of individual river systems and species, enabling a clearer understanding of stressors to local populations and better prioritization of restoration efforts.

The Conasauga River is in northwest Georgia and southern Tennessee, with its upper watershed protected by the Chattahoochee and Cherokee National Forests. This river is of critical conservation priority due to its role in supporting globally rare species, including native fishes and freshwater mussels (Albanese et al., 2015; Elkins et al., 2019). Among the 28 freshwater mussel species inhabiting the Conasauga, four are listed as threatened or endangered in Georgia, while seven are federally recognized as threatened or endangered (Georgia Department of Natural Resources, 2024). Potential threats to mussel populations in the Conasauga stem from declines of host fish populations and changes in habitat due to human activities (Freeman et al., 2007; Freeman et al., 2017). The lower portions of the Conasauga River watershed are primarily

influenced by agriculture and urban development which introduce harmful contaminants that could affect freshwater mussels (Freeman et al., 2007).

Research on the Conasauga River has revealed that contaminants in both sediment and water originate from agricultural sources, as evidenced by the proximity of agricultural farms and the seasonal patterns of elevated contaminant concentrations (Lasier et al., 2016; Sharpe & Nichols, 2007). Lasier et al. (2016) detected aminomethyl phosphonic acid (AMPA), a byproduct of glyphosate pesticides, in both surface water and sediment, with concentrations peaking in May and declining by November. Although the detected glyphosate concentration (5.7 mg/L) was not directly toxic to aquatic invertebrates, the pesticide formulation includes surfactants, which can harm juvenile mussels at concentrations as low as 0.5 mg/L (Bringolf et al., 2007). Chronic exposure to low concentrations of current-use pesticides has also been documented in the Conasauga (Sharpe & Nichols, 2007). Moreover, Sharpe and Nichols (2007) identified agricultural animal waste as a potential source of excess nutrients, supported by stable isotope signatures observed in snails. The highest concentrations of total nitrogen and phosphorus recorded by Sharpe and Nichols (2007) occurred during summer months (June and August), coinciding with manure application on crop fields.

These agricultural contaminants pose significant risks to freshwater mussels. High contaminant concentrations align with the critical timing of glochidia (larvae) release in spring and summer, just before juveniles detach from fish hosts and burrow into sediment in late summer and fall (Haag, 2012). Contaminants in both water and sediment may affect multiple mussel life stages, including the highly sensitive juvenile stage, during which mussels spend their first 0-4 years buried in sediment (Cope, 2008; Haag, 2012).

In addition to agricultural contaminants, the Conasauga River Basin is also impacted by urban development and industry. There are nearly 150,000 people living in Murray and Whitfield counties, which are separated by the Conasauga River (U.S. Census Bureau, 2020). The average percentage of developed land use in the study area is approximately 13%, but ranges from 2% to 37% (current study). Developed land use is characterized by the prevalence of impervious surfaces, which significantly affect water quality. Runoff from these surfaces can modify water chemistry by altering parameters such as pH, hardness, and alkalinity, and by increasing total suspended solids (Muller et al., 2020). Runoff can also introduce contaminants—including metals (e.g., lead, zinc, copper), major ions (e.g., chloride, potassium, magnesium, calcium), and nutrients (e.g., nitrogen, phosphorus)—to which mussels are particularly sensitive (Muller et al., 2020). In acute laboratory toxicity test, unionids are often sensitive to chloride, potassium, sulfate, copper, nickel, and zinc (Wang et al., 2017). Additionally, *in situ* exposure studies have shown correlations between decreased growth and magnesium, sodium, and potassium concentrations in both water and sediment (Gibson et al., 2018; Kunz et al., 2021; Skorupa et al., 2024).

Previous laboratory research on the acute and chronic toxicity of metals, ions, ammonia, and pesticides on freshwater mussels has revealed that U.S. Environmental Protection Agency (U.S. EPA) water quality criteria are set at or above critical levels of toxicity for juvenile freshwater mussels (Bringolf et al., 2007; Gibson et al., 2016; Newton et al., 2003). While standardized laboratory experiments are valuable for assessing lethality of contaminants and establishing protective water quality criteria, they often lack the environmental realism needed to fully capture the complexity of natural ecosystems. By not assessing interactive effects of multiple stressors or cumulative effects of long-term exposure, many laboratory trials represent a

simplification of the challenges organisms face in the wild (Chapman, 2002). Combining standard laboratory approaches with *in situ* conditions, through the use of field collected sediments, may provide a more comprehensive understanding of the effects of contaminants on mussel populations (Archambault et al., 2017; Cope et al., 2021). This integrated approach allows researchers to better evaluate how contaminants influence mussel growth, survival, and overall population health within a watershed of concern, considering the variability of water and sediment chemistry, seasonal dynamics, and location specific factors throughout a basin.

In the current study, we assess the effects of contaminants in sediments collected from the upper Conasauga River on juvenile mussel growth and survival, with the goal to evaluate potential stressors to mussel persistence in the basin. Further, the mussel species (Alabama Rainbow, *Cambarunio nebulosus*) used in these experiments is a species of conservation concern in Georgia (Escobar, 2021). The objectives of this study are twofold: (1) to evaluate the effects of Conasauga River sediment on juvenile mussel growth and survival in a 28-day laboratory sediment toxicity test, and (2) to identify potential stressors in the sediment associated with human development, agriculture, and forested landscapes. These objectives were addressed through sediment collection and laboratory testing conducted in 2023 and 2024. This chapter focuses on the 2024 trial, while details of the 2023 trial are presented in Appendix A. We hypothesized that mussel growth and survival in the 2024 trial will be reduced in sediments collected from more developed and agricultural areas due to the presence of contaminants.

Methods

Juvenile mussel rearing

Juvenile Alabama Rainbow mussels were provided by the Alabama Aquatic Biodiversity Center (AABC) in Marion, Alabama. This species was selected because it is native and considered an at-risk species currently being evaluated for listing under the US Endangered Species Act (Federal Register Citation: 76 FR 59836). Adult mussels were collected for broodstock from the Coosa River basin during the summer of 2022 and 2023. Glochidia were infested on Cahaba Bass (*Microterus cahabae*; Baker, Blanton, & Johnston, 2013) during Spring of 2023 and 2024 and held in multitank aquatic habitat systems until transformed. Then, juvenile mussels from these systems were collected and grown for approximately 30-60 days in bucket recirculating systems where they were fed commercial algae.

Site Selection

During the 2024 sediment toxicity trial, juvenile mussels were exposed to a clean control sand and sediment collected from our 13 study sites which spanned the Upper Conasauga River from Tennessee State Route 317 south to Calhoun, Georgia (Figure 2.1). To randomly select site locations in the Conasauga River, we first created a stream network in MATLAB (The MathWorks, 2023) from 1/3rd arc-second Digital Elevation Models (DEMs) using the TopoToolBox, Image Processing Toolbox, and Mapping Toolbox (Schwanghart & Scherler, 2014). In ArcGIS Pro version 3.1.2, (ESRI, Redlands, CA, USA) we filtered out portions of the stream network that were above 1,200 feet in elevation, were 1st or 2nd order streams, and the mainstem of the Conasauga below the confluence of Drowning Bear Creek near Dalton, GA. This filtering was done to prioritize sampling above known areas of industrial contamination

downstream of Dalton and to increase the probability that the selected points would contain mussel habitat based on GA Department of Natural Resources mussel database. We then generated 100 random points on the refined stream network and delineated a watershed around each one based on the DEM's.

To characterize the status of the watershed around each point, we calculated percent land use, National Pollutant Discharge Elimination System (NPDES) density (per square km), and added the EPA's 303(d) assessment to each watershed. Percent land use was calculated from the 2021 National Landcover Database after we condensed the landscape classes from 16 unique classes to 6 (Dewitz & U.S. Geological Survery, 2021). The 6 land use classes used to calculate percent land use in each watershed were water (2 classes), forest (5 classes), barren (1 class), agriculture (2 classes), developed (4 classes), and wetlands (2 classes). We removed water, barren, and wetlands from the watershed characteristics because they made up approximately 1% of the watershed and were not of particular interest when considering land use impacts on mussel health.

We calculated composite site-level scores to stratify locations across a range of anthropogenic impacts. First, we standardized each watershed characteristic (percent land use by category, NPDES density, and 303(d) impairment status) by converting values to z-scores. For each of the 100 candidate sites, we then summed the z-scores of all standardized watershed variables to create a composite watershed impact score for each potential location. These composite scores were then ranked and divided into quantiles to represent a gradient from low to high potential anthropogenic impact. To ensure balanced representation across this gradient, we randomly selected sites from each quantile for further consideration. Final site selection for the

trial was narrowed based on physical access due to stream conditions (flow and depth) and landowner permission for actual site access (Table 2.1).

Sediment collection and analysis

For the 2024 toxicity trial, sediment was collected at 13 study sites accessed via the nearest bridge crossing or with landowner permission. At each site, composite 2-L samples were taken from depositional areas located at least 50 meters upstream of the access point. Using a stainless-steel spoon, the top 1–2 cm of fine sediment was collected, sieved to 2 mm, and homogenized. The processed sample was then evenly divided into six 250-mL amber glass bottles. Site environmental characteristics were recorded using the Physical Characterization/ Water Quality and Habitat Assessment data sheets from the U.S. EPA Rapid Bioassessment Protocol (Barbour et al., 1999). After each sampling event, all equipment was rinsed with stream water followed by methanol. Samples were stored on ice during transport and then refrigerated at 4 ℃ at the University of Georgia's Aquatic Biotechnology and Environmental Laboratory (ABEL). One bottle per site was analyzed for pesticides using gas chromatography–tandem mass spectrometry (GC-MS/MS) at the U.S. EPA National Exposure Research Laboratory (NERL) in Athens, GA. Another was analyzed for chromium (Cr), pH, calcium (Ca), potassium (K), magnesium (Mg), manganese (Mn), phosphorus (P), zinc (Zn), carbon (C), nitrogen (N), and total organic carbon (TOC) at UGA's Agricultural and Environmental Services Laboratories (AESL). The remaining four bottles per site were stored at 4 ℃ until used in the toxicity trial.

Toxicity Trial Specifications

The sediment toxicity trial was conducted following the American Society of Testing and Materials (ASTM) Standard Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates (2020). The trial was carried out over 29 days in a controlled laboratory environment using a flow-through system, with water maintained at an average temperature of 23 ± 1 °C and a photoperiod of approximately 16L:8D from ambient laboratory light. To accommodate the large number of samples, a staggered start was used. Two groups of mussels—each consisting of 280 individuals distributed across 28 beakers—were initiated on June 3 and June 4, respectively. For analysis, the initiation date for each beaker was designated as day 0, ensuring a uniform 28-day exposure period for all individuals. Mussels used in the trial were approximately 30–60 days old and measured ~1.5 mm in length.

Sediment from each of the 13 study sites was collected, stored in amber glass bottles, and kept refrigerated (4 °C) until use to prevent ammonia-related issues observed in a previous trial (Appendix A). Prior to use, sediment was homogenized, and 100 mL was transferred to 300 mL glass beakers. Beakers were then filled with 175 mL of overlying reconstituted water and placed randomly within the dilution table. Four replicate beakers were prepared per site. A washed and refrigerated sand control was included using the same handling and storage conditions as the site sediments. Reconstituted water was formulated by diluting deionized water to a target hardness of 90–110 mg/L as $CaCO_3$, following the moderately hard reconstituted water recipe described in Smith et al. (1997). The overlying water in each beaker was exchanged three times daily using the flow-through renewal system, with ~175 mL replaced per flush (Zumwalt et al., 1994).

Mussels were held within mesh cups constructed from 850 µm stainless steel mesh (walls) and 350 µm fabric mesh (bottom), secured with zip ties to ensure containment and

facilitate retrieval. To increase baseline survival and growth, we increased food rations from those detailed in the ASTM guide to 3 mL, 3 times a day with two water changes (early morning and late afternoon (Ning Wang, USGS Columbia Environmental Research Center, personal communication)). Water quality parameters—temperature, pH, ammonia, and dissolved oxygen (DO)—were measured daily in one replicate per site. Hardness, alkalinity, and conductivity were measured on days 0 and 28 of the trial for all replicates in each of the trial groups.

Growth was assessed using shell length and height, measured at both the start and end of the trial. Mussels were photographed under a microscope using an AmScope MU1400B or MU900 camera (0.8x or 0.63x magnification), interfaced with ImageJ software calibrated to a stage micrometer. Length was defined as the maximum distance between anterior and posterior shell margins, while height was measured from the umbo to the ventral margin. The microscope magnification level and camera resolution were recorded for each replicate to facilitate accurate pixel to μm conversion. Initial dry weights were estimated using a cohort of mussels preserved at the start of the trial (five replicates of 10 mussels each, preserved in 70% ethanol).

Due to the small size of the animals, mussel recovery was challenging. In beakers where fewer than 100% of mussels were recovered, a Rose Bengal stain in 70% ethanol was used to assist in locating remaining individuals. The primary endpoints for this trial were survival and growth. Survival was determined by visual assessment of gaping or empty shells and observation of foot movement within a 5-minute period. Surviving juveniles were counted and preserved in 70% ethanol for subsequent growth measurements. Animals that were dyed with Rose Bengal solution were counted as alive if the shell was closed and had tissue inside, and dead if the shell was empty and had no tissue inside. At the end of the trial, average shell length per replicate was

determined from microscope photographs, and average dry weight was measured after drying mussels for 24 hours at 60 ℃ followed by 24 hours in a desiccator at room temperature.

Statistical Analysis

Data were analyzed using R statistical programming language (R Core Team, 2024). Recovery was calculated as the number of animals recovered divided by the number of animals added at the start of the study (n = 10). Survival was calculated as the total number of animals that survived divided by the total number of animals that were recovered, multiplied by 100. The average percent change in length was calculated as the difference between the starting length and ending length, divided by the starting length, multiplied by 100. Direct measurement of initial dry weight was not possible as dry weights were obtained *post mortem*, so it was estimated using a predictive model based on a subsample of mussels (standards) collected at the beginning of the study.

To establish a relationship between length and dry weight, the initial length and dry weight of the standard mussels were measured, and a log-log linear regression model, using natural logarithms, was applied. The model:

$$\log(\text{Weight}) = \alpha + \beta \log(\text{Length})$$

where α represents the intercept or the expected weight (mg) when length (mm) is zero, and β the scaling exponent or rate at which mussel weight increases as length increases. The parameters for this equation were estimated using the measured values from the standard mussels. The model was used to predict the initial dry weight of mussels in the experiment based on their starting length, which was directly measured.

Data for mussel growth (i.e., average percent change in length or weight per replicate) and survival (i.e., average percent survival) were treated as separate response variables and analyzed independently. To test for differences among sites, we conducted one-way ANOVAs followed by Tukey's Honestly Significant Difference (HSD) post hoc tests. Model assumptions were evaluated using the Shapiro–Wilk test for normality of residuals and Levene's test for homogeneity of variances. Residual plots were also examined visually to identify any remaining patterns or violations of assumptions.

To evaluate the relationship between mussel growth and environmental predictors, we used a model selection approach based on multiple linear regression models. Candidate predictor variables were screened prior to inclusion in the global model. Given the relatively small sample size (i.e., 13 sites), we limited the number of predictors to reduce the risk of overfitting and maintain model stability. To reduce multicollinearity among predictor variables prior to model selection, I calculated Pearson correlation coefficients for all pairs of numeric variables. Pairs with a correlation coefficient of $|r| \geq 0.8$ were flagged as highly correlated. I then manually reviewed these highly correlated variables and grouped them into sets based on overlapping relationships. I selected a single representative variable from each group to be retained for model selection, prioritizing ecological relevance and support from literature (Appendix B). Variables were also excluded if they had low variability (standard deviation < 0.1) or a high proportion of values below detection limits (> 60%). All retained predictors were standardized (mean = 0, standard deviation = 1) to facilitate comparison of effect sizes.

To assess the potential toxicity of pesticides in sediment and water samples, we calculated toxic units (TUs) for each pesticide and site. Methods to calculate TU's generally follow those outlined in Schweiger et al. (2025). Pesticide concentrations in sediment (ng/g) and

decanted water (ng/L) were provided by the analytical lab. Any pesticides that were not detected at all 13 study sites were removed prior to analysis. We only used pesticides detected in both the sediment and water. For these shared pesticides, we retrieved 24 to 48-hour LC50 values specific to mollusks from the U.S. EPA ECOTOX database using the following filters: endpoint = LC50, taxa = mollusks, exposure media = freshwater, test type = laboratory, and duration = 1–2 days (U.S. EPA 2024a). For those detected in sediment, we estimated porewater concentrations using the organic carbon–water partitioning model:

$$Cpw = \frac{Csediment}{(Koc \times Foc)}$$

where *Cpw* is the estimated porewater concentration (mg/L), *Csediment* is the sediment concentration (ng/g), *Koc* is the organic carbon–water partition coefficient (L/kg) obtained from the EPA CompTox database and *Foc* is the fraction of organic carbon (TOC/100) for each site (U.S. EPA 2024b).

Toxic units were then calculated for each pesticide at each site by dividing the measured (or estimated) concentration by its corresponding LC50. TU values were summed across pesticides within each site to produce a cumulative toxicity metric (TU_{sum}) for both water and sediment. These unitless TU_{sum} values were used as predictor variables in subsequent statistical modeling to evaluate relationships with mussel growth and survival. Consistent with all other sediment predictor variables, TU_{sum} was standardized (mean = 0, standard deviation = 1) for use in models. There was a single site (Sumac 2) where the water concentrations of pesticides were not able to be measured. In an effort to still evaluate the effects of all other predictors on the growth observed at this site, we retained Sumac 2 in the dataset with an imputed value for the TU_{water} value.

We used the dredge function in R to systematically evaluate all combinations of the remaining environmental predictor variables (Bartoń 2024). We evaluated predictor variables in two separate analyses: landscape variables and sediment chemistry variables. We chose to separate landscape parameters from sediment chemistry parameters for analysis because they are often correlated, and because while landscape parameters are of interest, they do not evaluate potential toxicity mechanisms that could describe reduced growth. Reducing the number of variables also ensured that the dredge function could evaluate candidate models efficiently and appropriately, without exceeding the information available in the dataset. Candidate models were ranked by their Akaike Information Criterion corrected for small sample size (AICc) values, with lower AICc indicating better relative fit. The difference between each model's AICc and the minimum AICc (ΔAICc) was calculated. Models with $\Delta AICc \leq 2$ were considered well supported and were retained in the final set of top models for further interpretation.

Results

Sediment Collection and Analysis

We analyzed sediment from each site for 11 chemical analytes—Ca, K, Mg, Mn, P, Zn, and Cr (reported in mg/kg); C, N, and TOC (as percentages); and pH (Table 2.2)—as well as 213 common-use pesticides. The clean sand control was not analyzed chemically, so it was excluded from statistical models that used chemical predictors. However, it was retained for ANOVA comparisons to evaluate differences in mussel growth. Among the analytes, magnesium concentration was highest at Mill 3 (148.9 mg/kg) and lowest at Holly (17.9 mg/kg). Carbon concentrations were highest at Conasauga 2 (0.917%) and lowest at Sumac 1 (0.168%).

There was no clear relationship between pesticide concentrations in water and those estimated from sediment porewater (Figure 2.2). Most values clustered near zero, and concentrations did not show a consistent trend. This pattern was also reflected in toxic unit (TU) calculations: the correlation between summed TUs from water and those estimated from porewater was negative (Pearson's r = –0.44), indicating an inverse and weak relationship. For example, Mill 3 had the highest TU from estimated porewater (8.3×10^{-6}) but the lowest TU from measured water concentrations (8.13×10^{-6}).

We also evaluated the relationship between mussel growth and GIS-derived watershed characteristics, including percent land use and NPDES (National Pollutant Discharge Elimination System) permit density. On average, land use across sites consisted of 25.39% agricultural, 60.98% forested, and 13.02% developed land. Mills Creek had the highest proportion of agricultural land use (45.77%), Conasauga 3 was the most forested site (96.63%), and Town Branch had the highest percentage of developed land (36.60%). NPDES density was calculated as the number of permitted discharges divided by watershed area (permits/km^2) and ranged from 0 to 1.15, with a mean value of 0.18. Mill 3 had the highest NPDES density (1.15 permits/km^2), indicating the greatest potential for permitted point-source inputs.

Recovery, Growth, and Survival

There were no significant differences in initial mussel length among the sediment treatments from the 13 field sites and control sand (ANOVA, $F(13,41) = 0.64$, $p = 0.81$), suggesting that initial size was consistent across treatments and was unlikely to have influenced the observed differences in growth. We initially recovered 461 of the 560 mussels used in the study, which was an acceptable recovery rate per the ASTM guide (recovery = 82.32%). After

the use of the Rose-Bengal solution, we were able to recover 79 more mussels, increasing the final overall recovery rate to 96.42%. Survival across sediment treatments was uniformly high, with an average survival of 96.4 ± 9.4% (mean ± SD), and 82% of replicates exhibiting 100% survival. Given this limited variation, we did not perform statistical comparisons among sites and instead focused on growth end points for model selection. Juvenile mussels had consistent starting sizes across treatments, with an average initial length of 1.50 ± 0.10 mm and estimated weight of 0.276 ± 0.036 grams based on their starting lengths. By the end of the 28-day exposure, mussels reached an average length of 2.57 ± 0.34 mm and weight of 1.48 ± 0.64 grams.

ANOVA Results

We found a statistically significant difference in the average percent change in length between sediment treatments ($F(13,41) = 3.94$, $p = 0.0003$; df = site, residual; Figure 2.3). Mussels exposed to sediments from Holly, Sumac 2, Conasauga 2, and Conasauga 3 had significantly greater growth than those from Mill 3 ($p < 0.05$). These same four sites also supported significantly greater growth than the clean sand control. Assumptions of normality (Shapiro–Wilk test, $p = 0.675$) and homogeneity of variances (Levene's test, $p = 0.220$) were met, and no concerning patterns were observed in the residual plots. There were no significant differences in the average percent change in weight between sites ($F(13, 41) = 1.41$, $p = .20$; df = site, residual; Figure 2.4). Consistent with this result, no significant pairwise differences between sites occurred. Assumptions of normality (Shapiro–Wilk test, $p = 0.687$) and homogeneity of variances (Levene's test, $p = 0.237$) were met, and residual plots did not show any notable violations. The average percentage change in length for mussels at the 13 sites was also used as the response variable for model selection.

Variable Filtering

Sediment samples were analyzed for a total of 11 chemical variables and 214 common use pesticides. Prior to model selection, two pairs of highly correlated variables were identified. The first group included Mg and Ca (Pearson's r = 0.94); Mg was retained due to its previously reported negative association with mussel growth (Skorupa et al., 2024), where it was linked to differences in growth across sites. A second group included Mn and K (Pearson's r = 0.93); K was retained based on evidence that elevated potassium concentrations (>24 mg/L) can reduce growth in freshwater organisms (Kunz et al., 2021). In our study, potassium ranged from 3.86 to 31.25 mg/kg, exceeding both this threshold and the 15.97 mg/L concentration shown to negatively affect *C. nebulosus* in Gibson et al. (2018).

No sediment variables were excluded due to high proportion of values below detection limits, but nitrogen was excluded because of low variability among sites (standard deviation < 0.1). The GIS-derived predictors (e.g., NPDES density and percent land use) were retained despite some exhibiting pairwise correlations of $|r| \geq 0.8$. These variables had sufficient variability (standard deviation > 0.1) and a relatively low proportion of zeros (<60%) but were expected to be intercorrelated due to their compositional nature—they represent proportional land cover categories that together sum to 100%. Each provides distinct landscape context, so none were removed for collinearity. After filtering, the final retained predictors used for model selection were NPDES density, percent land use in the watershed (forest, agriculture, and development), Cr, pH, K, Mg, P, Zn, C, TOC, total TU_{water}, and total $TU_{porewater}$.

Top Multiple Regression Models

Three models were identified within the candidate set for landscape variables predicting the average percent change in length (ΔAICc $\leq$ 2; Table 2.3), although two were nested within the top-ranked model and offered no improvement in model fit. The best-supported model included only NPDES permit density as a predictor, and the final regression indicated a significant negative relationship between NPDES density and juvenile mussel growth (Figure 2.5). Specifically, higher NPDES density was associated with a reduced average percent change in length (p = 0.01; Table 2.4).

Ten models were identified within 2 ΔAICc units of the top-ranked model in the candidate set evaluating sediment chemistry variables and TUporewater. However, all models beyond the fourth were nested versions of simpler models and did not substantially improve model fit (Table 2.5). The top-ranked model (AICc = 103.50, weight = 0.15) included $TU_{porewater}$ and pH, both showing significant negative effects on mussel growth (Figure 2.7), with $\beta_{TU} = -3.35 \times 10^6$ (p = 0.02) and $\beta_{pH} = -13.49$ (p = 0.05) (FTable 2.6). The second-ranked model (ΔAICc = 0.03, weight = 0.14) included $TU_{porewater}$ ($\beta_{TU} = -4.14 \times 10^6$, p = 0.01) and carbon ($\beta_C$ = 27.30, p = 0.05), where $TU_{poreewater}$ is still negatively associate with growth while carbon is positively associated with growth (Figure 2.6). The third model (AICc = 103.83, ΔAICc = 0.33, weight = 0.12) showed growth positively related to carbon (β_C = 26.42, p = 0.03) and phosphorus (β_P = 0.97, p = 0.04) and negatively related to magnesium ($\beta_{Mg} = -0.27$, p = 0.001)(Figure 28). A fourth model within 2 ΔAICc units included carbon and magnesium as shared predictors but replaced phosphorus with potassium (β_K = 0.87, p = 0.04). Although this model explained a similar proportion of variance, it did not improve model fit and was interpreted as an alternative rather than superior explanation.

Collectively, these results highlight that increased $TU_{porewater}$ and magnesium consistently reduced mussel growth, while higher carbon, phosphorus, and potassium supported greater growth across top models.Together, these models suggest that pesticide toxicity in estimated porewater concentrations may help explain the differences in mussel growth. Together, these results suggest that Mg, C, and total $TU_{porewater}$ are predictors of mussel growth, while variables such as pH, K, and P also help explain some of the observed variation.

Discussion

The purpose of this study was to evaluate sediment and water chemistry conditions and effects of contaminants on the growth and survival of juvenile freshwater mussels during a 28-day sediment toxicity trial. We found that survival was uniformly high across sediment treatments (i.e., sediments from different sites), but that there was variation in growth among treatments. Sediment was chemically analyzed for 11 analytes and 213 pesticides. From this analysis we identified Mg, C, and estimated total $TU_{porewater}$ as strong predictors of growth, while NPDES density, pH, K, and P also explained some variation.

The high survival rates observed in this study are consistent with findings from other chronic sediment toxicity trials involving juvenile mussels (e.g., 82.6%; in Archambault et al., 2017). Unlike acute toxicity tests, which evaluate lethality over short timeframes and are often used to establish water quality criteria, chronic exposures are designed to assess sublethal endpoints such as growth and reproduction. Although acute tests are valuable for regulatory purposes, mussels in natural systems are perhaps unlikely to encounter the high contaminant concentrations used in such tests. Instead, they are more likely to experience prolonged, low-level exposure to complex mixtures of contaminants. These chronic exposures may not

immediately result in mortality but can cause cumulative sublethal effects that eventually lead to population-level declines (Strayer et al., 2004). Among sublethal endpoints, growth is particularly sensitive and informative, often serving as an early warning indicator of environmental stress. Monitoring growth can therefore help identify site-specific stressors and provide information where mussel declines occur.

There were no significant differences in the average percent change in weight among sites, likely due to the wide 95% confidence intervals calculated around each site's mean. While we directly measured the final dry weight of each replicate at the end of the trial, initial weights were estimated using length-based projections derived from a length–weight relationship established from individuals sacrificed at the start of the experiment. Due to this added uncertainty, we focused on the average percent change in length as the primary growth endpoint.

We found significant differences in length-based growth among sites, with mussels from Mill 3 and the clean sand control exhibiting significantly lower growth than those at four other sites (Figure 2.3). The reduced growth observed in the clean sand control suggests that mussels likely derive some nutritional benefit from components present in the natural sediments (White et al., 2022). This result also indicates that a clean, uncontaminated reference sediment collected from the field may serve as a more appropriate reference condition control than sand.

The lowest mussel growth was observed in sediments collected from site Mill 3, in Dalton, GA. The low growth at this site may also be driving the negative relationships of growth with Mg, $TU_{porewater}$ and NPDES density, because this site had the highest levels of these contaminants and NPDES density (Figures 2.5, 2.8, and 2.7). Because of this collinearity it is difficult to identify whether a single parameter is causative of reduced growth, or if additive

stressors may be a more reasonable cause. Despite this uncertainty prior evidence exists of the effects of some of these stressors on unionids.

Mill 3 had the highest concentration of magnesium detected in the sediment (148.9 mg/kg). Although toxicity research on magnesium in freshwater mussels is limited, the range observed in this study (17.9–148.9 mg/kg) overlaps with concentrations shown to reduce growth in other studies. For instance, Kleinhez et al. (2019) reported growth rate reductions in 10% of test organisms at 88 mg/L in 14-day chronic toxicity tests with juvenile mussels. Similarly, Skorupa et al. (2024) found a negative correlation between magnesium concentrations and juvenile mussel growth in an *in situ* exposure study. While Skorupa et al. (2024) attributed elevated major ion concentrations to deicing salts in the Northeast, such sources are unlikely in the present study. Instead, while underlying limestone may contribute some magnesium, we found a moderate positive correlation ($r = 0.52$) between sediment magnesium concentrations and the percentage of developed land in the watershed. Mill 3, located in Dalton, GA, had the highest measured magnesium concentration. This suggests that magnesium inputs may also stem from anthropogenic sources such as concrete, industrial and municipal wastewater, or fertilizers (Mueller et al., 2020). Future studies could use stable isotope analysis of magnesium (e.g., $\delta^{26}Mg$) following methods outlined in Nitzsche et al. (2019) to distinguish between geologic and anthropogenic sources.

We observed a positive relationship between mussel growth and the concentrations of carbon, phosphorus, and potassium in sediment. Similar associations have been reported in *in situ* studies, where nutrients such as total organic carbon (TOC), phosphorus (P), and potassium (K) in the water column were positively correlated with mussel growth (Haag et al., 2019). While Haag et al. (2019) measured nutrient concentrations in water rather than sediment,

nutrient-rich environments, particularly those with elevated carbon, may provide greater food availability or improved conditions for mussel growth.

Although we observed a positive effect of sediment potassium on mussel growth, previous studies have shown that elevated potassium in water can be harmful to freshwater mussels. Kunz et al. (2021) reported reduced juvenile mussel growth at water concentrations of 24 mg/L in exposures to effluent, while Gibson et al. (2018) observed adverse effects on *C. nebulosus* at concentrations as low as 15.96 mg/L. It's important to note that these were acute water-only exposures (Gibson et al., 2018) or water-and-sand exposures (Kunz et al., 2021) and cannot be directly compared to sediment concentrations from our study, which ranged from 3.86 to 31.25 mg/kg. To make such comparisons valid, porewater concentrations would need to be estimated or measured directly.

We found that there was no relationship between measured water and estimated porewater concentrations of pesticides. This suggests that waterborne and sediment-associated pesticide exposures may vary independently across sites, likely reflecting differences in compound-specific properties such as solubility, persistence, or sediment affinity (Fairbairn et al., 2015). Porewater concentrations and corresponding toxic units (TUs) were initially calculated to estimate exposure from sediment-bound pesticides, as juvenile mussels in this trial were buried in the sediment and likely experienced porewater exposure as a dominant pathway. While water concentrations were measured directly, porewater concentrations were estimated using sediment concentrations, organic carbon content, and published Koc values. Thus, although porewater TUs may offer a more biologically relevant exposure estimate in this experimental setup, they are model derived rather than empirically measured and should be interpreted with appropriate caution.

The total $TU_{porewater}$ had a significant negative effect on mussel growth. In general, we observed very low concentrations of pesticides at any given site, compared with concentrations found to be lethal or even have sublethal effects. For example, Bringolf et al. (2007) found that the median effective concentration (EC50) of atrazine was 4.3 mg/L in chronic water exposure with juvenile mussels. The highest estimated porewater concentration of pesticides we observed was orders of magnitude smaller, with Pyrazophos at 2.59 x 10-5 mg/L at Mill 3. However, our analysis aimed at understanding if there was an additive effect of all pesticides on mussel growth. Similar studies have shown that the cumulative effect of pesticides can affect sensitive aquatic insect diversity and richness (Schweiger et al., 2025). It is evident that the majority of the upper Conasauga watershed is impacted by agriculture and there is evidence of chronic exposure to low concentrations of current-use pesticides (Lasier et al., 2017; Sharpe and Nichols 2007). Interestingly, our highest $TU_{porewater}$ concentration was at a site with higher developed landscape than agricultural land use (Mill 3; Developed = 32.36%, Agriculture = 8.15%). Future work could further investigate the potential impacts of the observed sediment pesticides by expanding the range of taxa used to determine LC50 values and by including all detected pesticides in TU calculations, rather than limiting them to those found in both water and sediment, given the lack of correlation between them.

This study adds to the growing body of research on the role of contaminants in freshwater mussel declines. We found that certain contaminants, including magnesium and pesticides, were associated with reduced growth, and these effects were linked to broader landscape-level variables. In contrast, nutrients such as carbon, potassium, and phosphorus had a positive effect on juvenile mussel growth, potentially reflecting the dietary contribution of sediment through pedal feeding. Future research could aim to identify the specific chronic sediment concentrations

of magnesium that are lethal to juvenile mussels. Additionally, the existing dataset could be used to explore the additive or synergistic effects of multiple pesticide exposures on mussel growth and survival.

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Tables

Table 2.1. Summary of watershed characteristics for each study site. Assessment refers to the EPA 303(d) impairment status. Area is the watershed area in square kilometers (km^2). NPDES Density is the number of National Pollutant Discharge Elimination System permits per square kilometer. PerAgri, PerFor, and PerDev indicate the percentage of the watershed classified as agricultural, forested, and developed land use, respectively.

Stream Name	EPA 303(d) Assessment	Area (km^2)	NPDES Density	PerAgri	PerFor	PerDev
Mill 3	NotSupporting	114.65	1.15	8.15	58.31	32.36
Town Branch	Supporting	23.05	0.17	35.59	26.42	36.60
Mill 1	NotSupporting	86.07	0.48	23.08	58.15	18.29
Holly Creek	NotAssessed	95.95	0.00	4.03	91.69	4.14
Conasauga 1	NotSupporting	47.97	0.00	39.78	50.33	8.40
Spring	Supporting	34.78	0.35	34.38	53.82	11.00
Sumac 2	NotSupporting	61.37	0.08	8.90	85.27	5.51
Sumac 1	NotSupporting	24.49	0.08	33.76	51.70	14.26
Conasauga 2	NotSupporting	413.07	0.00	14.61	81.22	3.83
Coahulla	Supporting	138.99	0.06	39.25	44.94	15.26
Mills	Supporting	18.02	0.00	45.77	46.36	7.69
Conasauga 3	Supporting	279.21	0.01	1.42	96.63	1.85
Mill 2	NotSupporting	60.87	0.00	41.31	47.91	10.06

Table 2.2. Sediment chemistry results from 13 study sites in the Conasauga River watershed. Values represent pH as well as concentrations of contaminants (chromium, calcium, potassium, magnesium, manganese, phosphorus, and zinc, in mg/kg) and nutrients (carbon, nitrogen, and total organic carbon, in %) for each site.

		mg/kg							%		
Stream Name	pH	Cr	Ca	K	Mg	Mn	P	Zn	C	N	TOC
Mill 3	7.4	8.53	1225.05	8.88	148.9	45.4	8.6	4.73	0.33	0.03	0.22
Town Branch	7.0	24.36	202.65	4.83	49.42	42.67	4.29	2.66	0.26	0.03	0.2
Mill 1	7.21	6.38	160.48	6.03	38.29	30.84	5.29	1.95	0.17	0.04	0.16
Holly	5.71	10.61	115.91	6.35	17.93	10.68	5.14	1.35	0.34	0.05	0.22
Conasauga 1	6.82	8.49	162.56	5.44	28.35	24.72	6.19	1.46	0.2	0.03	0.19
Spring	6.76	11.21	340.66	10.35	75.84	25.77	5.89	1.04	0.6	0.03	0.24
Sumac 2	6.3	3.21	264.34	11.32	45.58	36.49	20.94	4.39	0.48	0.05	0.46
Sumac 1	6.91	9.83	148.3	3.86	32.61	14.83	5.85	1.27	0.17	0.03	0.13
Conasauga 2	6.67	5.56	161.93	5.75	24.79	19.35	5.3	1.32	0.92	0.07	0.55
Coahulla	6.87	24.83	961.53	31.25	95.66	114.44	21.14	3.74	0.53	0.04	0.46
Mills	7.28	76.39	639.52	13.27	81.85	60.72	13.23	3.65	0.32	0.04	0.23
Conasauga	6.69	4.11	236.79	5.28	22.42	15.53	11.63	3.12	0.3	0.04	1.17
Mill 2	7.33	24.18	548.75	15.29	84.89	66.81	8.54	1.11	0.21	0.03	0.18

Table 2.3. Output from the top 10 models ranked by AICc using the dredge function for landscape variables hypothesized to influence growth of *Cambarunio nebulosus* in sediments collected from 13 study sites in the Conasauga River in 2024. Each row represents a candidate model predicting mussel growth (average percent change and length), with the intercept representing the overall average percent change in length for the toxicity trial excluding the growth from the clean control sand. Predictors shown as standardized coefficient estimates and excluded predictors denoted as "-". Columns include the number of parameters (df), log-likelihood (logLik), AICc score, ΔAICc (difference from the top model), and Akaike weight (weight), indicating relative model support.

Model	cond((Int))	NPDES	PerAgri	PerDev	PerFor	df	logLik	AICc	delta	weight
2	73.883	-9.278	-	-	-	3	-48.346	105.358	0	0.3245
4	73.883	-10.751	-5.701	-	-	4	-46.198	105.395	0.037	0.3185
10	73.883	-8.558	-	-	4.853	4	-46.783	106.566	1.208	0.1774
5	73.883	-	-	-7.471	-	3	-49.954	108.575	3.217	0.0649
6	73.883	-7.7	-	-2.392	-	4	-48.148	109.295	3.937	0.0453
1	73.883	-	-	-	-	2	-52.162	109.523	4.166	0.0404
9	73.883	-	-	-	6.122	3	-50.765	110.197	4.839	0.0288
12	73.883	-11.995	-8.713	-	-3.135	5	-46.101	110.773	5.415	0.0196
8	73.883	-11.934	-6.294	1.56	-	5	-46.109	110.79	5.432	0.0195
14	73.883	-11.89	-	5.804	8.202	5	-46.133	110.838	5.48	0.0190

Table 2.4. Model estimates and significance values from the top candidate models identified by the dredge function for the landscape variables. AICc values are provided for model comparison. Estimates and associated p-values are shown for the predictor retained in the model.

		NPDES_Density	
Model	AICc	estimate	*p*
2	105.358	-28.374	0.0129

Table 2.5. Top 10 models ranked by AICc from the dredge analysis of sediment chemistry, TU_{water}, and $TU_{porewater}$, hypothesized to influence mussel growth in sediments from 13 Conasauga River sites in 2024. Columns for TU_{water}, chromium, and zinc are omitted as they did not appear in these models. Each row shows a candidate model predicting average percent change in length, with the intercept reflecting the overall mean growth excluding the clean sand control. Standardized coefficients are listed, with "–" indicating excluded predictors. Additional columns report the number of parameters (df), log-likelihood (logLik), AICc, ΔAICc, and Akaike weight (weight).

Model	Intercept	C	K	Mg	P	pH	TUpw	TOC	df	logLik	AICc	delta	weight	Model
97	73.88	-	-	-	-	-6.21	-7.42	-	4	-45.25	103.50	0.00	0.15	97
66	73.88	5.80	-	-	-	-	-9.17	-	4	-45.27	103.53	0.03	0.14	66
26	73.88	5.62	-	-10.44	5.67	-	-	-	5	-42.63	103.83	0.33	0.12	26
14	73.88	5.23	6.40	-12.18	-	-	-	-	5	-42.75	104.08	0.58	0.11	14
13	73.88	-	7.98	-12.99	-	-	-	-	4	-45.65	104.30	0.80	0.10	13
65	73.88	-	-	-	-	-	-9.66	-	3	-47.89	104.45	0.95	0.09	65
10	73.88	6.63	-	-8.80	-	-	-	-	4	-45.83	104.66	1.16	0.08	10
98	73.88	4.54	-	-	-	-4.87	-7.52	-	5	-43.09	104.76	1.26	0.08	98
25	73.88	-	-	-10.72	6.76	-	-	-	4	-46.00	105.01	1.51	0.07	25
321	73.88	-	-	-	-	-	-8.80	4.82	4	-46.25	105.49	1.99	0.05	321

Table 2.6. Model estimates and significance values from the top two candidate models identified by the dredge function. AICc values are provided for model comparison. Estimates and associated p-values are shown for each predictor retained in the models: Carbon, Magnesium, Phosphorus, and Potassium. "-" indicates that the variable was not included in that model.

		TUpw		pH		Carbon		Magnesium		Phosphorus		Potassium	
Model	AICc	estimate	*p*	estimate	***p***	estimate	***p***	estimate	***p***	estimate	***p***	estimate	***p***
97	103.50	-3.35E+06	0.02	–13.49	0.05	-	-	-	-	-	-	-	-
66	103.53	-4.14E+06	0.01	-	-	27.30	0.05	-	-	-	-	-	-
26	103.83	-	-	-	-	26.42	0.03	-0.27	0.001	0.97	0.04	-	-
14	104.08	-	-	-	-	24.62	0.05	-0.31	0.001	-	-	0.87	0.04

Figures

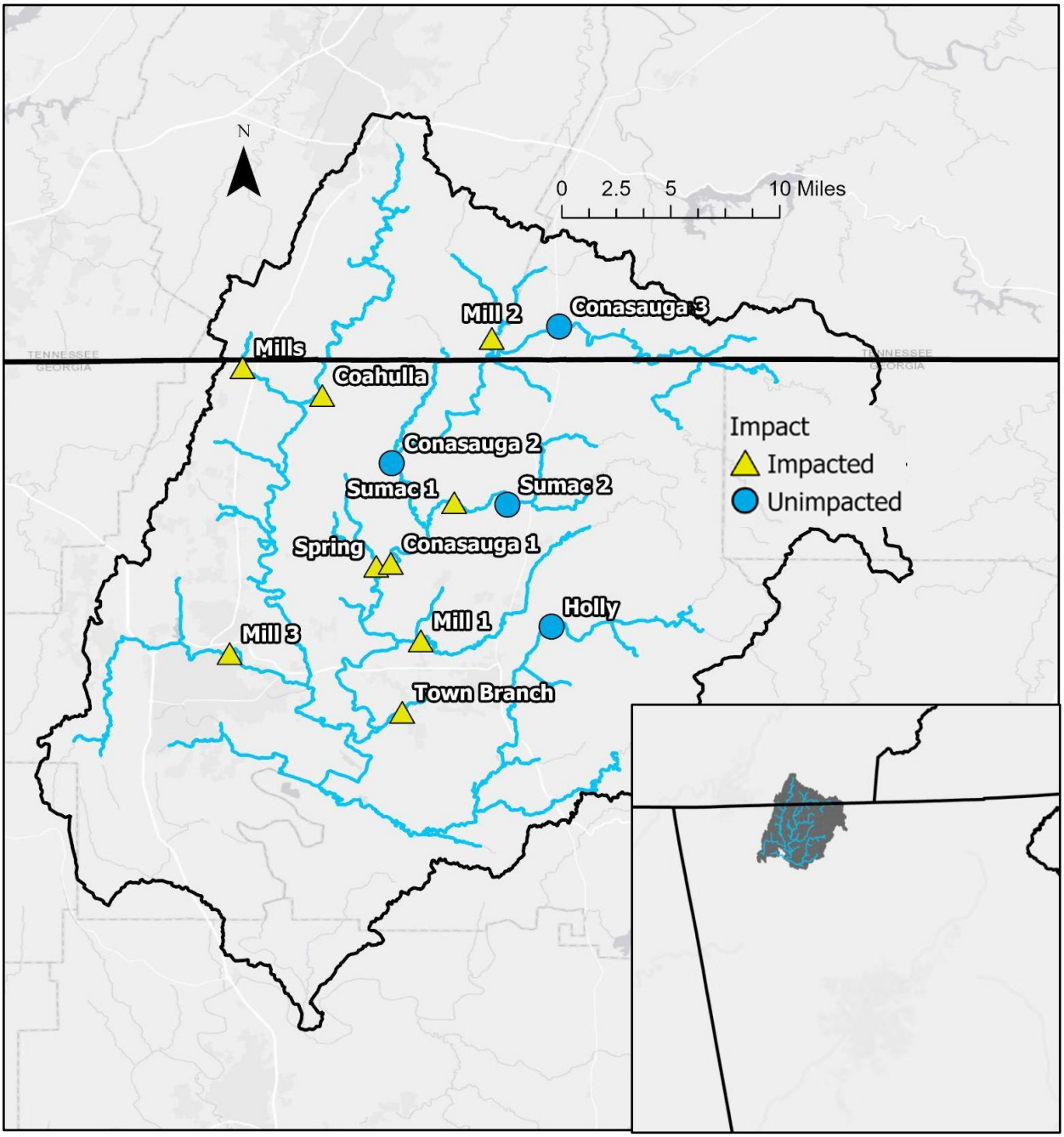

Figure 2.1. Thirteen randomly selected study sites and study area for the 2024 sediment toxicity trial. Impacted sites have less than 75% forested land cover present in the watershed. Unimpacted sites have greater than 75% forested land cover present in the watershed.

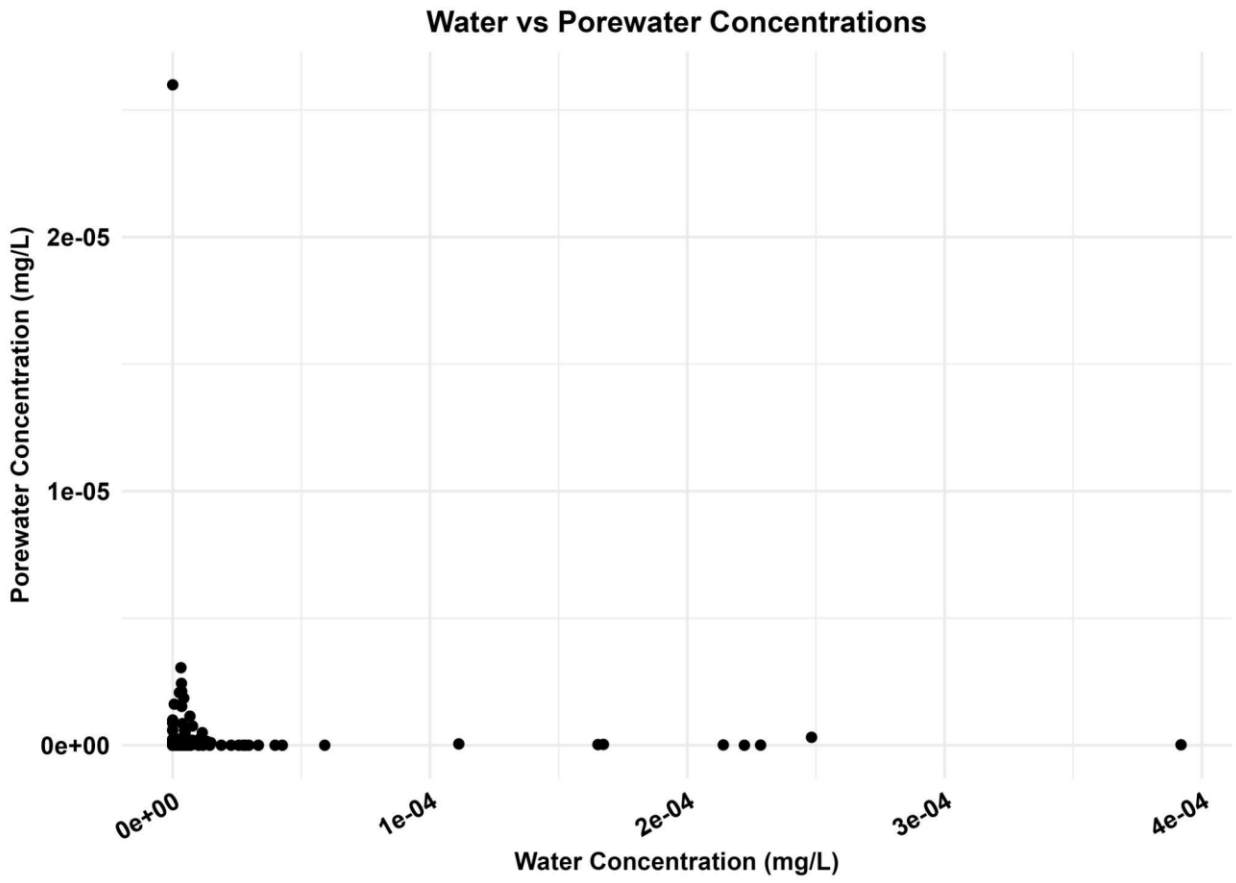

Figure 2.2. Comparison of pesticide concentrations measured in water (mg/L) and predicted porewater concentration (mg/L) across all sites. No clear relationship was observed between the two matrices, with most values clustered near zero and no consistent trend. This visual pattern aligns with a weak negative correlation between summed toxic units (TUs) from each medium (Pearson's r = –0.44).

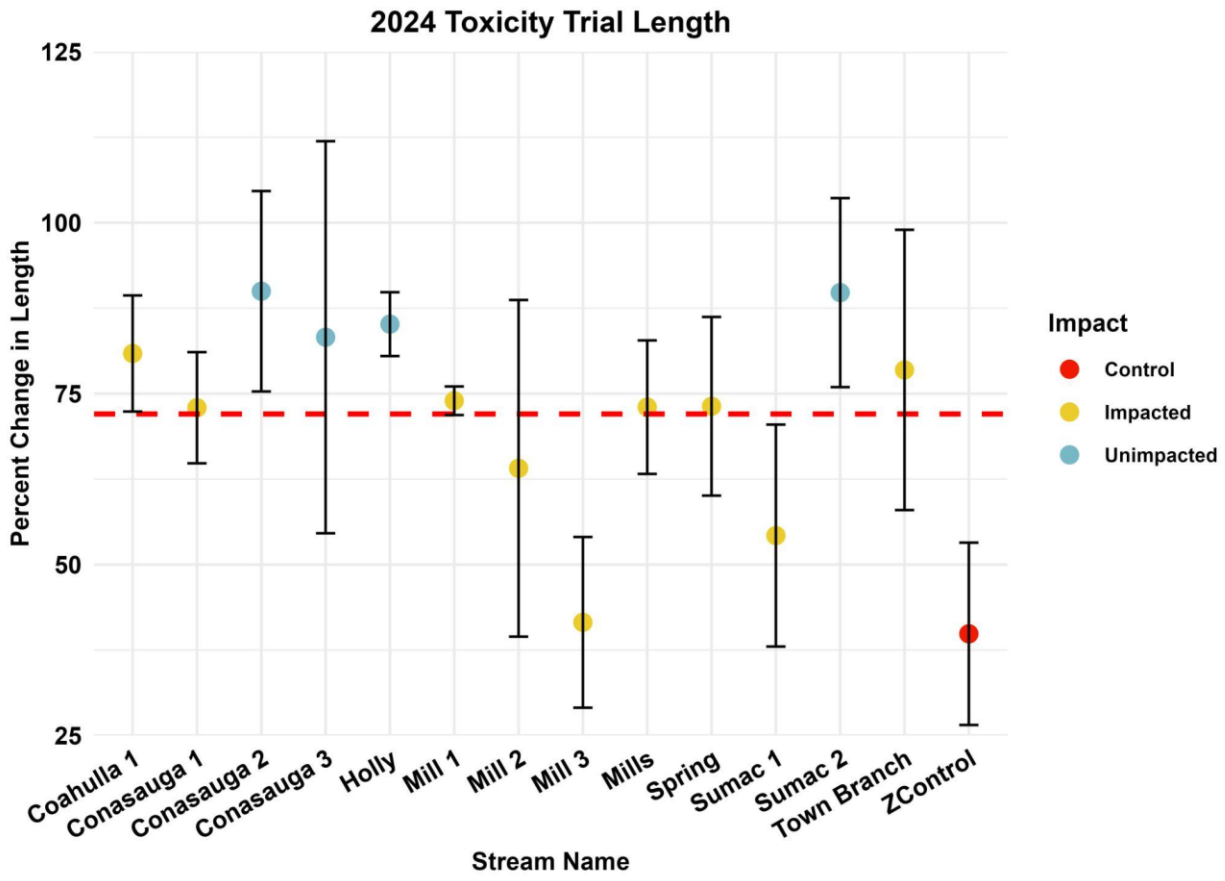

Figure 2.3. Average percentage change in mussel length for 13 study sites and control sand in experiments conducted during 2024. Red dashed line shows the overall mean (72%). Yellow bars (Impacted) have less than 75% forested land cover present in the watershed. Blue bars (Unimpacted) sites have greater than 75% forested land cover present in the watershed. The red bar is the control sand. Error bars show 95% confidence intervals for each site.

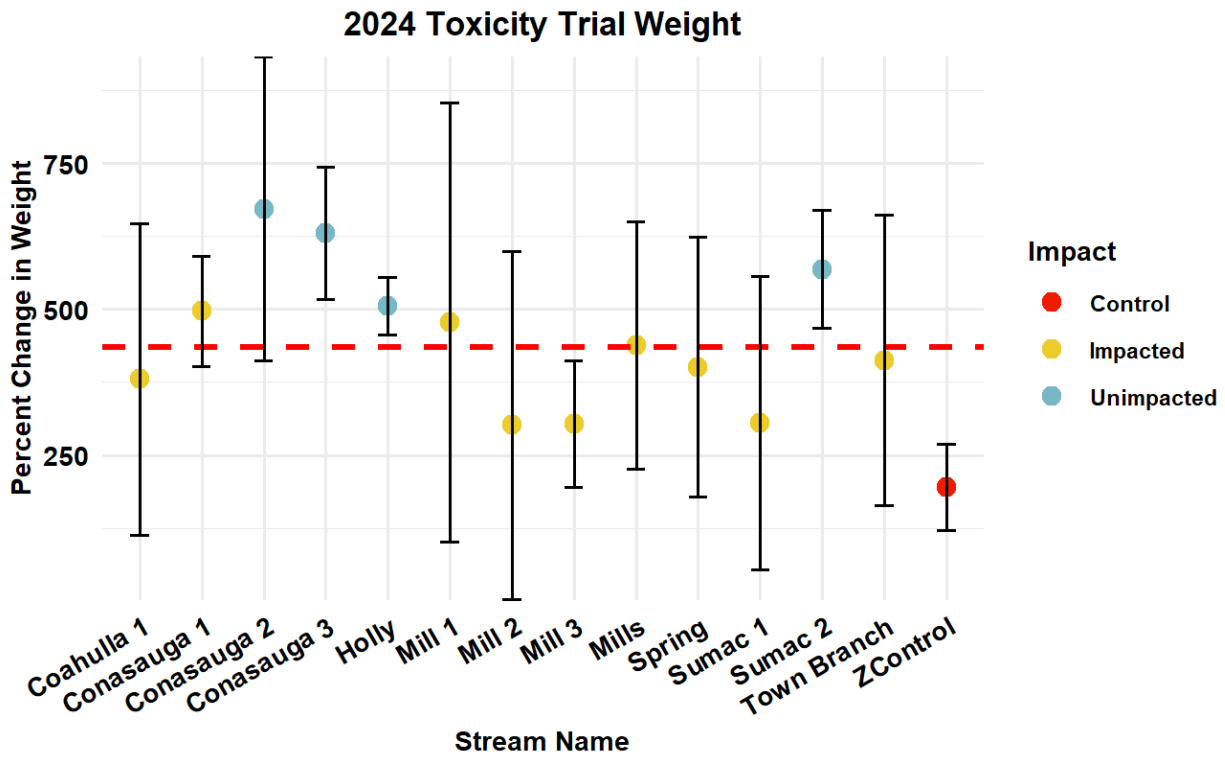

Figure 2.4. Average percentage change in mussel weight for 13 study sites and control sand in experiments conducted during 2024. Red dashed line shows the overall mean (435%). Yellow bars (Impacted) have less than 75% forested land cover present in the watershed. Blue bars (Unimpacted) sites have greater than 75% forested land cover present in the watershed. The red bar is the control sand. Error bars show 95% confidence intervals for each site.

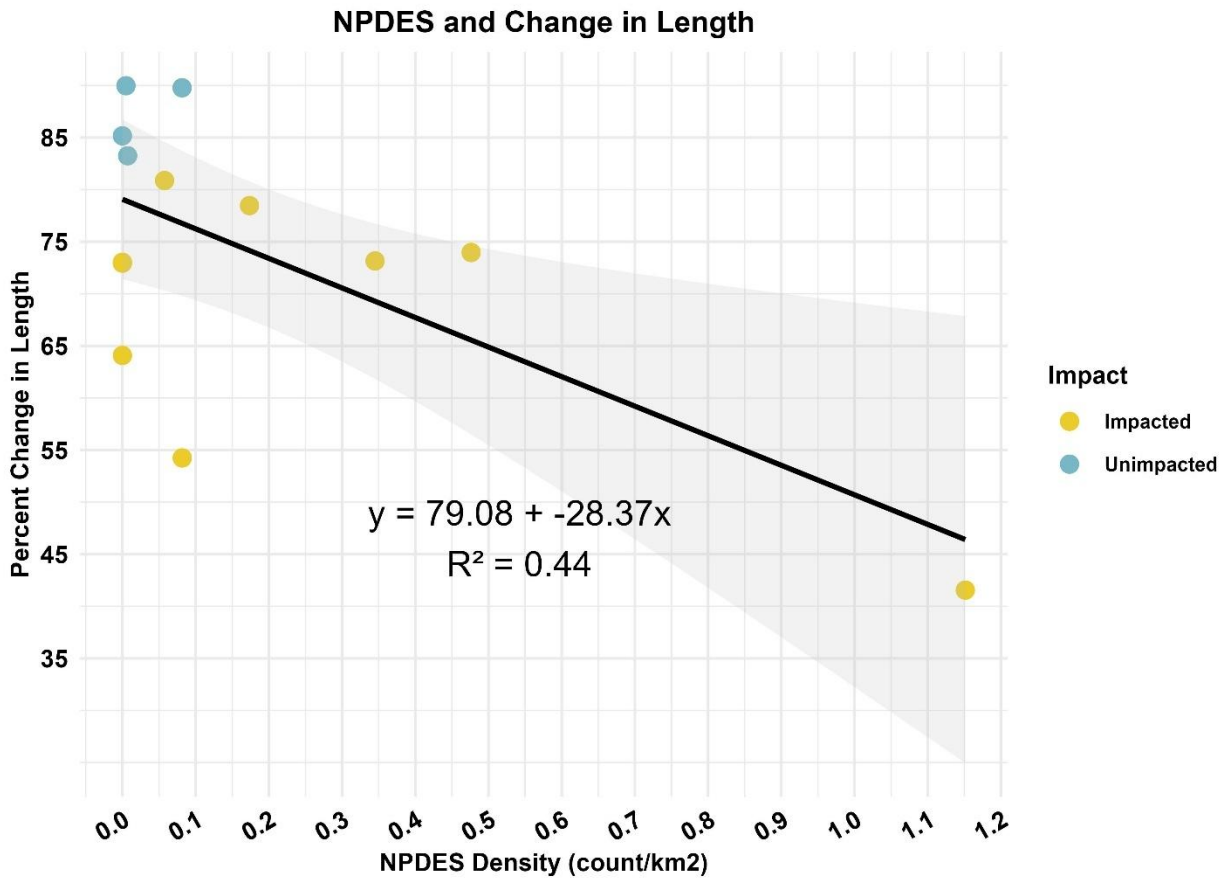

Figure 2.5. Relationship between NPDES permit density (count/km²) and average percent change in length of juvenile mussels from the 2024 sediment toxicity trial. Each point represents a study site, with blue points indicating watersheds with ≤75% forest land cover and yellow points representing watersheds with >75% forest cover. The solid line shows the fitted linear regression with 95% confidence intervals (shaded area), indicating a negative association between NPDES density and mussel growth ($y = 79.08 – 28.37x$, $R^2 = 0.44$).

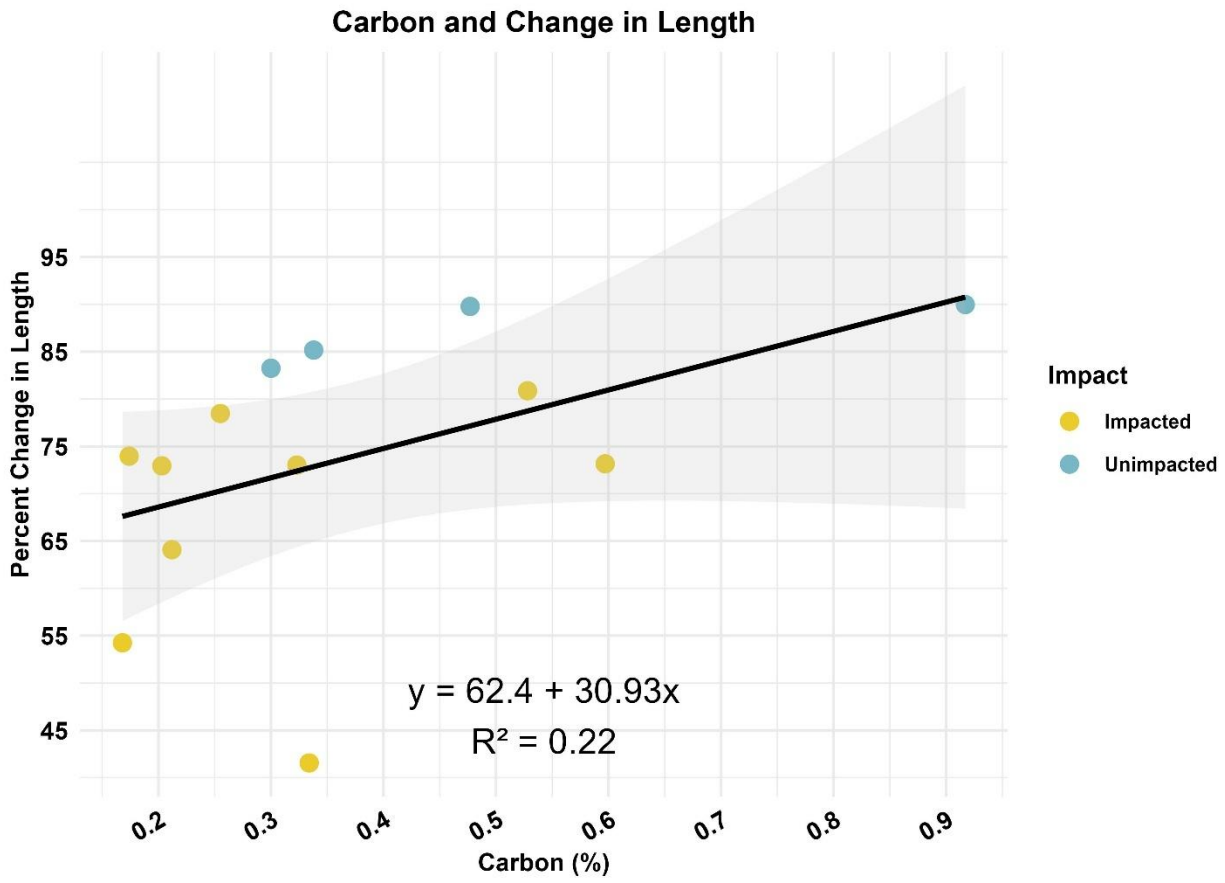

Figure 2.6. Relationship between sediment carbon (%) and average percent change in length of juvenile mussels from the 2024 sediment toxicity trial. Each point represents a study site, with blue points indicating watersheds with ≤75% forest land cover and yellow points representing watersheds with >75% forest cover. The solid line shows the fitted linear regression with 95% confidence intervals (shaded area), indicating a positive association between carbon and mussel growth ($y = 62.4 + 30.93x$, $R^2 = 0.22$).

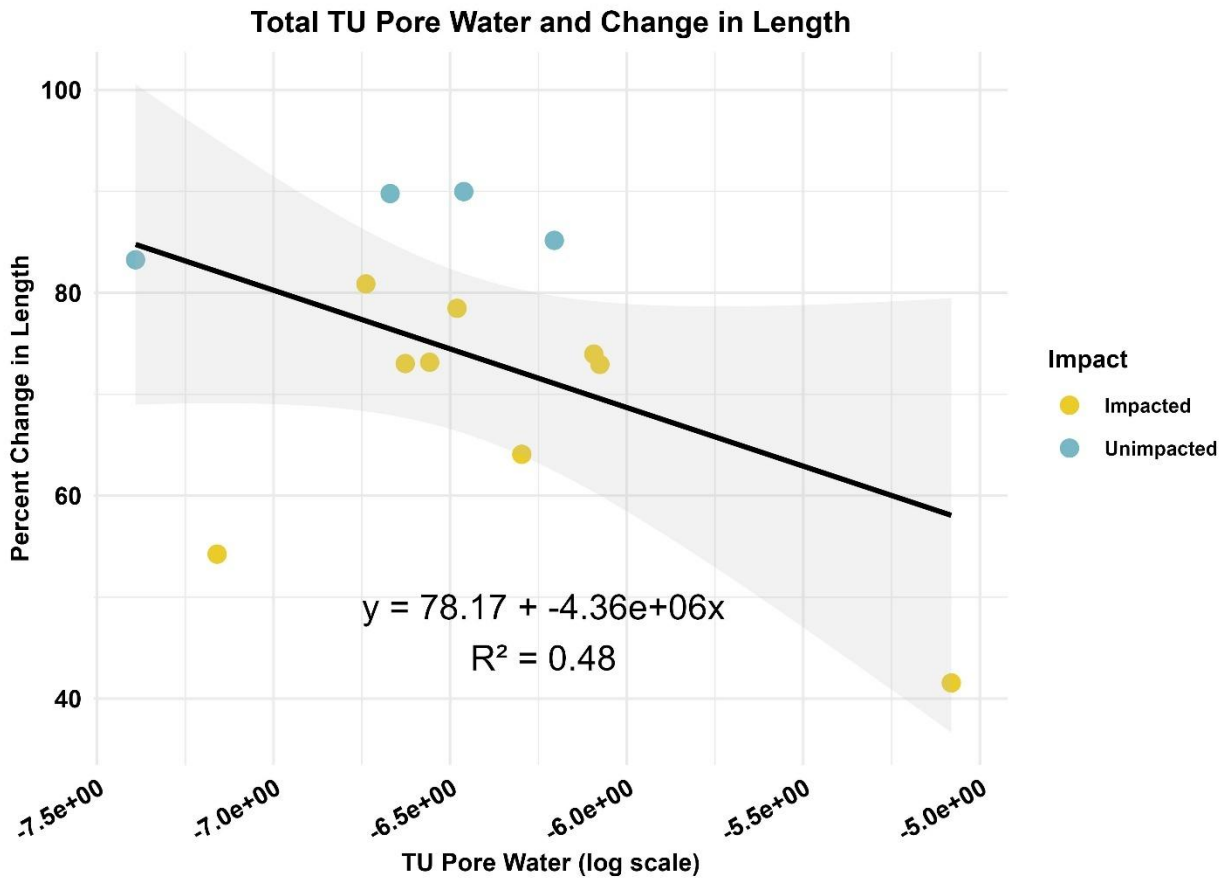

Figure 2.7. Relationship between total TU pore water (toxic unit sum calculated from estimated porewater concentrations; log scale) and average percent change in length of juvenile mussels from the 2024 sediment toxicity trial. Each point represents a study site, with blue points indicating watersheds with ≤75% forest land cover and yellow points representing watersheds with >75% forest cover. The solid line shows the fitted linear regression with 95% confidence intervals (shaded area), indicating a negative association between TU pore water and mussel growth ($y = 78.17 – 4.36 \times 10^6 x$, $R^2 = 0.48$).

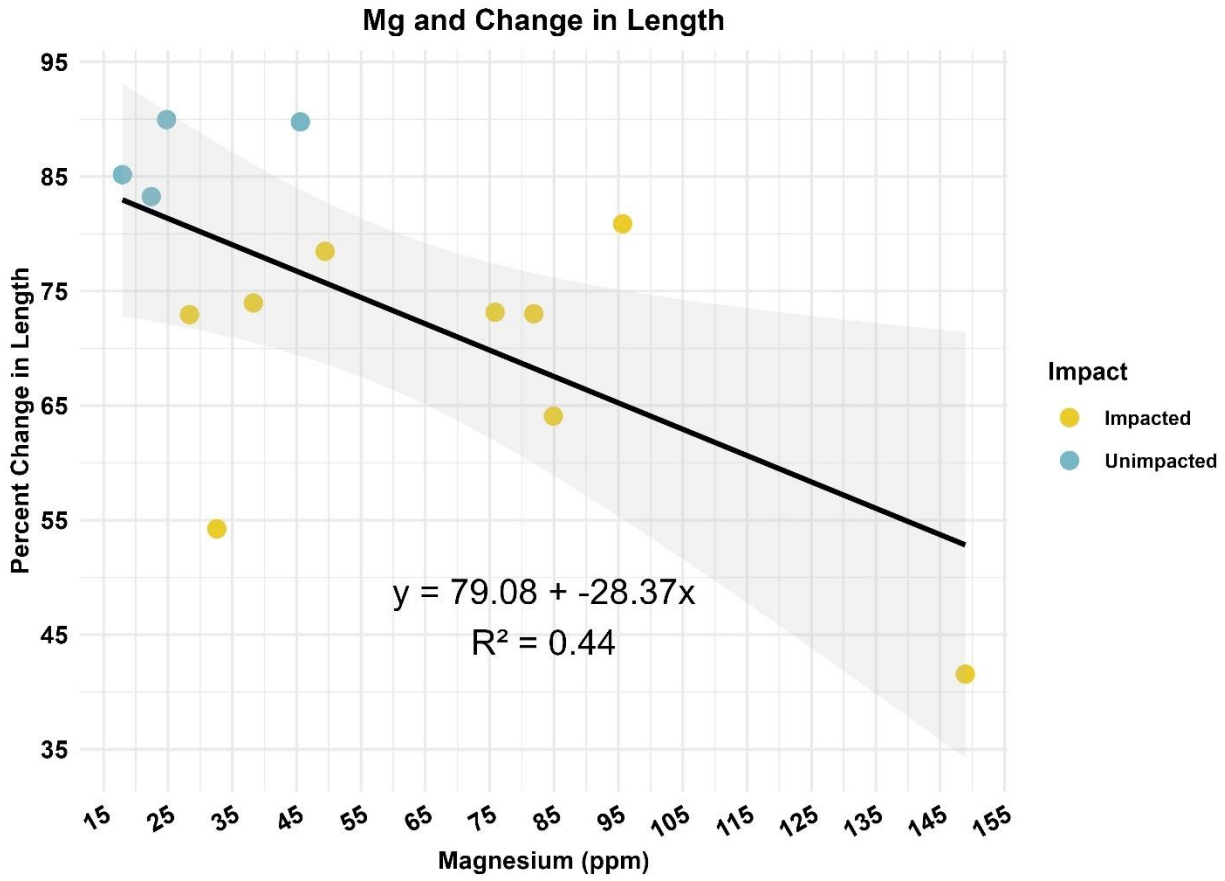

Figure 2.8. Relationship between sediment magnesium concentration (ppm) and average percent change in length of juvenile mussels from the 2024 sediment toxicity trial. Each point represents a study site, with blue points indicating watersheds with ≤75% forest land cover and yellow points representing watersheds with >75% forest cover. The solid line shows the fitted linear regression with 95% confidence intervals (shaded area), indicating a negative association between magnesium and mussel growth ($y = 79.08 – 28.37x$, $R^2 = 0.44$).

CHAPTER 3

CONTAMINANT, NUTRIENT, AND TEMPERATURE EFFECTS ON GROWTH OF JUVENILE MUSSELS IN THE CONASAUGA RIVER, GEORGIA[2]

[2] Martin, M.L., A.P. Escobar, K.F. Robinson, J.E. Kirsch, R.B. Bringolf, W.M. Henderson, S.T. Glassmeyer, M.J. Zapata, B.J. Irwin, P.D. Hazelton. To be submitted to a peer-reviewed journal (Freshwater Biology).

Abstract

Freshwater mussels are a highly imperiled group of unionid bivalves, sensitive to environmental changes driven by habitat degradation, pollution, invasive species, and dam construction. Although these disturbances are often correlated with mussel declines, studies identifying the direct causes remain limited. To address this, we conducted an *in situ* exposure study with juvenile mussels in the Conasauga River, Georgia. In summer 2024, mussels were deployed at 13 study sites spanning a gradient of municipal and agricultural land use. From May to September, we measured growth and survival in relation to waterborne contaminants, food availability, and physicochemical conditions (e.g., temperature). Water samples were collected at each site for chemical analysis and water quality assessment. Multiple linear regression models revealed significant effects of both chlorophyll-*a* and temperature on mussel growth. Chlorophyll-*a* had a nonlinear relationship with growth, showing a positive effect up to 3.97 µg/L, beyond which higher concentrations were associated with reduced growth. No significant relationships were found between waterborne contaminants and mussel growth. These findings highlight the complexity of nutrient dynamics in freshwater systems and suggest that food-related variables are influential on mussel growth compared to contaminant effects, for the scale and exposures achieved in this study. By linking these patterns to watershed land-use, this study improves our understanding of the environmental conditions shaping mussel populations and informs future conservation strategies.

Introduction

Freshwater mussels, or unionids, are a diverse and imperiled taxon belonging to the phylum Mollusca. North America is home to approximately 300 of the 950 total species of

unionids found worldwide (Graf & Cummings, 2021). The distribution of freshwater mussels, fishes, and crayfish in the U.S. is concentrated in the Southeast, specifically Alabama, Tennessee, and Georgia (Elkins et al., 2019). Since the 1960's, there have been marked declines in freshwater mussel populations. These declines have been linked to habitat degradation and fragmentation due to channelization and dams, loss of host fish, pollution, and invasive species (Downing et al., 2010; Dudgeon, 2019; Haag, 2019). However, there are systems that have nearly total faunal loss with no known causes (Cope et al., 2021; Haag et al., 2019).

The Conasauga River is a 93-miles long, un-impounded river in the headwaters of the Coosa River basin and is prioritized for the protection of globally rare native fishes and freshwater mussels (Albanese et al., 2015). While the upper reaches of the watershed are protected by the Chattahoochee and Cherokee National Forests, the lower reaches are impacted by agriculture and development. The Conasauga river is a quintessential example of the rich aquatic biodiversity found in the southeast; however, several species are considered imperiled (Elkins et al., 2019). There are approximately 28 species of freshwater mussels found here, 11 of which are state or federally threatened or endangered (Georgia Department of Natural Resources, 2024). Previous studies in the area have found that land-use practices could be contributing to harmful contaminants found in the watershed with influences from agriculture like poultry production and row crops, forestry, urban development, and industrial effluent (Freeman et al., 2007; Lasier et al., 2016).

Freshwater mussels have been shown to be highly sensitive to contaminants like heavy metals (copper, nickel, zinc) (March et al., 2007; Wang, Kunz, et al., 2020), major ions (potassium, magnesium, and sodium) (Kunz et al., 2021; Wang, Dorman, et al., 2020; Wang et al., 2017), excessive nutrients (nitrate/nitrite) (Wang, Dorman, et al., 2020), pesticides

(glyphosate) (Bringolf et al., 2007), and ammonia (Wang et al., 2008). While extensive research has documented freshwater mussel sensitivity to contaminants through laboratory toxicity testing, *in situ* exposures further validate these findings by demonstrating consistent patterns in their sensitivity under natural conditions (Archambault et al., 2017; Cope et al., 2021; Haag et al., 2019; Skorupa et al., 2024). Laboratory studies are useful in setting water quality criteria for the protection of species, but they lack both complexity and environmental realism of exposures that mussels face in the wild.

There are multiple routes of contaminant exposure to freshwater mussels, and these routes vary by life stage (Cope et al., 2008). The present study utilizes juvenile mussels which spend the first 0-4 years of life buried in the sediment thus facing exposure to contaminants through the sediment, pore water, and diet (Cope et al., 2008). In natural settings, mussels can be exposed to contaminant mixtures, compared to single contaminant testing in the lab. In addition to this they can experience added stressors of unsuitable temperatures or inadequate food that may make the contaminant effects worse. (Cope et al., 2021; Haag et al., 2019; Skorupa et al., 2024). Finally, mussels are long lived and nearly sedentary, leading to chronic exposures and bioaccumulation of contaminants (Brown et al., 2005; Haag & Rypel, 2011).

In addition to contaminants, stream conditions like temperature, water chemistry, and food abundance all impact growth. Denic et al. (2015) found that growth rates of juvenile mussels increased with increasing stream temperatures and organic carbon concentrations. Similarly, Haag et al. (2019) showed that reduced mussel growth was related to cool, unproductive streams, with low Total Organic Carbon (TOC). Cool water temperatures like this can exacerbate the effects of contaminants. Other studies have exhibited a synergistic effect of incompatible stream conditions (e.g., low water temperatures combined with low chlorophyll-*a*

concentrations) and major ion concentrations (e.g., magnesium) on growth of juvenile mussels (Skorupa et al., 2024). These studies are useful at capturing the complexity of stream conditions, the nuance of specific mussel populations, and allow us to see in real time how effects on growth could ultimately lead to population declines.

To assess habitat suitability of various sites within the Conasauga River, we evaluated how observed stream/watershed conditions and contaminant concentrations impacted the Alabama Rainbow mussel – a state species of concern (*Cambarunio nebulosus* [Conrad, 1834]). To do this, we incorporated *in situ* deployment of juvenile Alabama Rainbow across 5 months at 13 sites in the Conasauga river that represented a gradient of forested, agricultural, and developed dominant land use. We hypothesized that growth and survival would be inversely correlated with both agricultural and municipal contaminants and land use, whereas physical water quality measures (such as temperature and productivity) would be positively correlated with growth and survival over the course of the deployment period.

Methods

Juvenile mussel rearing

Juvenile Alabama Rainbow were provided by the Alabama Aquatic Biodiversity Center (AABC) in Marion, Alabama. This species was selected because it is native and considered an at-risk species currently being evaluated for listing under the US Endangered Species Act (Federal Register Citation: 76 FR 59836). Adult mussels were collected for broodstock from the Coosa Basin during the Summer of 2022 and 2023. Glochidia were infested on Cahaba Bass (*Micropterus cahabae* [Baker, Blanton, and Johnston, 2013]) during spring of 2023 and 2024 and held in multitank aquatic habitat systems until transformed. Then, juvenile mussels from

these systems were collected and grown for approximately one year in bucket recirculating systems where they were fed commercial algae.

Site Selection

Thirteen study sites for the *in situ* deployments of mussels spanned the Upper Conasauga River from Tennessee State Route 317 south to Calhoun, Georgia (Figure 3.1). To randomly select site locations in the Conasauga River, we first created a stream network in MATLAB (The MathWorks, Inc., 2023) from 1/3rd arc-second Digital Elevation Models (DEMs) using the TopoToolBox, Image Processing Toolbox, and Mapping Toolbox (USGS 2023; Schwanghart and Scherler 2014). In ArcGIS Pro version 3.1.2 (ESRI, Redlands, CA, USA), we filtered out portions of the stream network that were above 1,200 feet in elevation, were 1st or 2nd order streams, or part of the mainstem of the Conasauga below the confluence of Drowning Bear Creek near Dalton, GA. This filtering was done to increase the probability that the selected points would contain mussel habitat, while also focusing on the upper watershed. We then generated 100 random points on the refined stream network and delineated a watershed around each one based on the DEM's.

To characterize the status of the watershed around each point, we calculated percent land use, National Pollutant Discharge Elimination System (NPDES) density, and added the EPA's 303(d) assessment to each watershed. Percent land use was calculated from the 2021 National Landcover Database after we condensed the landscape classes from 16 unique class to 6 (Dewitz and USGS 2021). The 6 land use classes used to calculate percent land use in each watershed were water (2 classes), forest (5 classes), barren (1 class), agriculture (2 classes), developed (4 classes), and wetlands (2 classes). We removed water, barren, and wetlands from the watershed

characteristics because they made up approximately 1% of the watershed and are not of particular interest when considering land use impacts on mussel health.

We calculated composite site-level scores to stratify locations across a range of anthropogenic impacts. First, we converted percent land use by category, NPDES density, and 303(d) impairment status to z-scores to standardize these watershed characteristics. For each of the 100 candidate sites, we then summed the z-scores of all standardized watershed variables to create a composite watershed impact score for each potential location. These composite scores were then ranked and divided into quantiles to represent a gradient from low to high potential anthropogenic impact. To ensure balanced representation across this gradient, we randomly selected sites from each quantile for further consideration. Final site selection was further narrowed based on physical access due to stream conditions (flow and depth) and landowner permission for actual site access, resulting in 13 site locations where silos were successfully deployed (Table 3.1).

Water collection and chemical analysis

In situ exposures began on May 13th, and lasted until September 5th. We recorded specific conductance, dissolved oxygen, turbidity, and pH at each site during each month of the exposure, using a YSI ProDSS multiparameter water quality meter (Yellow Springs Instruments, Yellow Springs, Ohio, USA). Environmental characteristics of each site were recorded using the Physical Characterization/Water quality and Habitat Assessment field data sheets from the US EPA Rapid Bioassessment Protocol (Barbour et al., 1999). A 3-L composite sample of water was collected throughout the water column at each site and split across five 500 mL brown plastic bottles. One 500 mL sample of water was filtered through a Whatman 0.7 um glass fiber filter

for chlorophyll-*a* analysis. These filters were placed in screw top vials and wrapped in aluminum foil to keep light from degrading the chlorophyll. All samples, including the filters, were kept on ice in the field and during transportation to the University of Georgia Aquatic Biotechnology and Environmental Laboratory (ABEL). Water samples were held untouched in the 500 mL bottles from the field and refrigerated (4 °C) for an average of three days before analysis. Water samples were analyzed for hardness, pH, boron (B), calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), phosphorus (P), silicon (Si), zinc (Zn), chromium (Cr), nickel (Ni), chlorophyll-*a*, and nitrogen (reported as $NO_3 + NO_2$ - N). Monthly water samples were analyzed at UGA's Agricultural and Environmental Services laboratories (UGA AESL), and one sample from each month was kept frozen for further analysis at UGA ABEL.

Silo construction

Silos were constructed following the methods outlined in Haag et al. (2019), and instruction provided by Monte McGregor (personal communication, Kentucky Department of Fish & Wildlife). Silos are molded concrete domes that are 32 cm in diameter and 13 cm deep. The center consists of a cavity created using a 4-inch PVC pipe with a drain fixed to the bottom. The mussels are held in an insert constructed from a 3-inch PVC pipe with 1 mm window screen covers on both ends. The insert sits inside the cavity of the silo and is held in place with zip ties during deployment. Three legs were constructed with bent steel rods cast within the bottom of the silo to hold it approximately 5 cm off the streambed (Figure 3.2). As water passes over the silos, water is pulled up through the central cavity of the domed silo because of the Bernoulli Principle (Figure 3.2) (Patterson et al., 2018). This current allows waste to be carried away while

food and oxygen are supplied to the animals inside. It is likely that animals are moved due to the upwelling current, and while the effect of this is unknown, the consistent construction design of the silos may mediate the effect among sites to some extent.

In situ silo deployment

Mussels were kept in aerated coolers during transport to field sites and held at approximately 23°C. We acclimated mussels to the ambient water temperatures recorded at each field site changing the holding temperature 1-2°C per hour, until the temperatures were the same. Mussels were deployed in silos during May 13th of 2024 through September 5th 2024. Three silos containing 10 mussels each were placed at all 13 sites. We placed silos within the thalweg despite this habitat having greater stream velocity than preferred mussel habitat to necessitate adequate water flow. Silos containing acclimated mussels were placed on stable sediments, which were composed of mostly gravel and sand in runs of the stream. Water depths of at least 0.5 m deep were selected to increase the probability that silos would stay submerged. Mussels were haphazardly assigned to each silo insert, and the average initial length of these mussels was 19.12 ± 1.75 mm (mean ± SD). Silos were deployed for approximately 112 days and then retrieved in September of 2024. During the deployments, water temperature was measured every 2 hours using temperature loggers (iBee-G AlphaMach, Québec, Canada). At the completion of the *in situ* study, mussels were transported on ice to the UGA ABEL where they were frozen at -18°C.

Mussel length was measured monthly throughout the *in situ* exposures. To minimize time spent in the field, we photographed mussels at each silo and determined their lengths from these images later in the lab. Lengths were assessed by converting pixel measurements from

photographs to millimeters using an Olympus TG-6 camera and the PVC inserts in which mussels were held. To generate a conversion scale, we first measured the diameter of 10 inserts using calipers and used the average diameter to scale each image individually for conversion of pixels to mm. To validate this method, all 10 mussels from a single insert at each site were also measured manually with calipers, and the resulting lengths were compared to the photographic measurements to confirm they were within 0.5 mm.

Statistical Analysis

Data for mussel growth (mm/day) and survival (e.g., average percent survival) were treated as response variables and analyzed separately. We tested differences in growth among sites using ANOVA. Using a Tukey's Post Hoc Test, we tested to see which pairs of sites had significant differences in the change of average length. We examined if the assumptions of the model were met using a Shapiro-Wilk test to assess the normality of the residuals and a Levene test was used to test the homogeneity of variance. We also plotted the residuals to look for remaining patterns.

To evaluate relationships between mussel growth and environmental predictors, we used a model selection approach based on multiple linear regression models. Candidate predictor variables were screened prior to inclusion in the global model. Given the relatively small sample size (i.e., 13 sites), we limited the number of predictors to reduce the risk of overfitting and maintain model stability. To reduce multicollinearity among predictor variables prior to model selection, I calculated Pearson correlation coefficients for all pairs of numeric variables. Pairs with a correlation coefficient of $|r| \geq 0.8$ were flagged as highly correlated. I then manually reviewed these highly correlated variables and grouped them into sets based on overlapping

relationships. I selected a single representative variable from each group to be retained for model selection, prioritizing ecological relevance and support from literature. Variables were also excluded if they had low variability (standard deviation < 0.1) or a high proportion of values below detection limits (> 60%; Table 3.2). All retained predictors were standardized (mean = 0, standard deviation = 1) to facilitate comparison of effect sizes.

We used the dredge function (Bartoń, 2024) in R to systematically evaluate all combinations of the remaining environmental predictor variables. We evaluated predictor variables in two separate groups (landscape variables and water chemistry variables). Reducing the number of variables also ensured that the dredge function could evaluate candidate models efficiently and appropriately, without exceeding the information available in the dataset. Candidate models were ranked by their Akaike Information Criterion corrected for small sample size (AICc) values, with lower AICc indicating better relative fit. The difference between each model's AICc and the minimum AICc (ΔAICc) was calculated. Models with $\Delta AICc \leq 2$ were considered well supported and were retained in the final set of top models for further interpretation.

Results

Sediment/Water Collection and Analysis

In monthly water samples, concentrations of Cu, Mo, Zn, Cr, and Ni were nearly always below the detectable limit and showed little variability for the duration of the study and were thus excluded from additional analysis. The highest observed concentration of chlorophyll was in July at Mill 2 (16.87 µg/L). Ten of the 13 study sites (Mill 1, Holly, Conasauga 1, 2, and 3, Spring, Sumac 1 and 2, Coahulla, and Mills) had observed chlorophyll concentrations of 0 µg/L

in May. The remaining three sites (Mill 3, Town Branch, and Mill 2) had chlorophyll levels of 2.25, 2.53, and 8.57 µg/L, respectively, in May. Mills Creek had the lowest average chlorophyll concentrations (0.48 µg/L) during the study period, while Mill 2 had the highest (6.74 µg/L). The temperatures observed during this trial ranged from a low of 16°C at Holly Creek in May, to a high of 31°C at Conasauga 2 in July. The lowest average monthly temperature observed was at Mills Creek (20.8°C). Mills Creek also had the lowest average temperature for the study period (20.97°C). The highest average monthly temperature was at Conasauga 1 (26.60°C). Conasauga 1 had the highest average temperature during the study period (25.29°C).

We also evaluated the relationship between mussel growth and GIS-derived watershed characteristics, including percent land use and NPDES (National Pollutant Discharge Elimination System) permit density. On average, land use across sites consisted of 25.39% agricultural, 60.98% forested, and 13.02% developed land. Mills Creek had the highest proportion of agricultural land use (45.77%), Conasauga 3 was the most forested site (96.63%), and Town Branch had the highest percentage of developed land (36.60%) (Table 3.1). NPDES density was calculated as the number of permitted discharges divided by watershed area (permits/km^2) and ranged from 0 to 1.15, with a mean value of 0.18. Mill 3 had the highest NPDES density (1.15 permits/km^2), indicating the greatest potential for permitted point-source inputs.

Recovery, Survival, and Growth

There were no significant differences in initial mussel length among sites (ANOVA, $F(12,26) = 0.26$, $p = 0.99$; df = site, residual), indicating that starting size was consistent across treatments and is unlikely to have influenced observed patterns of growth. We recovered 380 of

the 390 mussels used in the silo study. The 10 mussels that were not recovered came from a single silo at Sumac 1. This silo was likely vandalized sometime between monthly checks in June and July. The overall survival for the trial was 92%. The only mortality observed in recovered mussels was at Conasauga 3 in mid-August, two weeks before the trial ended. Vandalism of all three silos at this site is assumed as temperature recorders indicated a change in temperature from 75ºC to 91 ºC during a 8 hour period when the silos were assumed to be removed from the water and left on the bank. Survival was not used as a response variable in statistical analysis because it was uniformly high with little variability. The average starting length of all mussels was 19.2 mm and the average ending length was 24.6 mm. The overall average growth was approximately 0.04 mm/day (Figure 3.3). Town Branch had the lowest average growth (0.010 ± 0.001 mean ± SE), while Conasauga 2 had the highest average growth (0.090 ± 0.007, mean ± SE).

ANOVA Results

We found statistically significant differences in average mussel growth (mm/day) between sites using a one-way ANOVA ($p < 0.001$). The assumption of homogeneity of variance was met (Levene's test, $p = 0.77$), but the Shapiro-Wilk test indicated a violation of the normality assumption ($p = 0.003$). However, ANOVA is generally robust to moderate deviations from normality when group sizes are balanced and variances are equal (Schmider et al., 2010). Visual inspection of a Q-Q plot and residual diagnostics confirmed only mild departures from normality (Figure 3.4). Based on this, we proceeded with post-hoc comparisons using Tukey's Honestly Significant Differences (HSD). These tests revealed that Mill 3 exhibited significantly higher growth than Town Branch, Mill 1, Holly, Spring, and Sumac 2 (all $p < 0.001$). For

comparison, we also conducted a Kruskal-Wallis test, which supported the ANOVA results ($p = 0.001$). Dunn's test with Bonferroni correction further showed that Town Branch had significantly lower growth than Mill 3, Conasauga 1, and Conasauga 2, while Mills had lower growth compared to Mill 3 and Conasauga 2. Together, these results indicate that mussel growth varied meaningfully among sites, with Mill 3 consistently exhibiting the highest growth and Town Branch among the lowest.

Variable Filtering

Water samples were analyzed for a total of 23 chemical variables. Prior to model selection, two groups of highly correlated variables ($|r| \geq 0.8$) were identified. The first group included water chemistry parameters—hardness, specific conductivity, Ca, Mg, and pH. From this group, Mg was retained based on its negative association with mussel growth observed by (Skorupa et al., 2024), where it accounted for one of the largest site-level deviations. A second group included sodium and nitrogen; nitrogen was retained because previous studies in the Conasauga River found nitrate concentrations associated with eutrophication (mean = 0.7 mg NO_3-N/L; Lasier et al., 2016), and in the present study, nitrogen concentrations ranged as high as 1.15 mg/L. Although nitrate concentrations in this study were well below published LC50 values for freshwater mussels (357–937 mg/L; Soucek and Dickinson (2012)), there could be sublethal effects or interactive stress.

Al, Fe, and P were excluded due to low variability (standard deviation < 0.1). Ni was removed due to a high proportion of values below detection limits (>60%). Mn, B, Cu, Mo, Zn, and Cr were excluded due to a combination of low variability and a high proportion of zeros. The GIS-derived predictors (e.g., NPDES density and percent land use) were retained despite some

exhibiting pairwise correlations of $|r| \geq 0.8$. These variables had sufficient variability (standard deviation > 0.1) and a relatively low proportion of zeros (<60%) but were expected to be intercorrelated due to their compositional nature—they represent proportional land cover categories that together sum to 100%. Each provides distinct landscape context, so none were removed for collinearity.

Initial exploratory graphing suggested a nonlinear relationship between chlorophyll-*a* and mussel growth. As a result, a quadratic term (chlorophyll-a^2) was included in models where chlorophyll-*a* was selected as a potential predictor. After filtering, the final retained predictors for landscape-level variables were NPDES density, percent land use in the watershed (forest, agriculture, and development). The water chemistry variables retained were magnesium, nitrogen, chlorophyll-*a* (and its quadratic term), temperature, dissolved oxygen, and turbidity.

Top Multiple Regression Models

Model selection results for growth (mm/day) and landscape-level variables indicated that the top-ranked model was the null model (df = 2, AICc = –50.85, weight = 0.34), suggesting no strong support for any single GIS predictor in explaining variation in juvenile mussel growth. Several models were within 2 ΔAICc units of the top model (Table 3.3). These included models with NPDES density ($\beta = 0.01$), percentage agricultural land use ($\beta = –0.01$), and percentage forest cover ($\beta = 0.008$), but the effect sizes were small and did not substantially improve model fit (ΔAICc = 1.76–2.36; weights = 0.10–0.14). Model weights were generally low across this set, further indicating weak support for individual predictors.

The model selection process for the water quality variables identified two well-supported models within the candidate set (ΔAICc ≤ 2; Table 3.4). The top-ranked model included

chlorophyll-*a*, its quadratic term (chlorophyll-a^2), and temperature, while the second model included only temperature. The full model revealed a significant positive effect of chlorophyll-*a* ($\beta = 0.03$, $p = 0.008$) and temperature ($\beta = 0.016$, $p = 0.004$), as well as a significant negative effect of the quadratic term chlorophyll-a^2 ($\beta = -0.014$, $p = 0.02$).

The inclusion of the quadratic term indicates that there is a nonlinear relationship in which mussel growth peaked at intermediate chlorophyll concentrations. Growth peaked at a chlorophyll-*a* concentration of approximately 3.97 µg/L as estimated from the fitted quadratic model (Figure 3.5); however, growth was inhibited at the Mills River site that had high chloraphyll-*a* concentrations greater than 6 µg/L. The second supported model also showed a significant positive effect of temperature ($p < 0.01$) but had a higher AICc and lower explanatory power. The top model including temperature, and the linear and quadratic terms for chlorophyll explained approximately 83% of the variation in growth ($\beta_{Chloro} = 0.03$, $p = 0.008$; $\beta_{Chloro^2} = -0.01$, $p = 0.020$; $\beta_{Temp} = 0.01$, $p = 0.004$; Table 3.5). These results suggest that temperature is a strong positive predictor of mussel growth, while chlorophyll has a non-linear effect, with growth declining at high concentrations. None of the models containing contaminant variables fell within the $\Delta AICc \leq 2$ candidate model set.

Discussion

We observed uniformly high survival across all sites during the *in situ* exposures. High survival during relatively short time durations (i.e., weeks to months) is common for *in situ* trials with freshwater mussels, even in streams that exhibit degraded conditions, contamination, or have experienced mussel population declines (Haag et al., 2019; Skorupa et al., 2024). These findings suggest that acute toxicity or lethal stressors were not present during the deployment

period. In contrast, we detected significant variation in mussel growth across sites with Mill 3 exhibiting the highest growth and Town Branch and Mills Creek among the lowest. Growth is commonly used as a sublethal endpoint in field trials due to its sensitivity to subtle environmental stressors; differences in growth are typically influenced by factors such as food availability, nutrient levels, contaminants, and temperature (Denic et al., 2015; Haag et al., 2019; Skorupa et al., 2024).

In this study, temperature best explains the variation in growth among sites. We observed temperature ranges in this study from 16°C to 31°C. This temperature range has been shown to have effects on freshwater mussel growth, physiology, and reproduction (Archambault et al., 2014; Denic et al., 2015; Sangsawang et al., 2019). In laboratory experiments, Archambault et al. (2014) found that median lethal temperatures (LT50) for two species of juvenile freshwater mussels ranged from 29.9 to 35.6°C. We did not observe sustained temperatures within this range. The only recorded temperatures within this range were from July 7th, 8th, and 9th at the three Conasauga mainstem sites for a maximum of 6 hours on any given day. On average, the temperature was at or above 30°C for approximately 1-2 hours. Our study supports evidence that growth generally increases with temperature (Ganser et al., 2013; Skorupa et al., 2024); however, some studies have found that detrimental effects of temperature accumulate over time and eventually lead to death for organisms pushed beyond their thermal tolerance (Ganser et al., 2013).

Our study used 1-year-old juvenile mussels, a life stage known to be more sensitive to thermal stress than adults. While the high temperatures observed in our study were not sustained long enough to elicit the levels of mortality reported in some previous research, our findings still align with the broader pattern of life stage–specific thermal sensitivity. Acute studies have shown

that the thermal tolerance of glochidia is lower than juveniles, while chronic studies have shown that adults have higher thermal tolerance than juveniles. Pandolfo et al. (2010) showed that glochidia have a lower 24-h average LT50 of 31.6°C, compared to juveniles with a 96-h LT50 of 34.7°C. While Ganser et al. (2013) reported juvenile mussel mortality at 25.3–30.3°C over 28 days, later studies by the same authors (Ganser et al., 2015) found low adult mortality across species and treatments at temperatures up to 35°C in similar trials. Finally, it should be noted that thermal tolerance can be influenced by genetic lineage. Denic et al. (2015) found that stock origin had a significant effect on survival in streams of variable temperatures. Our results reinforce the importance of considering life stage and individual variability when evaluating mussel responses to thermal stress, particularly under environmental conditions that may not exceed established thermal thresholds but could pose chronic challenges.

Our findings are consistent with the general pattern observed in laboratory studies, such as Carey et al. (2013), which identified 26°C as the optimal temperature for growth in captivity. In our field study, the highest growth occurred at sites with average temperatures between 23 and 25°C. Additionally, previous research has shown low growth in cooler streams and predicted no growth at temperatures of 17.87 - 20.27°C (Haag et al., 2019). In the present study, the sites where the lowest growth was observed occurred in slightly warmer temperatures that ranged from 20.97 - 22.79°C.

While water temperature is clearly an important determinant of growth in freshwater mussels, the combination of temperature and food availability appears to be similarly important (Skorupa et al., 2024; White et al., 2022). We saw a non-linear effect of chlorophyll, with an inflection point of growth beyond chlorophyll concentrations of 3.97 µg/L. Other studies have shown positive relationships with chlorophyll concentrations, within temperature ranges like

those we observed (16–26°C); however, we observed chlorophyll levels nearly 3-fold higher than in a recent study (Skorupa et al., 2024 = 2.82 ug/L, present study = 6.74 ug/L). Our results of reduced growth at high chlorophyll concentrations were largely driven by values at only one site but further highlight the need to understand food abundance and quality for freshwater mussel growth.

Other studies have found positive effects of increased feeding beyond what was previously recommended in hatchery settings (White et al., 2022). It is hard to draw a direct comparison between the present study and White et al. (2022) because the latter study was in a mussel hatchery and used fine particulate organic matter (FPOM) as the metric for food abundance. However, White et al. (2022) found that the growth in the hatchery was lower than the growth observed in streams with similar food abundance, even when temperature effects are accounted for, showing that there are complex natural conditions in the stream that determine mussel growth that are not accounted for in hatchery settings. A possible explanation for our finding is that elevated chlorophyll may signal reduced food quality or impose higher energetic costs associated with food sorting, reduced clearance rates, or increased pseudofeces production (Bartsch et al., 2017; Li et al., 2022; Riisgård et al., 2011). Moreover, high levels of suspended solids in streams have been shown to reduce clearance rates and hinder reproductive processes such as sperm uptake (Gascho Landis et al., 2013), further emphasizing how natural environmental complexity influences mussel performance. In order to confidently assess the non-linear relationship between chlorophyll and mussel growth, more growth data would need to be assessed at chlorophyll levels past the inflection point we observed at 3.9 µg/L.

Most of the contaminants we measured were excluded from the global model due to low variability (standard deviation < 0.1), which was often driven by concentrations below detection

limits. Low detectability may reflect low contaminant inputs during the sampling period or modest contributions from point sources such as industry or urban runoff, which are less prominent compared to agricultural and forested land uses in the watershed. Among the contaminants retained in the model (e.g., Mg, K, Si, N), we did not observe strong associations with mussel growth compared to temperature and chlorophyll-*a*. These findings suggest that under the conditions observed in this study, waterborne contaminant concentrations were not major contributors to variation in growth, though this does not preclude effects through other exposure routes (e.g., sediment-associated contaminants or episodic events not captured in monthly samples).

This study contributes to existing efforts to understand stream conditions that may be causing the decline in freshwater mussel populations seen globally. We found that temperature and chlorophyll typically increase growth in mussels but may be harmful past optimal levels. Future studies could expand upon the chemical analysis of sediment and water samples to identify contaminants that affect growth and survival or use endpoints like hemolymph or tissue analysis to observe the effects that contaminants have on mussel physiology (Fritts et al., 2015; Putnam et al., 2023; Rogers et al., 2017). Finally, the present study covers the stream conditions present in the summer of 2024 while mussel declines have been occurring for nearly 65 years (Haag, 2019). This study could be repeated, or previous water quality and sediment analysis data could be utilized to identify if the conditions observed during the study are representative of the system at different timepoints. Results found here could also be compared with mussel sampling data to determine if patterns exist between contamination and population decline.

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Tables

Table 3.1. Summary of watershed characteristics for each study site. Assessment refers to the EPA 303(d) impairment status. Area is the watershed area in square kilometers (km^2). NPDES Density is the number of National Pollutant Discharge Elimination System permits per square kilometer. PerAgri, PerFor, and PerDev indicate the percentage of the watershed classified as agricultural, forested, and developed land use, respectively.

Stream Name	Assessment	Area (km^2)	NPDES Density	PerAgri	PerFor	PerDev
Mill 3	NotSupporting	114.65	1.15	8.15	58.31	32.36
Town Branch	Supporting	23.05	0.17	35.59	26.42	36.60
Mill 1	NotSupporting	86.07	0.48	23.08	58.15	18.29
Holly Creek	NotAssessed	95.95	0.00	4.03	91.69	4.14
Conasauga 1	NotSupporting	47.97	0.00	39.78	50.33	8.40
Spring	Supporting	34.78	0.35	34.38	53.82	11.00
Sumac 2	NotSupporting	61.37	0.08	8.90	85.27	5.51
Sumac 1	NotSupporting	24.49	0.08	33.76	51.70	14.26
Conasauga 2	NotSupporting	413.07	0.00	14.61	81.22	3.83
Coahulla	Supporting	138.99	0.06	39.25	44.94	15.26
Mills	Supporting	18.02	0.00	45.77	46.36	7.69
Conasauga 3	Supporting	279.21	0.01	1.42	96.63	1.85
Mill 2	NotSupporting	60.87	0.00	41.31	47.91	10.06

Table 3.2. Water chemistry variable averages from the 5 monthly water samples taken during the silo deployment. Boron, Copper, Molybdenum, Zinc, Chromium, Nickel were excluded because they were always below detectable limits, except for nickel which was only detected at Mills Creek.

	YSI			University of Georgia Agricultural & Environmental Services Laboratories													
								parts per million (ppm)									
Site Name	D.O. (ppm)	Spec Con (µS/cm)	Turbid (NTU)	pH	Chloro-a (µg/L)	Temp (°C)	Hard	Al	Ca	Fe	K	Mg	Mn	Na	P	Si	$NO_3^- + NO_2^-$ (as N)
Mill 3	8.12	199.32	11.09	8.0	3.8	23.82	114.5	0.04	32.03	0.10	0.90	8.37	0.00	2.66	0.01	3.91	0.35
Town Branch	6.93	286.96	6.20	7.8	1.3	21.14	149.1	0.00	32.08	0.10	1.42	16.74	0.11	3.28	0.00	3.67	0.63
Mill 1	8.07	243.90	5.32	7.8	1.0	21.41	108.1	0.03	25.03	0.10	1.48	11.07	0.00	4.00	0.01	3.58	0.81
Holly	8.84	43.64	1.81	7.3	1.1	23.32	16.5	0.00	4.88	0.07	1.23	1.04	0.00	1.74	0.00	3.58	0.07
Conasauga 1	8.32	99.16	7.43	7.7	2.1	25.30	42.8	0.03	11.63	0.13	1.05	3.33	0.00	1.74	0.00	2.93	0.23
Spring	7.97	270.24	5.96	8.0	0.6	22.93	130.7	0.04	29.38	0.12	1.40	13.92	0.00	1.94	0.01	3.82	0.35
Sumac 2	8.57	146.12	3.01	7.8	0.6	22.80	63.6	0.03	17.04	0.28	1.41	5.11	0.00	2.71	0.01	4.41	0.64
Sumac 1	8.59	208.20	5.66	7.9	0.8	22.78	90.1	0.04	22.80	0.21	1.52	8.06	0.00	2.59	0.02	4.17	0.83
Conasauga 2	8.72	81.44	3.46	7.7	1.3	25.05	35.8	0.00	9.92	0.08	0.81	2.68	0.00	1.52	0.00	2.79	0.18
Coahulla	6.90	263.82	11.46	7.9	1.0	23.94	118.2	0.24	35.94	0.21	3.14	6.91	0.01	3.39	0.08	3.69	0.45
Mills	8.77	317.42	3.28	8.2	0.5	20.98	156.7	0.19	43.74	0.12	1.90	11.52	0.02	3.53	0.06	4.44	0.99
Conasauga 3	8.92	35.56	0.68	7.4	0.7	24.40	13.8	0.00	4.16	0.06	0.38	0.83	0.00	1.28	0.00	2.68	0.01
Mill 2	6.56	288.28	8.97	8.0	6.7	22.97	138.6	0.04	37.40	0.12	2.11	10.97	0.03	2.79	0.02	4.80	0.28

Table 3.3. Output from the top 10 models ranked by AICc using the dredge function for landscape variables; NPDES density and percent land use (agriculture, developed, and forested). These variables are hypothesized to influence growth of *Cambarunio nebulosus* in silos deployed at 13 study sites in the Conasauga River in 2024. Each row represents a candidate model predicting mussel growth (average percent change and length), with the intercept representing the overall growth (mm/day) for the silo study. Predictors shown as standardized coefficient estimates and excluded predictors denoted as "-". Columns include the number of parameters (df), log-likelihood (logLik), AICc score, ΔAICc (difference from the top model), and Akaike weight (weight), indicating relative model support.

Model	Intercept	NPDES_Density	PerAgri	PerDev	PerFor	df	logLik	AICc	delta	weight
1	0.05	-	-	-	-	2	28.03	-50.85	0.00	0.34
2	0.05	0.01	-	-	-	3	28.88	-49.10	1.76	0.14
3	0.05	-	-0.01	-	-	3	28.82	-48.98	1.87	0.13
9	0.05	-	-	-	0.01	3	28.58	-48.49	2.36	0.10
5	0.05	-	-	0.00	-	3	28.06	-47.45	3.40	0.06
6	0.05	0.02	-	-0.02	-	4	30.20	-47.39	3.46	0.06
10	0.05	0.01	-	-	0.01	4	29.81	-46.62	4.23	0.04
15	0.05	-	-0.84	-0.57	-1.08	5	32.20	-45.83	5.02	0.03
4	0.05	0.01	-0.01	-	-	4	29.39	-45.79	5.06	0.03
11	0.05	-	-0.01	-	0.00	4	28.83	-44.65	6.20	0.02

Table 3.4. Output from the top 10 models ranked by AICc using the dredge function. Each row represents a candidate model predicting mussel growth (mm/day), with included predictors shown as standardized coefficient estimates and excluded predictors denoted as "-". Columns include the number of parameters (df), log-likelihood (logLik), AICc score, ΔAICc (difference from the top model), and Akaike weight (weight), indicating relative model support.

Model	Intercept	Chloro	Chloro2	D.O.	Mg	N	K	Temp	Turb	df	logLik	AICc	delta	weight
68	0.06	0.04	-0.01	-	-	-	-	0.02	-	5	39.18	-59.78	0.00	0.25
65	0.05	-	-	-	-	-	-	0.02	-	3	34.18	-59.70	0.08	0.24
225	0.05	-	-	-	-	-	-0.02	0.02	0.01	5	38.18	-57.78	1.99	0.09
169	0.05	-	-	-	-0.02	-	-0.02	-	0.02	5	38.14	-57.71	2.07	0.09
72	0.06	0.04	-0.01	0.01	-	-	-	0.01	-	6	41.71	-57.41	2.37	0.08
66	0.05	0.01	-	-	-	-	-	0.02	-	4	35.18	-57.36	2.42	0.07
97	0.05	-	-	-	-	-	-0.01	0.02	-	4	34.87	-56.74	3.04	0.05
193	0.05	-	-	-	-	-	-	0.02	0.01	4	34.71	-56.43	3.35	0.04
73	0.05	-	-	-	0.01	-	-	0.03	-	4	34.46	-55.92	3.86	0.04
81	0.05	-	-	-	-	0.01	-	0.03	-	4	34.42	-55.85	3.93	0.04

Table 3.5. Model estimates and significance values from the top two candidate models identified by the dredge function. AICc values are provided for model comparison. Estimates and associated p-values are shown for each predictor retained in the models: chlorophyll-*a* (Chloro), its quadratic term (Chloro²), and temperature (Temp). "-" indicates that the variable was not included in that model.

		Chloro		Chloro2		Temp	
Model	AICc	estimate	*p*	estimate	*p*	estimate	*p*
68	-59.78	0.03	0.008	-0.01	0.020	0.01	0.004
65	-59.70	-	-	-	-	0.02	0.002

Figures

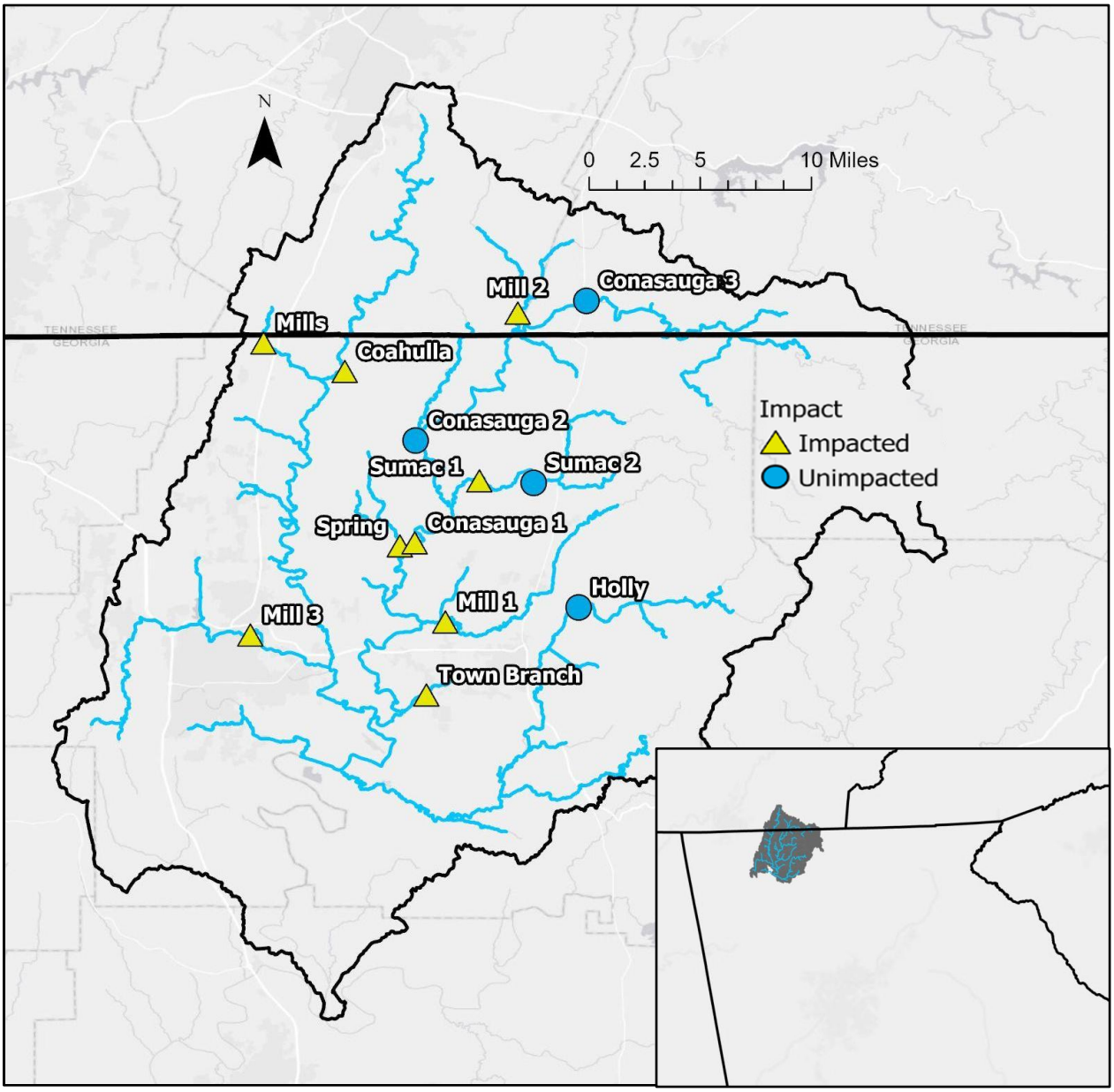

Figure 3.1. Thirteen randomly selected sites in the Upper Conasauga River watershed for the 2024 silo study. Impacted sites have less than 75% forested land cover present in the watershed. Unimpacted sites have greater than 75% forested land cover present in the watershed.

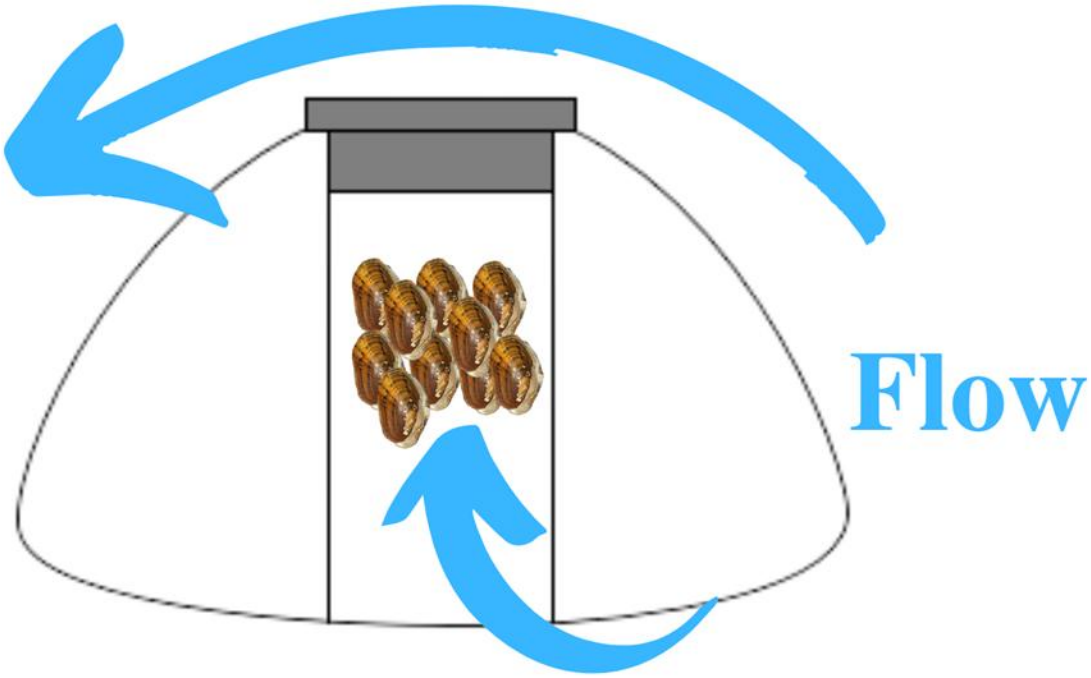

Figure 3.2. Conceptual diagram of a concrete mussel silo used for *in situ* deployments. Arrows illustrate directional water flow through the silo, allowing mussels to be exposed to ambient stream conditions while remaining contained for monitoring of growth and survival.

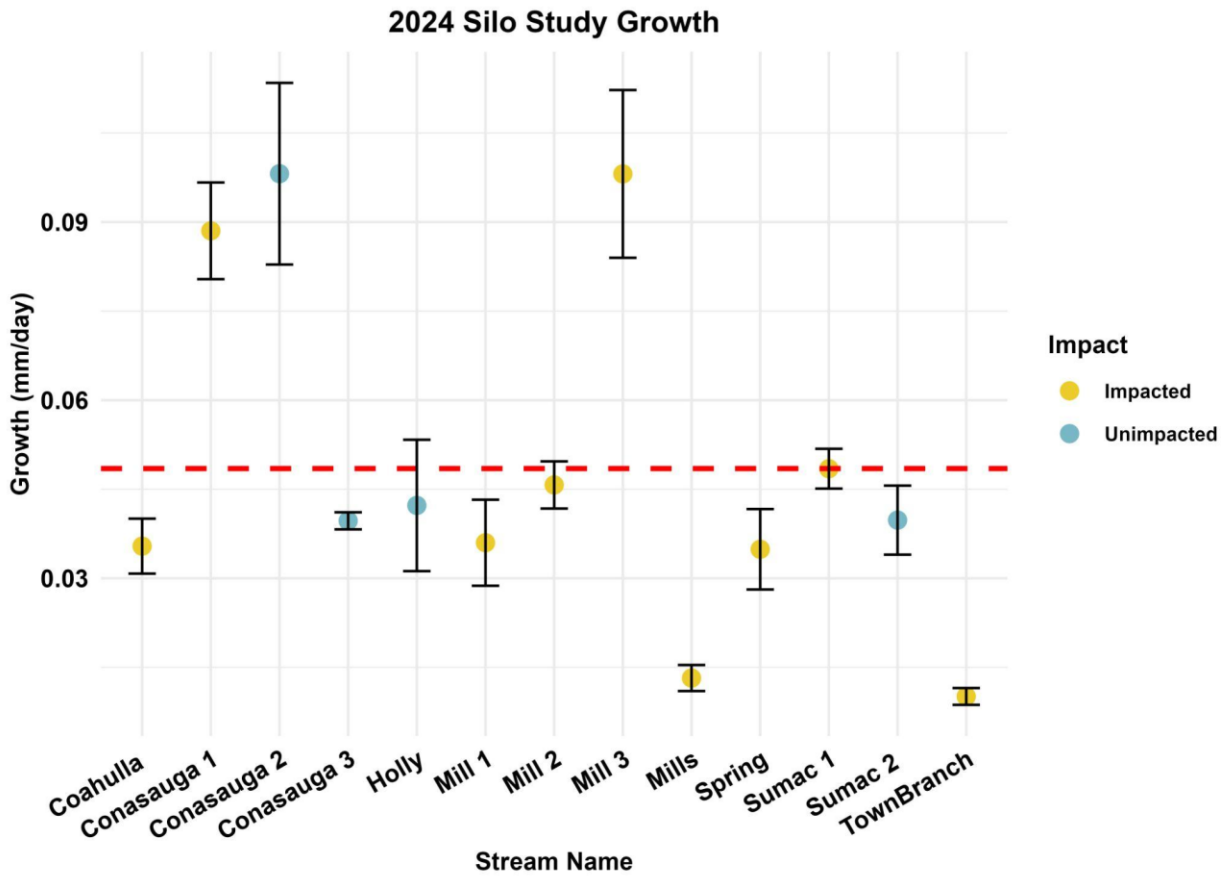

Figure 3.3. Mean growth (mm/day) of mussels for 13 study sites. Red dashed line shows the overall mean growth rate (0.04 mm/day). Yellow points (Impacted) have less than 75% forested land cover present in the watershed. Blue points (Unimpacted) sites have greater than 75% forested land cover present in the watershed. Error bars show 95% confidence intervals for each site.

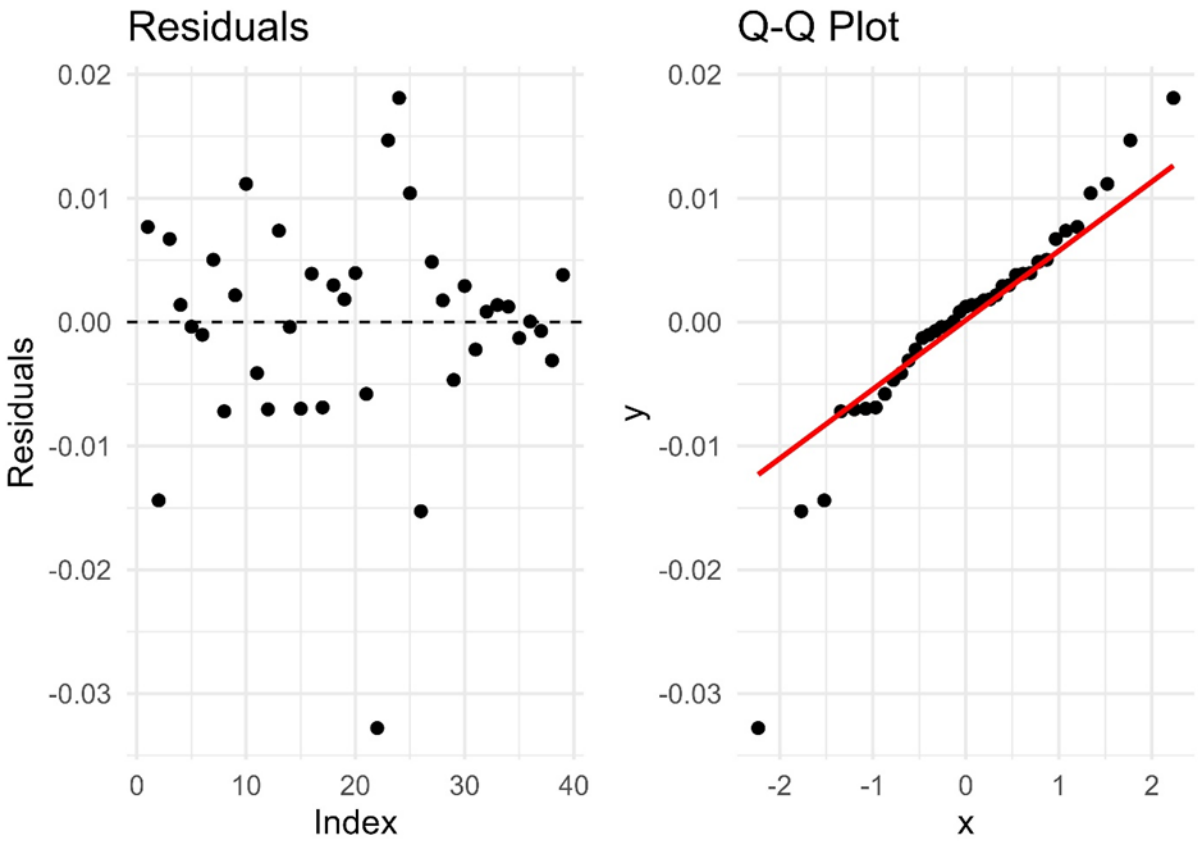

Figure 3.4. Residual diagnostic plots for the ANOVA model testing differences in mussel growth (mm/day) across stream sites. The left panel shows residuals plotted against observation index, with no obvious pattern or trend, suggesting acceptable linearity. The right panel is a Q-Q plot comparing standardized residuals to a theoretical normal distribution; while most points fall along the reference line, slight deviations at the tails indicate mild non-normality. Despite this, the model meets the assumptions of homogeneity of variance and is considered robust to these departures.

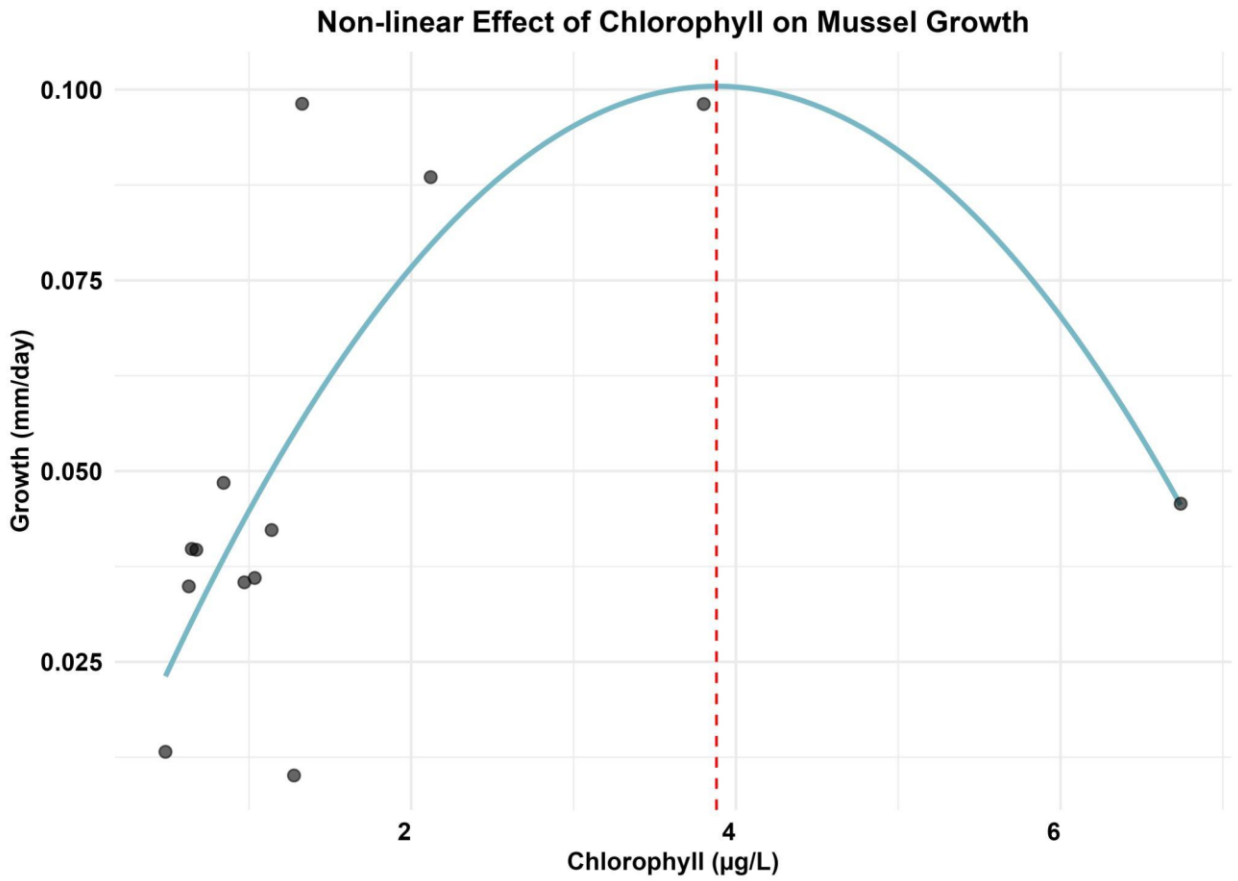

Figure 3.5. Predicted mussel growth (mm/day) across chlorophyll-a concentrations based on a quadratic regression model, with observed site-level growth shown as black points. The red dashed line indicates the estimated chlorophyll-a concentration (~3.97 µg/L) at which growth is predicted to peak.

CHAPTER 4

CONCLUSION

The Conasauga River in northwest Georgia and southern Tennessee is a free-flowing headwater of the Coosa River basin and a conservation priority due to its high biodiversity, including globally rare native fishes and freshwater mussels (Albanese et al., 2015). Given the ecological importance of this system, more information is needed on the potential drivers of species decline—particularly contaminants linked to agricultural and developed land use. This study aimed to evaluate both lethal and sublethal effects of agricultural and municipal contaminants on freshwater mussels by combining *in situ* water exposures with laboratory sediment toxicity tests. We used field-collected sediments from a gradient of impacted sites across the watershed, focusing on the vulnerable juvenile life stage and exposing mussels to environmentally relevant contaminant mixtures.

We found that survival was uniformly high in both the sediment toxicity trials and the *in situ* silo deployments, which is common in studies using these exposure methods that often focus instead on sublethal effects of contaminants and other stressors. However, it is not always clear whether low growth should be interpreted strictly as negative or high growth as inherently positive, since this remains an active area of research. In some systems, slower growth rates have been associated with increased longevity in freshwater mussels, potentially reflecting life history trade-offs under certain environmental conditions (Haag & Rypel, 2011). This highlights the

complexity of interpreting growth responses and the need to consider broader ecological and physiological contexts when evaluating habitat quality.

We found that magnesium, pesticide concentrations, NPDES density, and excessive chlorophyll-a levels had significant negative effects on juvenile mussel growth. While our regression models provide useful insight into the factors affecting juvenile mussel growth, they also have important limitations. In many cases, the relationships we observed seem to be driven by a single site, which could have an outsized influence on the slope estimates and overall model fit. Mill 3 consistently had the highest values for several predictors linked to reduced growth, including magnesium, TU pore water, and NPDES density, and showed the lowest growth across all sites. Because these stressors were all elevated at the same location, it is hard to say whether one factor is primarily responsible or if they together represent a general signature of pollution at this site. As a result, the patterns we found between individual predictors and mussel growth might partly reflect the influence of this one highly impacted site. This highlights the need to be cautious in interpreting these results and drawing conclusions about specific causes.

In contrast, carbon, phosphorus, potassium, temperature, and moderate chlorophyll-a concentrations were positively associated with growth. Interestingly, we observed notable differences between field and lab results at the site level. For example, Mill 3 showed the highest growth in the silo study but the lowest in the sediment toxicity trial, even after accounting for temperature and food availability. This contrast suggests that favorable environmental conditions, such as optimal temperature and food abundance, may mask the sublethal effects of sediment-bound contaminants—highlighting the importance of integrating both field and laboratory approaches to fully assess habitat suitability.

In the silo study, mussels were primarily exposed to the water column, with only intermittent contact with sediment that accumulated between monthly sampling events. This limited sediment exposure could explain the higher growth observed at Mill 3 in the field, compared to the consistent exposure to contaminated sediments in the 28-day lab trial. However, similar growth outcomes have been reported across silo and sediment cage methods (Haag et al., 2019), suggesting that under certain conditions, exposure route may not drastically alter outcomes. More research is needed to better understand the relationship between these methodologies and their effectiveness in identifying stressors to benthic organisms.

Future work could focus on refining toxicity thresholds for magnesium and investigating the cumulative effects of pesticide mixtures, which were linked to growth suppression in this study. Incorporating tools such as stable isotope analysis, hemolymph biomarkers, and longer-term survival tracking could help clarify how early growth responses translate into fitness and population-level outcomes. Continued integration of field and lab approaches will be critical for informing evidence-based reintroduction, restoration, and listing decisions.

This work provides valuable insight for USFWS and GA DNR regarding the condition of Conasauga tributaries and the mainstem, particularly for informing reintroduction planning and understanding contaminant impacts on *Cambarunio nebulosus*, a state species of concern. It also contributes to the broader Species Status Assessment process by helping predict how other mussel species may fare in the Conasauga. It highlights the importance of tributaries like Holly Creek, where we observed high growth in the sediment toxicity trial and low contaminant concentrations in the sediment. Holly Creek continues to host an abundance of freshwater mussel diversity. More broadly, these findings highlight potential areas for stream restoration and where conservation actions may have the most impact. There are rarely river systems that have single

causes of decline, rather there is a complex mixture of contaminants and environmental conditions that over time can contribute to mussel declines. Each system may need unique conservation action to address specific problems from such contaminants and stream conditions.

Beyond the Conasauga River, this research contributes to our understanding of the ongoing global decline of freshwater mussels. Unionids require relatively clean water and sediment, intact fish communities, natural flow regimes, and adequate food to persist (Freshwater Mollusk Conservation Society, 2016). This study is one example of how we can begin to assess the underlying causes of decline in freshwater populations—a necessary first step in reversing it.

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APPENDIX A

MUSSEL GROWTH AND SURVIVAL IN SEDIMENT TOXICITY TRIAL 2023

Abstract

Freshwater mussels are some of the most imperiled taxa in the world. Threats to mussel populations have been attributed to numerous causes, including habitat degradation or loss from dams, pollution, and invasive species. We conducted laboratory exposure trials to assess the effects of multiple stressors from contaminated sediment on freshwater mussel survival and growth. We conducted substrate-exposure studies in the laboratory using sediment collected from throughout the Conasauga River, GA watershed following established sediment toxicity test conditions. During the summer of 2023, sediments were collected at 19 study sites representing a gradient of expected municipal and agricultural contamination and varying land use practices. Juvenile mussels (2023 average start length ~3 mm) were exposed to these sediments at the University of Georgia's Aquatic Biotechnology and Environmental Laboratory. In 2023, average percent survival was 89%. Average percentage change in length was 8%, while the average percent change in weight was 58%. We did not see an effect from land use on growth, but the trial start group had a significant effect on growth endpoints. We think this is likely due to high ammonia levels measured in exposure beakers from the handling of field collected sediments prior to the start of the trial.

Methods

Juvenile mussel rearing

Juvenile Alabama Rainbow were provided by the Alabama Aquatic Biodiversity Center (AABC) in Marion, Alabama. This species was selected because it is native and considered an at-risk species currently being evaluated for listing under the US Endangered Species Act (Federal Register Citation: 76 FR 59836). Adult mussels were collected for broodstock from the Coosa Basin during the Summer of 2022 and 2023. Glochidia were infested on Cahaba Bass (*Micropterus cahabae* [Baker, Blanton, and Johnston, 2013]) during Spring of 2023 and 2024, and held in multitank aquatic habitat systems until transformed. Then, juvenile mussels from these systems were collected and grown for approximately 1 year in bucket recirculating systems where they were fed commercial algae.

Site Selection

Land use types in the Conasauga River watershed include row crops, dairy production, poultry farming, carpet manufacturing, residential development, and forest (Freeman et al., 2007; Konwick et al., 2008; Lasier et al., 2016). We randomly selected 20 sites (Figure 1) spanning the upper Conasauga River from Tennessee State Route 317 south to Calhoun, Georgia. To select sites in the Conasauga, we first created a stream network in MatLab from 1/3rd arc-second (~ 10m) Digital Elevation Models (DEMs) using the TopoToolBox, Image Processing Toolbox, and Mapping Toolbox (Schwanghart & Scherler, 2014; The MathWorks, 2023). In ArcGIS Pro version 3.1.2, we filtered out portions of the stream network that were above 1,200 feet in elevation, were 1st and 2nd order streams, and the mainstem of the Conasauga below the confluence of Drowning Bear Creek near Dalton, GA (Environmental Systems Research Institute, 2023). This filtering of locations was done to ensure that the generated points were

more likely to be sites containing mussel habitat, and this was cross-referenced with mussel observation data provided by the Georgia Department of Natural Resources. We then generated 100 random points on the refined stream network and delineated a watershed around each one based on the DEM's. To characterize the status of the watershed around each point, we calculated the percentage of land use, National Pollutant Discharge Elimination System (NPDES) density, and added the U.S. EPA's 303(d) assessment to each watershed. Land use was calculated from the 2021 National Landcover Database after we condensed the landscape classes from 16 unique classes to 6 (Dewitz & U.S. Geological Survery, 2021). The 6 land use classes in each watershed were water (2 classes), forest (5 classes), barren (1 class), agriculture (2 classes), developed (4 classes), and wetlands (2 classes). We removed water, barren land cover, and wetlands from the watershed characteristics because they respectively made up approximately 1% of the watershed and are not of particular interest when considering potential effects of land use on mussel health. Each land use class was summarized as a percentage contribution to the total. Using normalized z scores, we created a rank for all 100 points, combining all their watershed characteristics, and split them by their rank into quantiles. We randomly selected five sites from each quantile, to try and capture the full range of watershed characteristics in the basin. We used MS Access version 2308 (Microsoft, Redmond, Washington, USA) for data management. We were not able to access one of the 20 sites selected in 2023 because of the water depth so it was excluded from analysis. For the 2023 toxicity trial, juvenile mussels were exposed to sediment collected from our 19 study sites and clean sand control (Figure A.1).

Sediment Collection 2023

Sediment samples were collected from 19 study sites over a three-day sampling period during June of 2023. All sites were accessed from the nearest bridge crossing. The top 1-2 cm of

fine sediment was collected using a stainless-steel spoon and bowl, and sieved to 2-mm, then stored in 1-L amber glass containers. A total of 2-L of sediment was collected from each site in depositional areas that were at least 50 meters upstream of the bridges used to access the stream. Environmental characteristics of each site were recorded using the Physical characterization/ water quality and Habitat Assessment field data sheets from the U.S. EPA Rapid Bioassessment Protocol (Barbour et al., 1999). After each sampling event, all equipment was washed with water from the stream then rinsed with methanol. Samples were refrigerated before being transported on ice to University of Georgia Aquatic Biotechnology and Environmental Laboratory (ABEL). Sediment samples were held untouched in the 1-L bottles from the field and refrigerated (4 ℃) for approximately one month until the trial began. We homogenized one of the two 2-L bottles of sediment and placed it in beakers with reconstituted water for the exposures. Additionally, we kept 200 mL of sediment for later chemical analysis. Sediments for chemical analyses were stored in 100 mL amber glass jars and kept refrigerated (4 ℃) at ABEL until they were analyzed for atrazine and glyphosate pesticides, their surfactants, and by-products. Samples were analyzed for pesticides at the U.S. EPA National Exposure Research Laboratory (NERL) in Athens, GA. All remaining sediment samples were frozen (-18°C) until further use.

Toxicity Trial 2023

Toxicity tests were conducted in adherence to the American Society of Testing and Materials (ASTM) Standard Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates (2020). Toxicity tests were conducted in the laboratory for 42 days using a flow-through system with an average temperature of 23°C ± 1°C and a photoperiod of approximately 16L:8D from ambient laboratory light.

The 2023 trials used a staggered start approach where each replicate was exposed to sediments for 28 days total. Juvenile mussels were sorted into three size groups (small, medium, and large) based on the relative sizes of the cohort. Due to the low numbers of available mussels in the small size group, all mussels used in the 2023 trial were from the medium or large size group. All replicates were randomly assigned to either the medium or large size group, and 10 mussels were added to each beaker. For the exposures, deionized water was formulated to dilute hardness of 90-110 mg/L as $CaCO_3$ using the reformulated moderately hard reconstituted water described in Smith et al. (1997). Sediment samples from each site were homogenized and 100 mL of sediment was added to 300 mL beakers with 175 mL of overlying reconstituted water before being placed in the dilution table on day -1, 1, and 3 of the exposure trial. The sand control sediment was washed and refrigerated with the collected sediment prior to exposures. The initial plan was to add mussels to 2 replicates from each site and 2 replicates from the sand controls to the dilution table on day 0, 2, and 4 of the trial (40 beakers per day, 6 replicates per site, 120 beakers total). However, due to issues with high ammonia, mussels were added to 40 beakers on day 0, 38 beakers on day 7, 32 beakers on day 9, 9 beakers on day 11, and 1 beaker on day 14. The initiation day for each beaker was retained as day 0 for calculating the exposure length.

Shell length and height for each mussel was measured at the start and end of the trial using a microscope with an AmScope MU1400B or MU900 camera set at 0.8x or 0.63x magnification interfaced with digitizing software (ImageJ) that was calibrated to a stage micrometer. Length was measured as the longest distance between the anterior and posterior ends of the mussel shell, while height was measured as a straight line from the umbo to the ventral shell margin. Mussels were photographed, and the level of magnification was recorded

for each replicate. A subsample of 210 mussels were preserved in 70% ethanol at the start of the trial (7 replicates of 3 size classes, 10 mussels per replicate) for initial dry weight determinations.

Six replicate beakers, each containing 10 juvenile mussels, were run for each treatment of sediment (from 19 study sites) and the control sand. Beakers were randomly distributed throughout the water renewal system and the overlying water was exchanged three times a day with approximately 175 mL per flush (Zumwalt et al., 1994). Mussels in each beaker were held in a mesh cup, constructed from 850 μm stainless steel mesh on the walls and 350 μm fabric mesh on the bottom secured with a zip tie to ensure that mussels were contained and able to be easily retrieved at the end of the trial.

All beakers were fed 2 mL of non-viable algal mixture (1 mL of Nannochloropsis concentrate and 2 mL of Shellfish Diet concentrate into 1.8 L of water) twice daily, after the first and last water change. In a single replicate from each site, temperature, pH, ammonia, and DO were measured daily. These water quality parameters, as well as hardness, alkalinity, and conductivity, were measured on days 0 and 28 of the exposures for each batch respectively.

The measured endpoints of the trail were survival and growth. Survival was determined by observing if shells were empty or gaped and if there is foot movement within a 5-minute period. Surviving juveniles were counted and preserved in 70% ethanol until growth measurements were recorded. At the end of the trials, average shell length was measured per replicate for all individuals from microscope photographs. Average dry weight per replicate was measured after mussels were dried for 24 hours at 60°C and then brought to room temperature in a desiccator for 24 hours.

Statistical Analysis

Data were analyzed using R statistical programming language (R Core Team, 2024). Recovery was calculated as the number of animals recovered divided by the number of animals added at the start of the study (n=10). Survival was calculated as the total number of animals that survived divided by the total number of animals that were recovered, multiplied by 100 to make it a percentage. The average percent change in length was calculated as the difference between the starting length and ending length, divided by the starting length, multiplied by 100. To calculate the average percent change in weight, we first had to calculate the initial weight using initial lengths and the standard dry weights collected from the subsample at the start of the trial. Direct measurement of initial dry weight was not possible, as dry weights were obtained by euthanizing mussels in ethanol. Initial weights were estimated using a predictive model based on a subsample of mussels (standards) collected at the beginning of the study.

To establish a relationship between length and dry weight, the initial length and dry weight of the standard mussels were measured, and a log-log (natural log) linear regression model was applied. The model:

$$\log(\text{Weight}) = \alpha + \beta \log(\text{Length})$$

where α represents the intercept or the expected weight (mg) when length (mm) is zero, and β the scaling exponent or rate at which mussel weight (mg) increases as length increases. The parameters for this equation were estimated using the measured values from the standard mussels. The model was used to predict the initial dry weight of mussels in the experiment based on their starting length, which was directly measured.

Data for mussel growth (i.e., average change in length/weight per replicate) and survival (e.g., % survival) were treated as response variables and analyzed separately. Land use

percentage was used as an explanatory variable. We recorded survival outcomes as binary data and analyzed it using a generalized (binomial) linear mixed effect model where the mussels that were alive at the end of the trial were counted as "successes" while dead mussels were "failures". We treated the percentage of agricultural land use and the toxicity trial start group as fixed effects, while the study site was treated as a random effect. Growth endpoints were recorded as average percent change in length and weight for each replicate. The relationship between growth end points and sediment variables from the toxicity trial was analyzed using a linear mixed effect model where average percent change in weight or length was the response variable, land use percentage and trial start group were fixed effects, and study site was treated as a random effect. Significance was considered at an α-level of 0.05 for all analyses.

Survival model: glmm((Alive, Died) ~ PercentAgriculture + Group + (1|Site))

Growth model**:** lme(PercentChangeGrowth) ~ PercentAgriculture + Group + (~1|Site)

Results

Sediment collection and analysis 2023

The handling of sediments collected in 2023 likely resulted in ammonia concentrations exceeding recommended levels based on ASTM guidelines (ASTM 2020), which advise that ammonia should not exceed 50% of the temperature- and pH-dependent Ammonia Chronic Criterion Magnitude (U.S. Environmental Protection Agency, 2013). We observed ammonia concentrations (2.81 mg/L) that were 5x higher than the recommended concentrations (0.55 mg/L) for our observed temperatures of 23 °C and average pH of 7.6. Sediments were only analyzed for pesticides and in the Gas chromatography/quadrupole time-of-flight mass spectrometry (GC-qToF) sediment screen, we did not find quantifiable amounts of any of the tested pesticides.

Toxicity Trial Results 2023

The average recovery for the toxicity trial in 2023 was 88.25%. The average percent survival for the trial was 89.1%. There were no significant differences in survival among study sites. Average percentage of agricultural land use in the watershed did not have a significant effect on survival. However, we did find that survival for the four trial groups was significantly different from each other (Table A.1), which was unexpected and led to the experimental changes made during 2024. The average percent change in length was 8.5% (Figure A.2). There were no significant differences in average percentage change in length between study sites. The average percentage agricultural land use in the watershed did not have a significant effect on average percent change in length. However, we did find that the average percentage change in length for the four trial groups were significantly different from each other (Table A.2). The average percentage change in weight for the trial was 58.3% (Figure A.3). There were no significant differences in the average percent change in weight between study sites. The average percentage agricultural land use in the watershed did not have a significant effect on the average percentage change in weight. However, we did find that the average percentage change in weight for the four trial groups was significantly different from each other (Table A.3).

Discussion

For the 2023 toxicity trial, survival was high for all treatments, and we did not see a difference in the growth endpoints between treatments. There was no observed effect of percentage land use on growth, nor were there any pesticides detected in sediments collected during June of 2023. However, we did see that there were significant differences in growth between the four trial start groups. Ammonia has been shown to be incredibly lethal to early life stages of mussels even at low concentrations (Newton et al., 2003; Wang et al., 2008). We

observed elevated levels of ammonia in exposure chambers from trial groups 2, 3, and 4 after sediment samples had initially been handled to start trial group 1. This spike in ammonia could have been caused by the increase in temperature from being taken out of refrigeration, or from mixing of the sediment to homogenize it to start trial group 1. While we waited to add animals to the exposure chambers until after the ammonia levels in the water had dropped to an acceptable level, there is still a possibility that interstitial ammonia was elevated. The methodology for sediment toxicity trial with juvenile mussels is relatively new and is still being updated (ASTM 2020). Future studies were adjusted to avoid the elevated ammonia observed in 2023.

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Tables

Table A.1. Parameter estimates from a generalized linear mixed model, estimating the effects of percentage land use and trial group on survival in experiments conducted during 2023. Estimates provided on the logit scale. P-values < 0.05 are considered significant.

	Estimate	Std Error	z-value	p-value
Intercept (Trial Start 1)	3.317	0.281	11.82	<0.001*
Percent Agriculture	-0.007	0.005	-1.273	0.203
Trial Start 2	-0.922	0.311	-2.964	0.003*
Trial Start 3	-1.832	0.291	-6.294	<0.001*
Trial Start 4	-1.191	0.412	-2.895	0.004*

Table A.2. Parameter estimates from a linear mixed model, estimating the effects of percentage land use and trial group on percent change in weight in experiments conducted during 2023. P-values < 0.05 are considered significant.

	Estimate	Std Error	t-value	p-value
Intercept (Trial Start 1)	11.77	1.18	9.95	<0.001
Percent Agriculture	0.02	0.03	0.66	0.5
Trial Start 2	-4.99	1.34	-3.71	<0.001
Trial Start 3	-5.27	1.40	-3.76	<0.001
Trial Start 4	-9.31	2.17	-4.29	<0.001

Table A.3. Parameter estimates from a linear mixed model, estimating the effects of percentage land use and trial group on percent change in weight in experiments conducted during 2023. P-values < 0.05 are considered significant.

	Estimate	Std Error	t-value	p-value
Intercept (Trial Start 1)	73.32	5.349	13.71	<0.001*
Percent Agriculture	0.148	0.157	0.943	0.358
Trial Start 2	-24.45	6.039	-4.049	<0.001*
Trial Start 3	-22.14	6.298	3.515	0.001*
Trial Start 4	-52.12	9.738	-5352	<0.001*

Figures

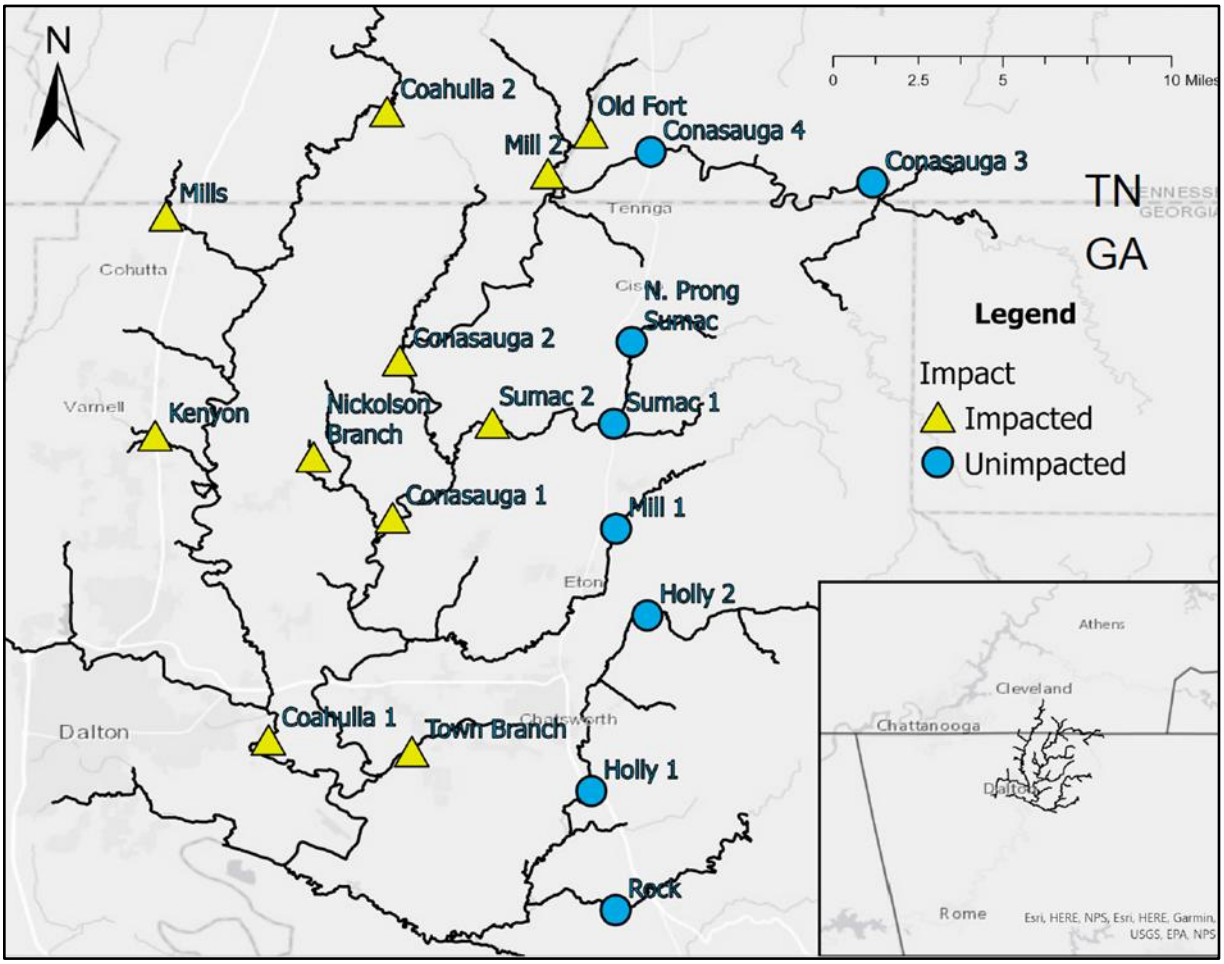

Figure A.1. Nineteen randomly selected study sites and study area for 2023 sediment toxicity trial. Impacted sites have less than 75% forested land cover present in the watershed. Unimpacted sites have greater than 75% forested land cover present in the watershed.

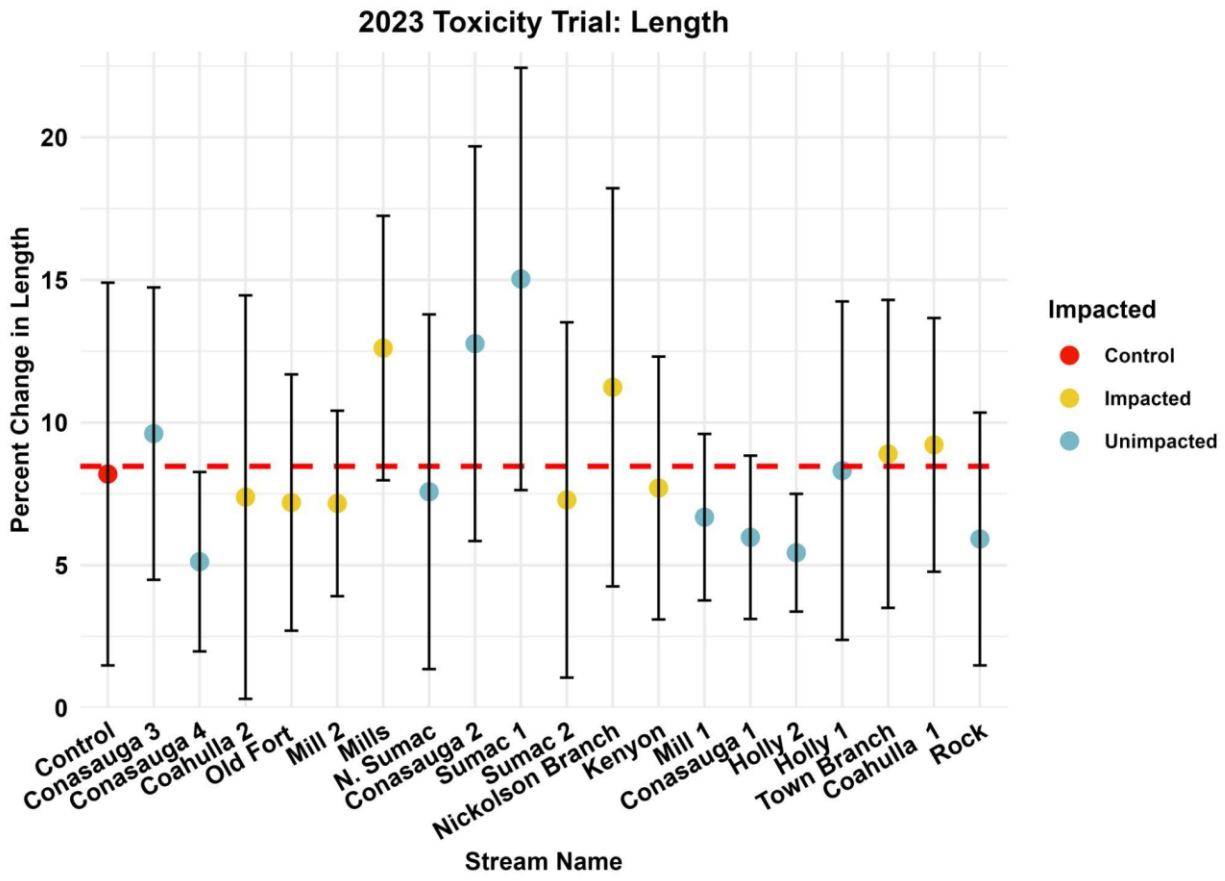

Figure A.2 Average percentage change in length for 19 study sites and control sand in experiments conducted during 2023. Red dashed line shows the overall mean (8.47%). Yellow points (Impacted) have less than 75% forested land cover present in the watershed. Blue points (Unimpacted) sites have greater than 75% forested land cover present in the watershed. The red point is the control sand. Error bars show 95% confidence intervals for each site.

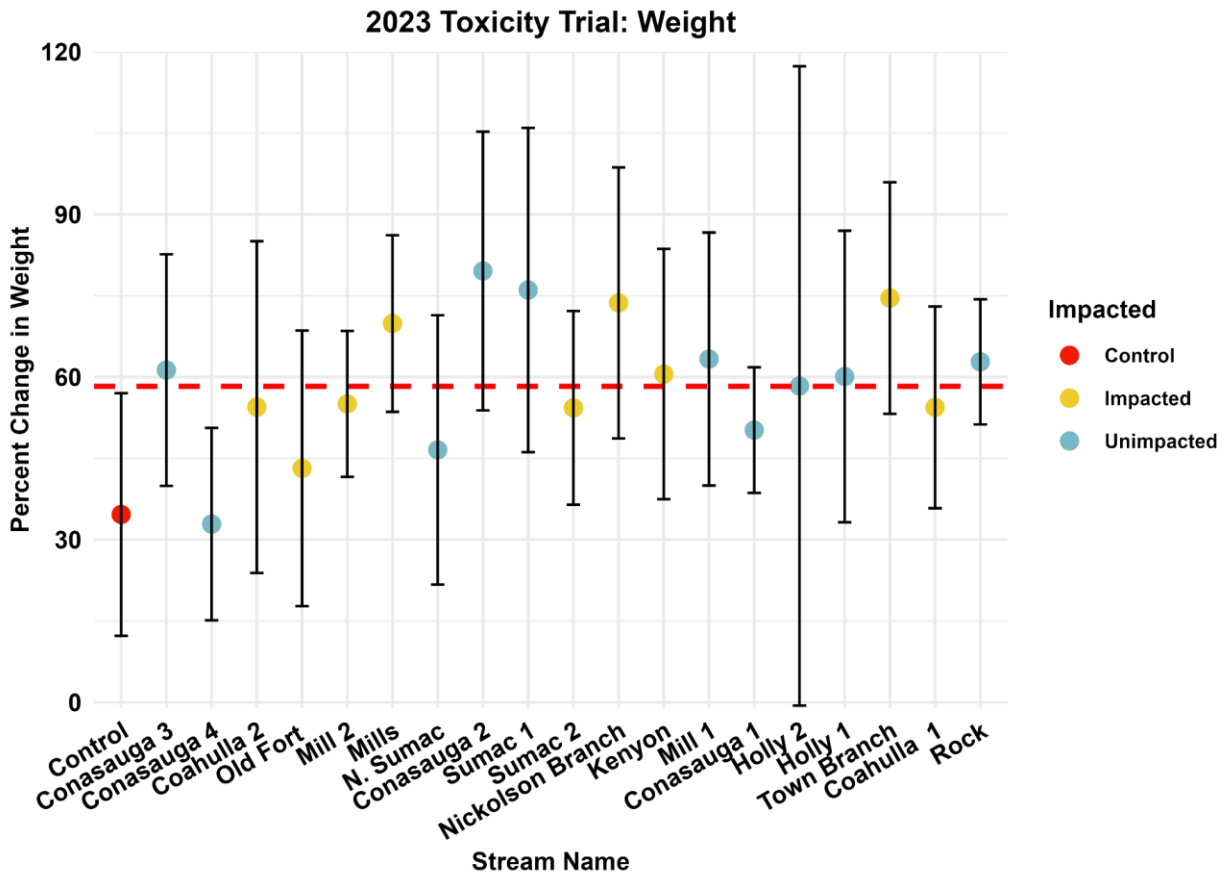

Figure A.3 Average percentage change in weight for 19 study sites and control sand in experiments conducted during 2023. Red dashed line shows the overall mean (58.29%). Yellow points (Impacted) have less than 75% forested land cover present in the watershed. Blue points (Unimpacted) sites have greater than 75% forested land cover present in the watershed. The red point is the control sand. Error bars show 95% confidence intervals for each site.

APPENDIX B VARIABLE SELECTION TABLE

Variable	Definition	Range	Unit	Hypothesis	Citation
Impact	Categorical variable. "Unimpacted" means 75% or more forested land cover in watershed sites. “Impacted” means 75% or less forested land cover in watershed. For data visualization not analysis	Impacted, Unimpacted	-	I expect sites that are categorized as "Impacted" to have less growth than sites that are categorized as "Unimpacted" for the toxicity trial where all other variables are kept the same. I do not expect the same relationship to hold in the silo study because of variability in natural conditions.	NA
NPDESDensity	The number of National Pollution Discharge Elimination System permits present in the watershed divided by the area of the watershed.	0 - 1.15	permits / km2	negative correlation between growth and the density of NPDES permits in the watershed. (As the density of NPDES permits increases, growth will decrease)	NA

Assessment	EPA 303(d) stream assessment	Supporting, Not Supporting, Not Assessed	-	I expect there to be less growth at sites that are in the "Not Supporting" category	NA
PerAgri	Reclassified NLCD raster to include all "agricultural" categories	1.42 - 45.77	%	I think there can be increased growth up to a certain percentage of Agricultural land use in the watershed. Agricultural land use does increase the productivity of a stream because of the nutrients it adds, but could also decrease mussel growth because of pesticides use, erosion of soil, alteration of riparian zone, and cattle access to the river. From Haag et al. 2019 "PC1 described a gradient of higher row crop agriculture, pesticides, nitrate/nitrite, and karst influence. Mussel growth was negatively correlated with PC1"	Haag et al 2019
PerFor	Reclassified NLCD raster to include all "forested" categories	26.41 - 96.63	%	I think there is a positive correlation between the percentage of forested landcover in the watershed and growth	
PerDev	Reclassified NLCD raster to include all "developed" categories	1.85 - 36.59	%	I think there is a negative correlation between percent developed land use in the watershed and growth. (Contaminants, heat, alteration in riparian zone, road run off, human recreation)	

Hardness	The amount of magnesium and calcium found in a 500mL sample of water	8.83 - 183.6	parts per million	Water hardness significantly influences the toxicity of certain contaminants like chromium (Wang 2023). Water hardness can decrease the toxicity of potassium (Kunz et al. 2021)	Wang et al 2023, Kunz et al 2021
pHWater	500 mL water sample analyzed for pH	7.1 - 8.3	-	Water pH would have to be very acidic to cause a negative effect (~ 5.0 and lower). We did not observe this. Can influence the toxicity of ammonia to juvenile mussels (Cope et al. 2008). Acidic water (6.0 - 4.5) can cause death in glochidia, and decrease survival of juvenile mussels	Cope et al 2008, Taskinen et al 2011
Aluminium_ppm	500 mL water sample analyzed for aluminum	0 - 0.97	parts per million	Increased aluminum concentration (0.5 mg/L), we saw concentrations nearly double that, decrease juvenile mussel survival (Taskinen et al. 2011)	Taskinen et al 2011
Boron_ppm	500 mL water sample analyzed for Boron	0 - 0.04	parts per million	Too low to have an effect. Hall et al 2014 saw effects starting at 56.1 mg/L of Boron in water-only exposures, our are much lower than that	Hall et al 2014
Calcium_ppm	500 mL water sample analyzed for calcium	2.54 - 46.73	parts per million	In the Bachelor Creek site, Calcium had the second highest deviance in growth rate attributed to it (negative correlation)	Skorupa et al 2024

Iron_ppm	500 mL water sample analyzed for iron	0 - 0.51	parts per million	Juvenile mussel survival was lower in combined aluminum + iron exposures (Taskinen et al 2011) (Iron concentration in the Taskinen paper was about double what we observed (1.0 mg/L))	Taskinen et al 2011
Potassium_ppm	500 mL water sample analyzed for potassium	0 - 5.19	parts per million	Potassium is really toxic to aquatic life but we didn't not see high concentrations of potassium. The potassium levels we found in this study are a third the concentration of what Gibson et al. 2018 found for an EC50 for the SAME SPECIES (our is 5,190 ug/L and she saw effects at 15,966 ug/L), so we may not see an effect. Kunz et al. 2021 found a reduction in growth of mussels in a 7-day toxicity test with K concentrations of 24mg/L, we observed 5.19 mg/L). These are toxicity test	Gibson et al 2018, Kunz et al 2021
Magnesium_ppm	500 mL water sample analyzed for magnesium	0.6 - 20.48	parts per million	In the Farmington and Nissihit sites Skorupa et al. 2024 found that Magnesium had the second highest deviance of mussel growth attributed to it (negative correlation)	Skorupa et al 2024
Manganese_ppm	500 ml water sample analyzed for manganese	0 - 0.2	parts per million	High concentrations of Mn in water may be associated with Mn concentration in mussel tissue (and the decline of wild mussel assemblages) but we did not measure these variables.	Archambault et al 2017, Cope et al

					2021, Rogers et al 2017, des Oliveira et al 2018
Sodium_ppm	500 mL water sample analyzed each month at every site	1.14 - 9.96	parts per million	I found two papers Wang et al. 2018 and Farag et al. 2014 but they are about sodium chloride and sodium bicarbonate respectively I am not sure they are comparable. Ayla also states this about her silo study: "Na1 was important in limiting the growth rates of age-1 and age-2 mussels across sites." In the Ware River site, Na+ was the second highest deviance in mussel growth rate (negative correlation)	Skorupa et al 2024
Phosphorus_ppm	500 mL water sample analyzed each month at every site	0 - 0.25	parts per million	Nutrient enriched surface waters found throughout Pete's study sites (he observed a mean of 0.275 ppm vs our highest 0.25 ppm). Said these levels are associated with eutrophication.	Lasier et al 2016
Silicon_ppm	500 mL water sample analyzed each month at every site	2.5 - 6.19	parts per million		

Chlorophyll-a	500 mL of water was collected and filtered through a 0.45 um fiberglass filter	0 - 16.87	ug/L	Average percent chang in weight and length will be positively correlated with cholorphyll concentration. This does not apply for the toxicity trial because food was a controlled variable. Ayla found that values over 2.82 ug/L resulted in higher mussel growth. Our values exceed the values she found (our max is 16.87 ug/L).	Skorupa et al 2024
Total Nitrate (NO3) + Nitrite (NO2)	Total Nitrate (NO3) + Nitrite (NO2) as N in a 500 mL water sample	0 - 1.15	parts per million	In elevated concentrations, Nitrate could increase toxicity of other variables (like Cr). Lasier et al 2016 found elevated nitrate at a lot of sites, observed and average of 0.7 mg/L (associated with eutrophication) vs our highest being 1.15 mg/L. Soucek and Dickinson et al. 2012 found LC50's of 357 mg/L and 937 mg/L for two species of mussels (L. silquoidae and M.nervosa)	Ning et al 2017, Soucek and Dickinson 2012, Lasier et al 2016
AvgTemp	Continuously measured by temperature loggers at each site	16 - 31	C	Will increase growth to certain piont but can also make other contaminants more toxic. Critical thermal maxmimum for three species is betweeen 39.5 to 42.7 C (Galbraith 2012). The lethal thermal tolerance of juvenile mussels is 35 C (Fogelman et al 2023). At site 88 where all our mussel died the temp reached 35C, but they were also out of the water. Low water temps associated with defaunted streams Haag et al. 2019	Galbraith 2012, Fogelman et al. 2023, Haag et al 2019

pHSoil	pH of the soil	5.71 - 7.40	-		
Ca	Calcium concentration in sediment	116 - 1,225	mg/kg (ppm)	This does range quite a bit so it may be interesting to look at. In the Bachelor Creek site, Calcium had the second highest deviance in growth rate attributed to it (negative correlation)	Skorupa et al. 2024
K	Potasium concentration in sediment	3.86 - 31.25	mg/kg (ppm)	Based on the levels of potassium we observed there should be a reduction in growth in replicates that exceed 24 mg/L (Kunz et al 2021). Gibson et al. 2018 found that potassium at 15.966 ppm had effects on C. nebulosus and we have potassium at over double that at 31.25 ppm. (maybe we need to take into account that we added some potsium to the water to make it moderately hard)	Kunz et al 2021, Gibson et al 2018
Mg	Magnesium concentration in sediment	17.9 - 148.9	mg/kg (ppm)	Kleinhez et al. 2019 EC50 = 241 and 10% (EC10) growth rate reductions = 88mg/L, We measured magnesium levels within that range so their could be an effect. And also what Ayla found reflected in the Magnesium box in the water rows	Kleinhenz et al. (2019)
Mn	Manganese concentration in sediment	10.7 -114.4	mg/kg (ppm)	Mn in sediment will be negatively correlated with average percent change in weight and length. Again, I think it may be unlikely that	Archambault et al 2017

				our Mn concentrations were high enough to cause and effect. Archambault et al 2017 reported levels as high as 700 mg/kg	
P	Phosphorus concentration in sediment	4.29 - 21.14	mg/kg (ppm)	I haven't found a study that look at the effects of phosphorus in the soil, is this comparable to studies that measured phosphorus in surface waters?	
Zn	Zinc concentration in sediment	1.04 - 4.73	mg/kg (ppm)	436 ug/L was the 96-hr EC50 for juvenil C. nebulosus. I don't know if it exactly converts, but we observed Zn concentrations much higher than that. (0.436 ppm vs. our 4.73 ppm). Wang et al. 2020 found a signifigant reduction in length, dry weight, and biomass during a 4 week exposure at Zn concentration as low as 120 ug/L or 0.12 ppm. During the 12-week exopsure he saw effects at 15 and 30 ug/L for all the same endpionts (and at all higher levels of Zn)	Gibson et al 2018, Wang et al 2020
Carbon	Percent carbon in sediment	0.168 - 0.917	%		
Nitrogen	Percent Nitrogen in sediment	0.031 - 0.073	%	Nitrogen can turn into ammonia and nitrate, publication seem to focus on ammonia and nitrate, also seems to be pretty low levels in the soil	

TOC	Total Organic Carbon in sediment	0.129 - 1.167	%	Low TOC associated with defaunted streams Haag et al 2019 (and these streams had lower growth)	Haag et al 2019
chromium	Chromium concentration (total acid digestion) in sediment	3.21 - 76.39	mg/kg (ppm)	Chromium might not have an effect in the toxicity trial because temperature was kept low and consistent across treatments. Chromium could have an effect in the silo study if a site has high chromium and high temperatures, although this is a difficult comparison to make because Ning's study is a toxicity trial not a silo study.	Wang et al 2017