



Freshwater Mollusk Biology and Conservation

REGULAR ARTICLES

Pages 30-39

Upper thermal tolerance of the western ridged mussel (*Gonidea angulata*), western pearlshell (*Margaritifera falcata*), and western sculpin species (*Cottus* spp.)

Rachael Valeria, Alexa Maine, Christine Parent, and James J. Nagler

Pages 40-48

Characterization of the native freshwater mussel assemblage across a reach of the Rappahannock River, Virginia

Traci P. DuBose, Jason Rohweder, Catherine Gatenby, Brian Watson, and Teresa J. Newton

Pages 49-56

Genetic variation in an invasive clam (form b: *Corbicula largilliertii*) across Illinois, USA

Brenden Mattfolk, Julia W. Bondi, Nicholas J. Iacaruso, Jeremy Tiemann, Mark A. Davis, and Joel B. Corush

Pages 57-66

Method development for rapid streamside assessment of freshwater mussel (order Unionida) hemocyte concentration and morphometrics

Madison E. Mueller, W. Gregory Cope, Christopher B. Eads, Loretta M. Lutackas, and Dorian J. Hayes

Pages 67-77

Distribution of microplastics in freshwater mussels across a watershed

James Brandon Williams and Michael M. Gangloff



REGULAR ARTICLE

UPPER THERMAL TOLERANCE OF THE WESTERN RIDGED MUSSEL (*GONIDEA ANGULATA*), WESTERN PEARLSHELL (*MARGARITIFERA FALCATA*), AND WESTERN SCULPIN SPECIES (*COTTUS SPP.*)

Rachael Valeria^{1*}, Alexa Maine², Christine Parent³, and James J. Nagler³

¹ Idaho Department of Fish and Game, Salmon, ID 83467 USA

² Department of Natural Resources, Confederated Tribes of the Umatilla Indian Reservation, Walla Walla, WA 99362 USA

³ Department of Biological Sciences, University of Idaho, Moscow, ID 83843 USA

ABSTRACT

Freshwater mussels are ecologically and culturally important and globally have the highest extinction and imperilment rates of any animal. The Western Ridged Mussel (*Gonidea angulata*) and the Western Pearlshell (*Margaritifera falcata*) are experiencing die-offs across their native ranges, and increasing water temperature is a possible contributing factor. To host their larval life stage, *G. angulata* uses the gills of sculpins (*Cottus* spp.) and *M. falcata* uses the gills of salmonids (*Oncorhynchus* spp.). The purpose of this laboratory study was to determine the upper thermal tolerances of *G. angulata* and *M. falcata* and two sculpin species, Margined Sculpin (*Cottus marginatus*) and Paiute Sculpin (*Cottus beldingi*), by using sublethal acute and chronic survival tests. The acute test consisted of increasing the water temperature until critical thermal maximum (CTmax) temperature behaviors were observed. The mean CTmax temperature was 34.1°C for *G. angulata* and 33.2°C for *M. falcata*, and the mean for the two *Cottus* spp. was 28.9°C. Based on the acute test results, *G. angulata* and *M. falcata* have a higher CTmax temperature than their host fish. The chronic test gradually increased the water temperature surrounding the test organism to 2°C below the CTmax temperature for each species. This temperature was maintained for 7 d, during which survival was recorded. All mussels perished, whereas 58.3% of the sculpins survived the chronic test. Survival curves from the chronic test showed that *G. angulata* survived longer and are more tolerant to prolonged elevated temperatures than *M. falcata*. These thermal tolerance tests demonstrate that a thermal separation between mussel and host fish is possible, thus limiting mussel reproduction as climate change leads to warming surface waters. In addition, tolerance to prolonged elevated temperature varies between these western mussel species.

KEY WORDS: bivalve, teleost, climate, sublethal, Unionidae, Cottidae

INTRODUCTION

Increasing water temperatures from climate change are altering freshwater ecosystems. Average water temperatures, extreme summer temperature events, and patterns of drought are projected to increase in the future (IPCC 2022), further threatening populations of freshwater mussels that are already stressed. In the western USA, two mussel species, the Western Ridged Mussel (*Gonidea angulata*) and the Western Pearlshell (*Margaritifera falcata*), have experienced significant declines over the past 2 decades (Xerces Society 2015; Blevins

et al. 2020). These mussels historically were an important food source for Columbia Plateau Tribes such as the Confederated Tribes of the Umatilla Indian Reservation (CTUIR). The CTUIR considers freshwater mussels a First Food (foods of significant traditional and cultural importance). First Food designations are closely tied to tribal creation beliefs that, as the foods promised to take care of the Native people, so the Native people have a reciprocal responsibility to take care of the First Foods (CTUIR 2015). Mussel harvest remains a treaty right for the CTUIR, but it is not currently practiced because of declines in freshwater mussel populations, especially in ceded territory in southeastern Washington and northeastern Oregon.

*Corresponding Author: rachael.valeria@idfg.idaho.gov

Historically, *G. angulata* was found in the western USA (California, Nevada, Idaho, Oregon, and Washington) and British Columbia, Canada (Blevins et al. 2020). It is a medium-to-large mussel with a maximum total length of 170 mm (Owyhee River, Oregon; A. Maine, personal communication) and a lifespan of 50–60 yr. *Gonidea angulata* has disappeared from 43% of its historic range (Blevins et al. 2017; Xerces Society and CTUIR 2020), possibly due to industrial pollution, habitat loss, invasive species, loss of host fish, and climate change (Downing et al. 2010; Snook et al. 2019). *Margaritifera falcata* is found in the western USA from California north to British Columbia and southern Alaska and inland to western Montana, Wyoming, and Utah (U.S. Fish and Wildlife Service 2023). It is a medium-to-large mussel with a maximum length of 162 mm (Salmon River, Idaho; Vannote and Minshall 1982) and a lifespan estimated to be 60–100 yr (Bauer 2001; Haag 2012; Maine et al. 2022). *Margaritifera falcata* has experienced a 17% population decline across its range (Blevins et al. 2017; Xerces Society and CTUIR 2020).

Recently, mass die-offs of *G. angulata* and *M. falcata* have been observed in which hundreds to thousands of mussels were killed (Blevins et al. 2020; Richard et al. 2023). At a long-term monitoring site on the Middle Fork John Day River, Oregon, mussel beds previously containing 500 *G. angulata* per square meter were reduced to zero between 2005 and 2007 (A. Maine, unpublished). Many factors have been implicated in the recent declines of *G. angulata* and *M. falcata* populations in the western USA (Blevins et al. 2020), including elevated water temperatures that occur during the summer. Determining the upper thermal limits of *G. angulata* and *M. falcata* is a first step toward understanding how their life histories allow vulnerability to warming waters and is thus key for conservation efforts to sustain these populations.

Like many freshwater mussels, the larvae (glochidia) of both *G. angulata* and *M. falcata* colonize the gills and/or skin of teleost host fishes for part of their life cycle. Studies show that *G. angulata* glochidia use sculpin gills (*Cottus* spp.; Haley et al. 2007; Stanton and MacConnachie 2012; O'Brien et al. 2013) and *M. falcata* use salmonid gills (e.g., Rainbow Trout, *Oncorhynchus mykiss*, and Cutthroat Trout, *Oncorhynchus clarkia*; Murphy 1942; Meyers and Milleman 1977; Karna and Millemann 1978). Summer die-offs of *Cottus* spp. have been observed on the Middle Fork John Day River, Oregon, between 2005 and 2021 (A. Maine, unpublished) that correlated with high water temperatures. The loss of host fishes would prevent successful reproduction of these mussel species.

A species' thermal tolerance is defined by a temperature range, that, when exceeded, can have sublethal (e.g., abnormal behavior, reduced growth, impaired reproduction) or lethal consequences (Paaijmans et al. 2013; Westhoff and Rosenberger 2016; Cereja 2020). Laboratory thermal tolerance studies for fish species use two primary approaches: critical thermal (CT) or incipient lethal temperature (ILT) (Beitinger et al. 2000). The CT method is a dynamic assay, where an individual is

subjected to increasing (i.e., CTmaximum [CTmax]) or decreasing (i.e., CTminimum) temperatures until a sublethal endpoint is reached (Cowles and Bogert 1944; Paladino et al. 1980; Beitinger et al. 2000). The ILT method is a static assay, where an individual is suddenly exposed to and then held at a constant temperature for a specified amount of time, or until specific endpoints are reached. The CT method has the following two advantages over the ILT method: (1) there is gradual linear change in temperature over time until the predetermined endpoints occur; and (2) sample sizes can be smaller, which is useful for studies of species that are threatened or have small population sizes (Beitinger et al. 2000; Rodland et al. 2009). Although CT testing is common in fish, few CT studies have been done on invertebrates, including freshwater mussels (Galbraith et al. 2012).

Our first objective was to determine upper thermal tolerances for *G. angulata* and *M. falcata* by using the CTmax method. Our second objective was to determine the upper thermal tolerances for *C. marginatus* and *C. beldingi*, two western *Cottus* spp. whose ranges overlap with *G. angulata*, also using the CTmax method. The fish hosts for *M. falcata* were not tested because CTmax temperature data are available from previous studies for several salmonids (Heath 1963; Becker and Genoway 1979; Currie et al. 1998). Our third objective incorporated the CTmax temperature data for the mussels and fish in a chronic (7-d) thermal test with a survival endpoint to assess prolonged exposure to elevated water temperature.

MATERIALS AND METHODS

Mussel and Fish Collections

Gonidea angulata (61–88 mm total length) were collected in July 2024 via snorkeling in the Salmon River (Table 1). Upon capture, mussels were put in a draw-string net bag that was placed in a shaded, flowing area of the river. Stream temperature at the time of collection was 17°C. Mussels were layered in clean damp cloths in coolers without water and transported on the day of capture to the CTUIR Mussel Propagation Laboratory (Walla Walla, WA, USA). Ice was added to the coolers during transport, approximately every hour as needed, to maintain river water temperature. *Margaritifera falcata* (23–48 mm total length) were similarly collected and transported from the Grande Ronde River, Oregon, in July 2023 (Table 1). Stream temperature at the time of collection was 21.6°C.

Sculpins (*Cottus* spp.) >40 mm total length were collected by electrofishing from streams in the Walla Walla River basin in June and July 2023 (Table 1). Stream temperatures varied between 12 and 18°C during collection. Captured fish were placed in coolers with aerated stream water for transport to the lab. Ice packs added approximately every hour as needed to maintain stable water temperature. Sculpins were identified as Margined Sculpin (*Cottus marginatus*) and Paiute Sculpin (*Cottus beldingi*), based on external morphological traits (Whitney

Table 1. Collection locations, dates, water temperatures, and total counts (*N*) of mussels and fish used in this study from the Columbia River Drainage.

Species	Waterbody	Elevation (m)	Date(s)	Temperature (°C)	<i>N</i> (Acute)	<i>N</i> (Chronic)
<i>Margaritifera falcata</i>	Grande Ronde River, OR (45.232439, –118.396278)	1,034	July 3, 2023	17	54	43
<i>Gonidea angulata</i>	Salmon River, ID (45.41889, –116.1620000)	1,829	July 11, 2024	22	32	36
<i>Cottus marginatus</i>	South Fork Walla Walla River, OR (45.8576058, –118.2210385)	542	June 12, July 14, 2023	12, 16	11	4
<i>Cottus marginatus</i>	Mill Creek, WA (46.0818599, –118.2519255)	383	June 5, July 13 and 14, 2023	16, 21, 16	17	8
<i>Cottus marginatus</i>	Walla Walla River, OR (45.9455112, –118.3844809)	306	June 15, 2023	16	3	13
<i>Cottus beldingi</i>	South Fork Walla Walla River, OR (45.8576058, –118.2210385)	542	June 12, July 14, 2023	12, 16	14	10
<i>Cottus beldingi</i>	Mill Creek, WA (46.0818599, –118.2519255)	383	June 5, July 13 and 14, 2023	16, 21, 16	3	13

and Wydoski 2003; Page and Burr 2011) and geographical location (Young et al. 2022).

General Laboratory Husbandry

In the laboratory, mussels were held in polycarbonate “Cambro” pans (CamWear Cambro Pans, Huntington Beach, CA, USA; 53 × 32 × 15 cm [25.6 L]), with screened sides and a mixed sand–gravel substrate. Each individual mussel was measured and tagged with a unique number on either a homemade paper or vinyl shellfish tag (Floy Tag and Manufacturing, Seattle, WA, USA). The pans were held in a flow-through water bath (600–800 mL/s) supplied with well water at 13°C. The pans were maintained in a room with a 9:15 (light:dark) photoperiod (light period: 0730 to 1600 h). Mussels were fed 2 mL of Shellfish Diet 1800 and 1 mL of Nanochloropsis 3600 (both feeds from Reed Mariculture, Campbell, CA, USA) per liter of water once daily (modified from Wang et al. [2021]).

Sculpins were held individually in 1.5-L polycarbonate tanks, with gravel-sized substrate, in an Aquatic Habitats for Accelerated Bioresearch (AHAB) system (Pentair Aquatic Systems, Apopka, FL, USA) with dechlorinated tap water at 13°C and a flow rate of 15 mL/s. The AHAB system is a pre-fabricated multitank recirculating system used for fish and mussel propagation. The photoperiod used for sculpins was identical to that of the mussels. Sculpins were fed freshly thawed bloodworms (San Francisco Bay Brand, Newark, CA, USA) to satiation every other day (similar to Walsh et al. [1997]). Fish care and experimentation were conducted in accordance with Animal Care and Use Protocol IACUC-2022-63, reviewed and approved by the University of Idaho Animal Care and Use Committee.

Mussel Thermal Experiment Protocols

The CTmax method was used in acute trials for all species. The CTmax temperature was recorded as the temperature at

which “locomotory activity becomes disorganized and the animal loses its ability to escape conditions that will promptly lead to its death” (Paladino et al. 1980; Beiting et al. 2000). Protocols have been established for measuring CTmax temperature for ectothermic vertebrates (Hutchison 1961; Beiting et al. 2000; Cereja 2020), but few exist for invertebrate organisms, including for mussels (Rodland et al. 2009; Galbraith et al. 2012). The onset of valve gaping (i.e., valves open visibly wider than normal) has been used as a CTmax temperature behavioral endpoint for other freshwater mussel species (Galbraith et al. 2012), and gape duration and frequency were shown to change as water temperature increased (Rodland et al. 2009). To determine CTmax temperature behavioral endpoints for *G. angulata*, a pilot study was conducted on a few individuals where the water temperature was increased until lethality or close to lethality. Based on Galbraith et al. (2012) and the pilot study observations, the CTmax temperature was determined by observing at least two of the following behaviors: extreme valve gaping with parting of mantle tissue edges; no response from foot and no valve closure to probing with a glass stir rod; and rapid valve closure and opening (i.e., water rushing out quickly, “gasping”). These behaviors were consistent observations of upper thermal stress in *G. angulata* and therefore used as the CTmax temperature behavioral endpoints for this mussel. The acute thermal trials were conducted in an apparatus that gradually increased the water temperature (~2–2.5°C/h; Lee and Rinne 1980; Smale and Rabeni 1995) over a 7- to 8-h period until the CTmax temperature (Fangue et al. 2006) was reached. Mussels were visually checked (and probed when needed) for responses throughout the whole trial period. Before each trial, mussels were acclimated at 13°C for at least 7 d (McClanahan et al. 1986; Galbraith et al. 2012) and food was withheld 24 h before temperature treatment. Mussels were not fed during the trials. Dissolved oxygen levels were monitored with a dissolved oxygen probe (YSI Inc., Yellow Springs, OH, USA, and multiparameter sonde from In-Situ Inc.,

Fort Collins, Colorado, USA) during trials. Mussels were never used in more than one trial.

Three acute thermal trials were conducted with *G. angulata* in July 2024, with 12 mussels per trial (6 treatment and 6 control) for two of the trials and 8 mussels per trial for one trial (4 treatment and 4 control). For the treatment group, individual *G. angulata* were placed into 960-mL plastic containers (Tupperware, Dart Industries Inc., Orlando, FL, USA) filled with water at 13°C and without substrate. The containers were arranged within a Cambro pan, as a water bath, and filled with 13°C water placed on two magnetic stir plates. Two magnetic stir bars were placed in the bottom of the polycarbonate pan to provide water circulation. Each individual mussel container was equipped with a temperature logger (HOBO MX Temp 400, Onset Computer Corporation, Bourne, MA, USA) for continuous temperature recording and air supplied with an air stone. The Cambro pan was equipped with a submersible water heater (1000W PONG-DANG, KD Heater Co. Ltd., Gyeonggi-do, South Korea) connected to an electric temperature controller (Ranco, Grainger Inc., Lake Forest, IL, USA) to achieve a water temperature increase of 2–2.5°C/h. The control treatment consisted of *G. angulata* subjected to the same conditions but maintained at a constant water temperature of 13°C. Once the trial commenced, water temperature in the heat-treated group was increased until the CTmax temperature for each individual mussel was determined. Once the CTmax temperature was recorded for an individual, the mussel was removed from the test apparatus and returned to 13°C. Feeding was resumed the day after the trial, and mussels were monitored for 1 wk posttrial to observe any delayed mortality.

Five acute thermal trials, using 48 mussels, were conducted with *M. falcata* in July and August 2023. Sample size ranged from 6 (3 treatment and 3 control) to 16 mussels (8 treatment and 8 control) per trial. Testing and posttesting conditions were similar to those for *G. angulata*, but with several exceptions. Glass beakers (300 mL) were used for *M. falcata*, and five (four 25-W, one 125-W) submersible aquarium heaters (Hydor, Bassano del Grappa, Vicenza, Italy) were used to achieve the appropriate water temperature increase. To determine CTmax temperature behavioral endpoints for *M. falcata*, a pilot study like the one used for *G. angulata* was conducted. Based on Galbraith et al. (2012) and pilot study observations, the CTmax temperature for *M. falcata* was determined by observing at least two of the following behaviors: extreme valve gaping with parting of mantle tissue edges, no response from foot to probing with a glass stir rod, and no valve closure or discharging of mucus-like substance.

The chronic thermal trials were a survival (lethal) test, measuring the time to death at a temperature 2°C below the CTmax temperature for each species. When designing this experiment, we determined it would be beneficial to start below the CTmax temperature because mortality would likely occur quickly once it was reached. A custom-built chronic testing apparatus that held 48 1.5-L polycarbonate tanks (one mussel per tank) was used for these trials. Half of the tanks

were connected to a heated recirculating well-water supply (heat-treated group), and the other half were connected to a chilled recirculating water supply maintained at 13°C (control group). The chronic trials were performed on 38 naïve (not previously tested) *G. angulata* (19 treatment and 19 control) and 42 naïve *M. falcata* (21 treatment and 21 control) in August 2024 and August 2023, respectively. The water in the chronic testing apparatus was heated at the same rate as for the acute trials, but heating was terminated 2°C below the CTmax temperature for each species. This chronic temperature was constantly maintained for 7 d (168 h). Each individual tank was equipped with a temperature logger (HOBO MX Temp 400 or HOBO Pendant Light/Temp, Onset Computer Corporation). Pretrial acclimation conditions were the same as in the acute thermal trials. Mussels were not fed during the chronic thermal trial. Every day during the chronic trial period, the foot or valve opening of the mussel was probed gently with a glass stir rod at 0800 and 2000 h. Mortality was determined by (1) open valves with over extension of the anterior and posterior adductor muscles and (2) no response to probing. When mortality was observed, the mussel and logger were removed from the tank. This trial was conducted once for each species. Dissolved oxygen was measured twice every day in both treatment and control groups.

Sculpin Thermal Experiment Protocols

Four acute thermal trials, using a total of 48 *Cottus* spp., were conducted in June and July 2023. Each trial used 12 sculpins (6 treatment and 6 control). Overall, 16 *C. marginatus* and 8 *C. beldingi* were used in the heat treatments and 15 *C. marginatus* and 9 *C. beldingi* were used in the control groups. Sculpins were acclimated and tested like the mussels, with the following exceptions. The 1.5 L AHAB holding tanks, containing each fish with a layer of gravel-sized substrate, were moved directly into the Cambro pan water bath. The CTmax temperature behavioral endpoints for sculpin were determined by opercular flaring and failure of a fish to right itself within 3 s when it was rolled over on its side or back by using a glass stir rod (i.e., loss of equilibrium). Because sculpins do not consistently undergo violent muscular spasms (Kowalski et al. 1978; Walsh et al. 1997), as noted in other fish species when experiencing thermal stress, this response was not used. Once the CTmax temperature was attained for each individual, the tank and fish were returned to the AHAB system, where the water temperature was allowed to gradually decline to 13°C. Dissolved oxygen levels were monitored, similar to those for the mussels. As with the mussels, sculpins were never used in more than one trial.

The chronic testing apparatus and conditions used for sculpins were like that for the mussels, with a few exceptions. Every day at 0800 and 2000 h, *Cottus* spp. were checked for movement and probed with a glass stir rod if unmoving. If the fish did not move when probed and there was no opercular movement, this was considered mortality, which was the chronic test endpoint. A total of 48 naïve sculpins (25 *C. marginatus* and 23 *C. beldingi*) were

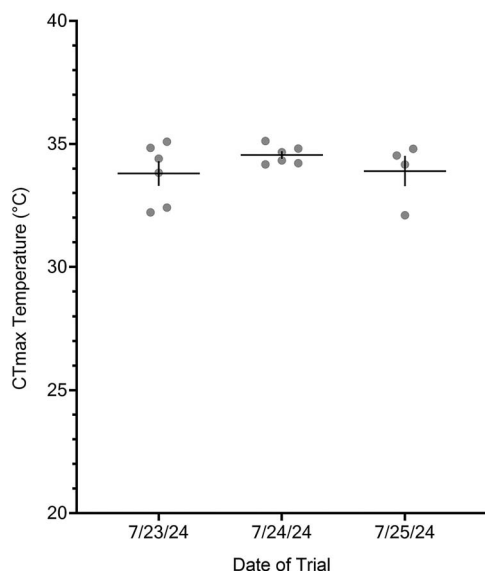


Figure 1. Critical thermal maximum (CT_{max}) water temperatures for *Gonidea angulata* determined in the acute thermal experiment. Each date represents a separate trial, and each shaded circle represents a single individual. The horizontal lines represent the mean, and the vertical lines represent the SE. The CT_{max} temperatures did not differ significantly among trials ($F(2, 13) = 0.985$, $P = 0.399$).

tested: 24 heat treated (10 *C. marginatus* and 14 *C. beldingi*) and 24 controls (15 *C. marginatus* and 9 *C. beldingi*) in July and August 2023. When mortalities were observed, the fish and temperature logger were removed from the tank. After 7 d, the water temperature was gradually returned to 13°C. During the trial period, and posttrial for 7 d, the fish were observed for signs of distress or mortality. This trial was conducted once, and as with the mussels, the sculpins were not fed during the trial. Dissolved oxygen levels were measured daily.

Statistical Analysis

Differences between the CT_{max} temperatures across all acute trials for a given species were assessed by one-way analysis of variance (normally distributed data) or Kruskal–Wallis (nonnormally distributed data) tests. Because the CT_{max} temperatures were not different across trials for each mussel species, a *t*-test was used to compare between species. Statistical significance was set at $\alpha < 0.05$ for all acute trials. The data from the chronic trials for all species were plotted using Kaplan–Meier survival curves (Kaplan and Meier 1958; Rich et al. 2010). We examined differences among survival curves by using the log rank test ($P < 0.05$). All statistical and graphical analyses were performed with Prism 10.6.0 (GraphPad Software, LLC, San Diego, CA, USA).

RESULTS

Acute Thermal Experiments

The mean CT_{max} temperatures for *G. angulata* ranged from 33.8 to 34.5°C (Fig. 1). The mean CT_{max} temperatures

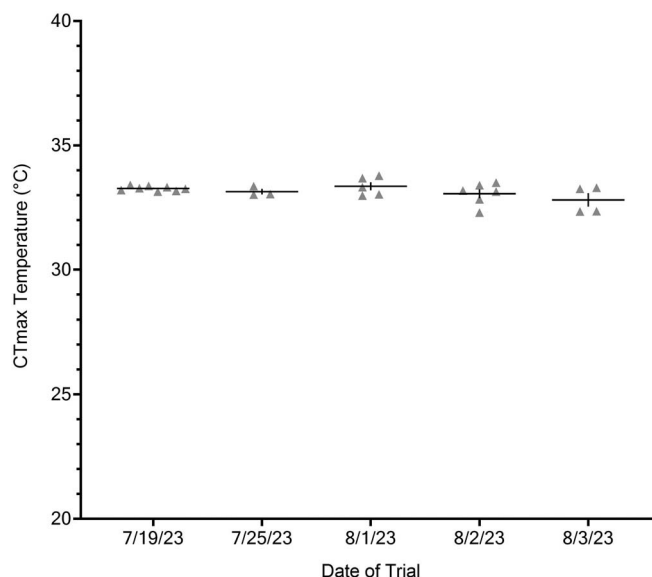


Figure 2. Critical thermal maximum (CT_{max}) water temperatures for *Margaritifera falcata* determined in the acute thermal experiment. Each date represents a separate trial, and each shaded triangle represents a single individual. The horizontal lines represent the mean, and the vertical lines represent the SE. The CT_{max} temperatures did not differ significantly among trials ($F(4, 21) = 1.77$, $P = 0.172$).

from the three trials were not significantly different ($F(2,13) = 0.98$, $P = 0.399$) from one another; therefore, a grand mean of $34.1 \pm 0.5^\circ\text{C}$ was calculated. The behavioral endpoints used to determine the CT_{max} temperature for *G. angulata* were consistent across individuals. Mussels extended their foot immediately when placed into the treatment containers. From 26 to 28°C, some mussels retracted their foot and closed their valves completely. *G. angulata* was slow to respond to probing when close to the CT_{max} temperature. Eventually, all mussels exhibited excessive valve gaping with retraction of mantle tissue and rapid valve closure. No mortalities were observed in any treatment group, and one mortality occurred in the control group. One additional mortality from the control group was observed during the 1-wk posttrial period. In the control group, the mean temperature was $14.6 \pm 0.2^\circ\text{C}$ through all trials and had 87.5% survival with a lack of CT_{max} temperature endpoints. Dissolved oxygen levels were between 87 and 93% saturation throughout the trials.

The mean CT_{max} temperatures for *M. falcata* in the five trials ranged from 32.8 to 33.4°C (Fig. 2). The CT_{max} temperatures were found to not differ significantly among trials ($F(4, 21) = 1.77$, $P = 0.172$); therefore, a grand mean of $33.2 \pm 0.1^\circ\text{C}$ was calculated. The response of *M. falcata* to increasing temperature during the acute thermal trials was consistent across individuals. Mussels were completely closed at the beginning of the trials, but by 30–31°C displayed foot extension. When reaching the CT_{max} temperature, *M. falcata* exhibited no response to foot touch by probing and extreme valve gaping with retraction of mantle tissue was observed. Many mussels also discharged a mucus-like substance. No

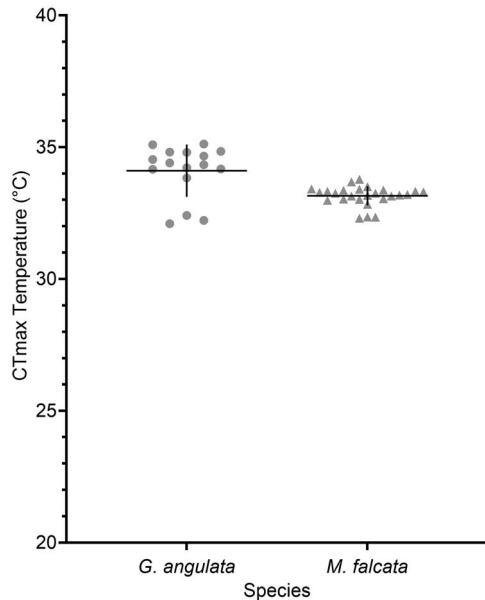


Figure 3. Comparison of critical thermal maximum (CTmax) temperatures of *Gonidea angulata* (gray circles) and *Margaritifera falcata* (gray triangles) from the acute thermal trials. Each point represents an individual mussel. The horizontal lines represent the mean, and the vertical lines represent the standard error. The CTmax temperatures for *G. angulata* and *M. falcata* were significantly different ($F(15, 25) = 7.34$, $P = 0.0017$).

mortalities were observed in either the heat-treated or control groups during the trials or after 1 wk posttrial. The control temperature was $15.10 \pm 0.01^{\circ}\text{C}$ (mean $\pm$ SE) during the trials, and both groups had 100% survival with a lack of CTmax temperature endpoints. Dissolved oxygen levels were $>80\%$ saturation throughout the trials. The CTmax temperatures for *G. angulata* and *M. falcata* were compared statistically and did differ significantly ($F(15, 25) = 7.34$, $P = 0.0017$; Fig. 3).

The mean CTmax temperature for *Cottus* spp. in the five trials ranged from 28.7 to 29.2°C (Fig. 4). The mean CTmax temperatures did not differ significantly ($F(3, 20) = 1.54$, $P = 0.235$) among trials or between *C. marginatus* and *C. beldingi* ($F(15, 7) = 1.33$, $P = 0.300$; Fig. 5). Therefore, a grand mean of $28.9 \pm 0.2^{\circ}\text{C}$ was calculated. All sculpins in the heat-treated group displayed erratic swimming behavior immediately after the tank was moved into the water bath, but by 3 h ($\sim 20^{\circ}\text{C}$) all fish were calm. Response to the increasing water temperature was notable at 26–27°C. At this temperature, sculpins exhibited more rapid opercular movement and began to push their mouths against the side of the tank and open their mouths widely. Some sculpins swam up and down the tank erratically. The erratic swimming behavior was almost immediately followed by a loss of equilibrium that defined the CTmax temperature endpoint. There was one mortality during the acute thermal experiment, in the heat-treated group, but no mortality occurred in the controls. There was no mortality 1 wk posttrial in either treatment group. The mean control temperature was $15.2 \pm 0.1^{\circ}\text{C}$. Dissolved oxygen levels were 89–126% saturation throughout the trials.

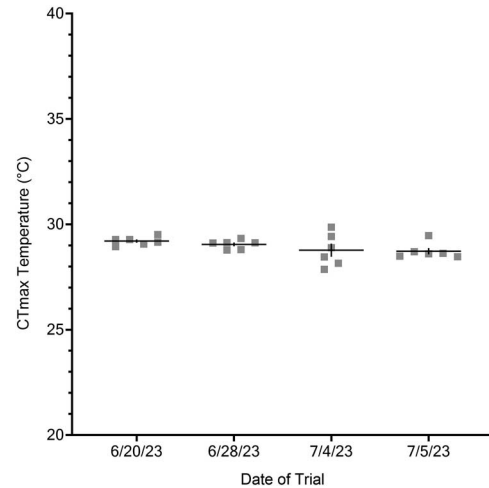


Figure 4. Critical thermal maximum (CTmax) water temperatures for *Cottus* spp. determined in the acute thermal experiment. Each date represents a separate trial, and each shaded square represents a single individual. The horizontal lines represent the mean, and the vertical lines represent the SE. The mean CTmax temperatures did not differ significantly among trials ($F(3, 20) = 1.54$, $P = 0.235$).

Chronic Thermal Experiments

For *G. angulata*, the chronic test temperature used was 32.1°C (2° below the CTmax temperature grand mean of 34.1°C calculated from the acute experiment), attained by 8 hours, and this point was considered the beginning of the trial (hour 0). The survival curve for *G. angulata* showed the first mortality occurred at hour 31 and by hour 139, all mussels were dead (Fig. 6). There was no mortality in the control group during testing or 1 wk posttrial. Dissolved oxygen measurements were 95–113% saturation during the *G. angulata* chronic thermal trial.

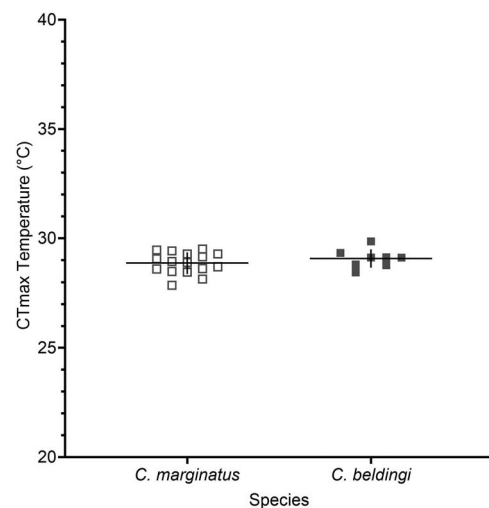


Figure 5. Comparison of the critical thermal maximum (CTmax) temperatures of *Cottus marginatus* (open gray squares) and *Cottus beldingi* (closed gray squares) from the acute thermal trials. Each point represents an individual fish. The horizontal lines represent the mean, and the vertical lines represent the SE. The mean CTmax temperatures did not differ significantly between *C. marginatus* and *C. beldingi* ($F(15, 7) = 1.33$, $P = 0.300$).

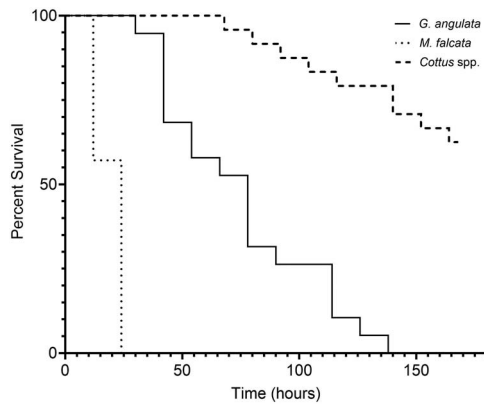


Figure 6. Kaplan–Meir survival curves for *Gonidea angulata* (black), *Margaritifera falcata* (dark gray), and *Cottus* spp. (light gray) from the chronic thermal experiments. Note, the temperature at the beginning (hour 0) of each trial was species specific (2°C below the critical thermal maximum [CTmax] temperature grand mean determined in the acute thermal trials).

For *M. falcata*, the chronic test temperature used was 31.2°C (2° below the CTmax temperature grand mean of 33.2°C calculated from the acute trials), attained by 7 h, and this point was considered the beginning of the trial (hour 0). The survival curve for *M. falcata* had nine mortalities by hour 13 and by hour 25, all remaining *M. falcata* were dead (Fig. 6). There was no mortality in the control group during testing or 1 wk post trial. Dissolved oxygen measurements were 90–111% saturation during the *M. falcata* chronic thermal trial. The survival curves for *G. angulata* and *M. falcata* were significantly different ($P < 0.0001$; Fig. 6).

For *Cottus* spp., the chronic test temperature used was 26.9°C (2° below the CTmax temperature grand mean of 28.9°C calculated from the acute trials), attained by 6 h, and this point was considered the beginning of the trial (hour 0). The survival curve for *Cottus* spp. showed one mortality by hour 69 followed by other additional mortalities, but 14 sculpins (62.5%) survived until the end of the treatment period (hour 168; Fig. 6). The heat-treated group had one mortality an hour after the 7-d trial was concluded. Among the nine mortalities, six were *C. marginatus* and three were *C. beldingi*. There was no mortality in the control group during testing the 1 wk posttrial. Dissolved oxygen measurements were 90–111% saturation during the *Cottus* spp. chronic thermal trial. The *Cottus* spp. survival curve was significantly different from the mussel survival curves ($\chi^2 = 91.04$, $df = 2$, $P < 0.0001$; Fig. 6).

DISCUSSION

Mussel Thermal Experiments

This study is the first to report the upper thermal limits for two freshwater mussels, *G. angulata* and *M. falcata*, native to the Pacific Northwest. The acute test found similar CTmax temperatures for the two mussel species, with *G. angulata* at 34.1°C and *M. falcata* at 33.2°C. There are a limited number of studies testing upper thermal limits in freshwater mussels

(reviewed in Fogelman et al. [2023]). One comparable study used the CTmax method for three species from Pennsylvania: *Alasmidonta varicose* (Brook Floater Mussel), *Elliptio complanate* (Eastern Elliptio Mussel), and *Strophitus undulatus* (Creeper Mussel) (Galbraith et al. 2012). These mussels were acclimated at 15 or 25°C before testing began. The CTmax temperature results were 39.5 and 41.1°C for *A. varicose*, 42.7 and 41.4°C for *E. complanate*, and 40.0 and 42.3°C for *S. undulatus* at each of the acclimation temperatures, respectively. The CTmax temperatures for these three mussel species are all considerably higher than our CTmax temperature results for *G. angulata* and *M. falcata*. Besides the considerable geographic diversity, the differences in CTmax temperatures between these freshwater mussels are likely because they are different species. Another possibility is that Galbraith et al. (2012) used two different acclimation temperatures (15 and 25°C), and there was a clear pattern of an effect of acclimation temperatures on the CTmax temperature. The higher acclimation temperature resulted in a higher CTmax temperature. In other studies, the effects of acclimation temperature on mussels have been mixed (Polhill and Dimock 1996; Pandolfo et al. 2012). However, it is possible that different acclimation temperatures could explain the differences in CTmax temperature for these different mussel species.

We used a 7-d chronic thermal experiment as a survival test. Although substantial mortality was not intended, no mussels of either species survived until the end of the test period. The survival curves were significantly different between the two mussel species with *G. angulata* showing more tolerance to prolonged elevated temperature than *M. falcata*. Several factors may explain these results. As with the acute tests, these are different mussel species. Also, the starting temperature for the chronic tests was 1°C different between the two mussel species; although this difference is small, it cannot be discounted. Finally, the *G. angulata* and *M. falcata* used in this study differed significantly in body size. *M. falcata* (length 23–48 mm) was considerably smaller than *G. angulata* (length 61–88 mm). Smaller mussels would warm more rapidly and have higher mass-specific metabolic rates. The physiological effect(s) of increasing water temperature likely impacted the smaller *M. falcata* more rapidly and resulted in mortality occurring earlier than for *G. angulata* (Spooner and Vaughn 2008; Ismail et al. 2016; Lopez et al. 2022).

Acute thermal testing identified novel behavioral endpoints for *G. angulata* and *M. falcata*. The extreme valve gaping response, as a CTmax temperature behavioral endpoint, has been previously reported (Galbraith et al. 2012) and occurs when adductor muscles relax, mantle edges and siphons part, and the foot is extended or partially extended. In unfavorable water conditions, mussels have been observed lying prone with the valves gaping and foot extended (Allen 1923; Galbraith et al. 2012). In our pilot experiments, most *G. angulata* individuals exhibited foot extension over the duration of the heat treatment, whereas *M. falcata* did not consistently display foot

extension. The extended foot extension time in *G. angulata* may be due to their affinity to burrow deeply into the sediment (Vannote and Minshall 1982; A. Maine, unpublished). Although both mussels in our study showed extreme valve gaping behavior and no response to foot touch, there were slight differences in behavior due to elevated temperature observed between the two species. When nearing their CTmax temperature, many of the *G. angulata* individuals displayed a pattern of slowly gaping wider, bringing in water and then rapidly closing valves, thus pushing out water forcefully. This discharge of water from the mantle chamber has been observed with vigorous probing (Allen 1923), but *G. angulata* displayed this behavior without stimulation. It was not observed in *M. falcata*. However, *M. falcata* consistently discharged a white mucus-like substance when nearing their CTmax temperature that was not consistently observed in *G. angulata*. Some mussel species can develop a mucus plug over mantle tissues to prevent direct exposure to the atmosphere during low water periods (Byrne and McMahon 1994), so mucus shedding could represent a mussel stress response.

Sculpin Thermal Experiments

This study is also the first to report the upper thermal limits for two sculpins, *C. marginatus* and *C. beldingi*, native to the Pacific Northwest. Acute thermal testing for these two *Cottus* spp. showed a CTmax temperature of 28.9°C that is similar to *Cottus* spp. native to the eastern USA. The CTmax temperatures for members of the family Cottidae from the eastern USA varied from 26.3 to 31.56°C: Mottled Sculpin (*Cottus bairdii*) at 30.37°C and 30.9°C, Tallapoosa Sculpin (*Cottus tallapoosae*) 30.32°C, Banded Sculpin (*Cottus caroliniae zopherus*) at 31.14 and 31.56°C, Ozark Sculpin (*Cottus hypselurus*) at 28.75°C, Pygmy Sculpin (*Cottus pygmaeus*) at 29.58°C, and Slimy Sculpin subspecies (*Cottus cognatus gracilia*) at 26.3 and 27.3°C (Kowalski et al. 1978; Otto and O'Hara Rice 1977; Walsh et al. 1997). As with mussels, the acclimation temperature is an important pretest variable for fish. The CTmax temperature of many fish species shows a positive correlation with acclimation temperature; thus, when the acclimation temperature is higher, the CTmax temperature tends to increase (Otto and O'Hara Rice 1977; Walsh et al. 1997; Beitinger et al. 2000). All the studies mentioned above from the eastern USA used an acclimation temperature of 15°C, representing a higher than the acclimation temperature that we used (13°C). If we repeated our study and exposed *C. marginatus* and *C. beldingi* to a higher acclimation temperature, we might find a higher CTmax temperature. Our chronic thermal test appears to be the first thermal test subjecting *Cottus* spp. to consistent higher temperatures for long periods of time. Some individual *Cottus* spp. from the chronic trial were able to survive 7 d at 26.9°C, whereas other individuals experienced mortality after hour 69. The survival of individuals *Cottus* spp. after 7 d of consistent high temperatures should be further studied.

Mussels and Host Fish

The upper thermal limits of *G. angulata* and *Cottus* spp. differed in the acute thermal tests. If a host fish is more thermally sensitive than the mussel that parasitizes it, the host fish may be absent when a female mussel releases her glochidia, thus halting mussel reproduction. Based on our acute test results, adult *G. angulata* tolerates higher temperatures than sculpin (CTmax temperature ~4°C warmer). If this occurred under natural environmental conditions, sculpins could be thermally stressed before *G. angulata*. One study found the same scenario with the endangered Dwarf Wedgemussel (*Alasmidonta heterodon*) and its host fishes Tesselated Darters (*Etheostoma olmstedii*) and Slimy Sculpin (*Cottus cognatus*; Galbraith et al. 2020). The CTmax temperature data showed that the fish hosts of *A. heterodon* were more temperature sensitive than the mussel. A similar pattern was evident in a literature review of CTmax temperatures of *M. falcata* salmonid hosts. The average CTmax temperatures of Coastal Cutthroat Trout (*Oncorhynchus clarkii clarkia*; Heath 1963), Coho Salmon (*Oncorhynchus kisutch*; Becker and Genoway 1979; Becker and Wolford 1980) and Rainbow Trout (*Oncorhynchus mykiss*; Strange et al. 1993; Currie et al. 1998; Chen et al. 2015), acclimated to 15°C, were similar between species, ranging from 28.7 to 29.9°C (reviewed in Beitinger et al. [2000]). These fish CTmax temperatures are several degrees lower than the CTmax temperature for *M. falcata*. Other comparisons of thermal tolerance data of mussels and their suspected or known host fishes also showed generally similar or slightly higher thermal tolerance for adult freshwater mussels (Pandolfo et al. 2012). Because *G. angulata* and *M. falcata* are likely host specialists, they are at risk of low or unsuccessful metamorphosis of larval glochidia on their host fishes if those hosts are thermally stressed by elevated water temperature.

In the chronic thermal tests, *G. angulata* and *Cottus* spp. were experimentally held at 2°C below their CTmax temperatures for 7 d, to provide initial data on how mussels and sculpins would react to sustained exposure to elevated water temperatures. It is important to note that maintaining the test organisms at a constant elevated water temperature for a week is a scenario these animals would not likely experience in the wild. Additionally, because test temperatures were 5°C different between mussels and *Cottus* spp., direct comparisons cannot be made. Nevertheless, the *Cottus* spp. survival curve is different from the *G. angulata* survival curve, and some sculpins survived the chronic test while all the mussels perished. Further chronic thermal studies, using the same test temperature for mussels and host fish, would be useful to better compare their long-term thermal tolerance.

In conclusion, this study established the upper thermal limits for two freshwater mussels, *G. angulata* and *M. falcata*, and two sculpins, *C. marginatus* and *C. beldingi*, by using acute and chronic thermal tests. We showed that *G. angulata* and *M. falcata* can withstand acute exposure to higher water temperature than the sculpin species tested. The chronic tests

demonstrate that *G. angulata* survived longer and was more tolerant to prolonged elevated temperature than *M. falcata*. Experimental thermal testing of aquatic organisms is important to inform predictions of their survival as water temperatures increase globally due to climate change (Zhu et al. 2021). These temperature tests on interacting species (rather than on a set of independent species) have the potential to inform how species interactions might be affected by environmental temperature changes. The determination of the upper thermal limits for the mussels and sculpins in this study can be used to determine imperilment risk within the native habitats they occupy. In addition, it will be useful for developing conservation plans for at-risk mussel and fish species and it will provide a reference for future studies on other imperiled species.

ACKNOWLEDGMENTS

We thank the Bureau of Indian Affairs Endangered Species Grant Program, the University of Idaho Randall Women in Science Grant Program, Kelly Creek Flycasters, and the University of Idaho for funding this project; CTUIR lab members, including Zach Seilo, Andy Johnson, and interns for help with lab maintenance, data collection, and apparatus construction; Lea Mederios from the University of Idaho for statistical and graphing assistance; Joel Sauder from Idaho Department of Fish and Game for collection assistance; Emily Blevins from the Xerces Society for collection locations; and James Kunz from U.S. Geological Survey for help with system design and lab husbandry.

LITERATURE CITED

- Allen, W. R. 1923. Studies of the biology of freshwater mussels. II. The nature and degree of response to certain physical and chemical stimuli. *Ohio Journal of Science* 23:57–82.
- Bauer, G. 2001. Framework and driving forces for the evolution of naiad life histories. Pages 233–255 in G. Bauer and K. Wachtler, editors. *Ecology and Evolution of the Freshwater Mussels Unionoidea*. Springer-Verlag, Heidelberg, Germany.
- Becker, C. D., and R. G. Genoway. 1979. Evaluation of the critical thermal maximum for determining thermal tolerance of freshwater fish. *Environmental Biology of Fishes* 4:245–256.
- Becker, C. D., and M. G. Wolford. 1980. Thermal resistance of juvenile salmonids sublethally exposed to nickel, determined by the critical thermal maximum method. *Environmental Pollution Series A, Ecological and Biological* 21:181–189.
- Beitinger, T. L., W. A. Bennett, and R. W. McCauley. 2000. Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. *Environmental Biology of Fishes* 58:237–275.
- Blevins, E., S. Jepsen, J. Brim Box, D. Nez, J. Howard, A. Maine, and C. O'Brien. 2017. Extinction risk of western North American freshwater mussels: *Anodonta nuttalliana*, the *Anodonta oregonensis/kennerlyi* clade, *Gonidea angulata*, and *Margaritifera falcata*. *Freshwater Mollusk Biology and Conservation* 20:71–88.
- Blevins, E., S. Jepsen, and S. Selvaggio (Xerces Society). 2020. PETITION TO LIST the Western Ridged Mussel *Gonidea angulata*. The Xerces Society for Invertebrate Conservation, Portland, Oregon.
- Byrne, R. A., and R. F. McMahon. 1994. Behavioral and physiological responses to emersion in freshwater bivalves. *American Zoologist* 34:194–204.
- Cereja, R. 2020. Critical thermal maxima in aquatic ectotherms. *Ecological Indicators* 119:106856.
- Chen, Z., M. Snow, C. S. Lawrence, A. R. Church, S. R. Narum, R. H. Devlin, and A. P. Farrell. 2015. Selection for upper thermal tolerance in rainbow trout (*Oncorhynchus mykiss* Walbaum). *Journal of Experimental Biology* 218:803–812.
- Cowles, R. B., and C. M. Bogert. 1944. A preliminary study of the thermal requirements of desert reptiles. *Bulletin of the American Museum of Natural History* 83:1–49.
- CTUIR (Confederated Tribes of the Umatilla Indian Reservation). 2015. *River Mussels through Time*, 2nd ed. Lynx Group, Salem, Oregon.
- Currie, R. J., W. A. Bennett, and T. L. Beitinger. 1998. Critical thermal minima and maxima of three freshwater game-fish species acclimated to constant temperatures. *Environmental Biology of Fishes* 51:187–200.
- Downing, J. A., P. Van Meter, and D. A. Woolnough. 2010. Suspects and evidence: A review of the causes of extirpation and decline in freshwater mussels. *Animal Biodiversity and Conservation* 33:151–185.
- Fangue, N. A., M. Hofmeister, and P. M. Schulte. 2006. Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, *Fundulus heteroclitus*. *Journal of Experimental Biology* 209:2859–2872.
- Fogelman, K. J., J. M. Archambault, E. Irwin, M. Walsh, S. Brewer, and J. A. Stoeckel. 2023. A review of lethal thermal tolerance among freshwater mussels (Bivalvia: Unionida) within the North American faunal region. *Environmental Reviews* 31:278–297.
- Galbraith, H. S., C. J. Blakeslee, and W. A. Lellis. 2012. Recent thermal history influences thermal tolerance in freshwater mussel species (Bivalvia: Unionida). *Freshwater Science* 31:83–92.
- Galbraith, H. S., C. J. Blakeslee, D. E. Spooner, and W. A. Lellis. 2020. A weight-of-evidence approach for defining thermal sensitivity in a federally endangered species. *Aquatic Conservation: Marine and Freshwater Ecosystems* 30:540–553.
- Haag, W. R. 2012. *North American Freshwater Mussels: Natural History, Ecology, and Conservation*. Cambridge University Press, New York.
- Haley, L., M. Ellis, and J. Cook. 2007. Reproductive timing of freshwater mussels and potential impacts of pulsed flows on reproductive success. PIER Energy Related Environmental Research Program, 100. California Energy Commission, Sacramento.
- Heath, W. G. 1963. Thermoperiodism in sea-run Cutthroat Trout (*Salmo clarki clarki*). *Science* 142:486–488.
- Hutchison, V. H. 1961. Critical thermal maxima in salamanders. *Physiological Zoology* 34:92–125.
- IPCC (Intergovernmental Panel on Climate Change). 2022. *Climate change 2022: Impacts, adaptation, and vulnerability*. Cambridge University Press. <https://www.ipcc.ch/report/sixth-assessment-report-working-group-ii/>
- Ismail, N. S., J. P. Tommerdahl, A. B. Boehm, and R. G. Luthy. 2016. *Escherichia coli* reduction by bivalves in an impaired river impacted by agricultural land use. *Environmental Science and Technology* 50:11025–11033.
- Kaplan, E. L., and P. Meier. 1958. Nonparametric estimation from incomplete observations. *Journal of the American Statistical Association* 53:457–481.
- Karna, D. W., and R. E. Millemann. 1978. Glochidiosis of salmonid fishes. III. Comparative susceptibility to natural infection with *Margaritifera* (L.) (Pelecypoda: Margaritanidae) and associated histopathology. *Journal of Parasitology* 64:528–537.
- Kowalski, K. T., J. P. Schubauer, C. L. Scott, and J. R. Spotila. 1978. Inter-specific and seasonal differences in the temperature tolerance of stream fish. *Journal of Thermal Biology* 3:105–108.
- Lee, R. M., and J. N. Rinne. 1980. Critical thermal maxima of five trout species in the southwestern United States. *Transactions of the American Fisheries Society* 109:632–635.
- Lopez, J. W., T. P. DuBose, A. J. Franzen, C. L. Atkinson, and C. C. Vaughn. 2022. Long-term monitoring shows that drought sensitivity and riparian land use change coincide with freshwater mussel declines. *Aquatic Conservation: Marine and Freshwater Ecosystems* 32:1571–1583.

- Maine, A., C. O'Brien, and B. McIlraith. 2022. Prepared for the CTUIR (Confederated Tribes of the Umatilla Indian Reservation). Master plan: Freshwater mussel conservation, supplementation, aquaculture, restoration, and research. Report to the Northwest Power and Conservation Council, Project 2002-037-00, Portland, Oregon. <https://nwcouncil.app.box.com/s/kitbrdd4frtdhvh4aus0wx1a8iqgefb>
- McClanahan, L. L., C. R. Feldmeth, J. Jones, and D. L. Soltz. 1986. Energetics, salinity and temperature tolerance in the Mohave Tui Chub, *Gila bicolor mohavensis*. *Copeia* 1986:45–52.
- Meyers, T. R., and R. E. Milleman. 1977. Glochidiosis of salmonid fishes. I. Comparative susceptibility to experimental infection with *Margaritifera* (L.) (Pelecypoda: Margaritanidae). *Journal of Parasitology* 63:728–733.
- Murphy, G. 1942. Relationship of the fresh-water mussel to trout in the Truckee River. *California Fish and Game* 28:89–102.
- O'Brien, C., D. Nez, D. Wolf, and J. B. Box. 2013. Reproductive biology of *Anodonta californiensis*, *Gonidea angulata*, and *Margaritifera falcata* (Bivalvia: Unionoida) in the Middle Fork John Day River, Oregon. *Northwest Science* 87:59–72.
- Otto, R. G., and J. O'Hara Rice. 1977. Responses of a freshwater sculpin (*Cottus cognatus gracilis*) to temperature. *Transactions of the American Fisheries Society* 106:89–94.
- Paaajmans, K. P., R. L. Heinig, R. A. Seliga, J. I. Blanford, S. Blanford, C. C. Murdock, and M. B. Thomas. 2013. Temperature variation makes ectotherms more sensitive to climate change. *Global Change Biology* 19: 2373–2380.
- Page, L. M., and B. M. Burr. 2011. Peterson Field Guide to Freshwater Fishes of North America, 2nd ed. HarperCollins Publishers, New York.
- Paladino, E. V., J. R. Spotila, J. P. Schubauer, and K. T. Kowalski. 1980. The critical thermal maximum: A technique used to elucidate physiological stress and adaptation in fishes. *Revue Canadienne de Biologie* 39:115–122.
- Pandolfo, T. J., T. J. Kwak, and W. G. Cope. 2012. Thermal tolerances of freshwater mussels and their host fishes: Species interactions in a changing climate. *Freshwater Mollusk Biology and Conservation* 15:69–82.
- Polhill, J. B., and R. V. Dimock. 1996. Effects of temperature and pO₂ on the heart rate of juvenile and adult freshwater mussels (Bivalvia: Unionidae). *Comparative Biochemistry and Physiology. A, Comparative Physiology* 114:135–141.
- Richard, J. C., E. Blevins, C. D. Dunn, E. M. Leis, and T. L. Goldberg. 2023. Viruses of freshwater mussels during mass mortality events in Oregon and Washington, USA. *Viruses* 15(8):1719. <https://doi.org/10.3390/v15081719>
- Rich, J. T., J. G. Neely, R. C. Paniello, C. C. J. Voelker, B. Nussenbaum and E. W. Wang. 2010. A practical guide to understanding Kaplan-Meier curves. *Otolaryngology - Head and Neck Surgery* 143:331–336.
- Rodland, D. L., B. R. Schöne, S. Baier, Z. Zhang, W. Dreyer, and N. A. Page. 2009. Changes in gape frequency, siphon activity and thermal response in the freshwater bivalves *Anodonta cygnea* and *Margaritifera falcata*. *Journal of Molluscan Studies* 75:51–57.
- Smale, M. A., and C. F. Rabeni. 1995. Hypoxia and hyperthermia tolerances of headwater stream fishes. *Transactions of the American Fisheries Society* 124:698–710.
- Snook, R., J. Magerøy, J. Pither, J. Curtis, L. Nield, and I. R. Walker. 2019. Modeling habitat suitability for the Western Ridged Mussel (*Gonidea angulata* Lea) in Okanagan Lake, British Columbia, Canada. *Northwest Science* 92:375–387.
- Spoooner, D. E., and C. C. Vaughn. 2008. A trait-based approach to species' roles in stream ecosystems: Climate change, community structure, and material cycling. *Oecologia* 158:307–317.
- Stanton, L., and S. MacConnachie. 2012. Exploratory surveys and directed studies of rocky mountain ridged mussel (*Gonidea Angulata* Lea, 1839) in British Columbia. Canadian Manuscript Report of Fisheries and Aquatic Sciences 3003. Fisheries and Oceans Canada, Nanaimo, British Columbia.
- Strange, R. J., R. B. Petrie, and J. J. Cech. 1993. Slight stress does not lower critical thermal maximums in hatchery-reared rainbow trout. *Folia Zoologica* 42:251–256.
- U.S. Fish and Wildlife Service. 2023. Species – Western Pearlshell. <https://www.fws.gov/species/western-pearlshell-margaritifera-falcata>
- Vannote, R. L., and G. W. Minshall. 1982. Fluvial processes and local lithology controlling abundance, structure, and composition of mussel beds. *Proceedings of the National Academy of Sciences of the United States of America* 79:4103–4107.
- Walsh, S. J., D. C. Haney, and C. M. Timmerman. 1997. Variation in thermal tolerance and routine metabolism among spring- and stream dwelling freshwater sculpins (Teleostei: Cottidae) of the southeastern United States. *Ecology of Freshwater Fish* 6:84–94.
- Wang, N., J. L. Kunz, D. K. Hardesty, J. A. Stevens, T. Norberg-King, E. J. Hammer, C. R. Bauer, T. Augspurger, S. Dunn, D. Martinez, M. C. Barnhart, J. Murray, M. Bowersox, J. Roberts, R. B. Bringolf, R. Ratajczak, S. Ciparis, W. G. Cope, S. B. Buczek, and J. Wardell. 2021. Method development for a short-term 7-day toxicity test with unionid mussels. *Environmental Toxicology and Chemistry* 40:3392–3409.
- Westhoff, J. T., and A. E. Rosenberger. 2016. A global review of freshwater crayfish temperature tolerance, preference, and optimal growth. *Reviews in Fish Biology and Fisheries* 26:329–349.
- Whitney, R. S., and R. R. Wydoski. 2003. *Inland Fishes of Washington*. University of Washington Press, Seattle.
- Xerces Society and CTUIR (Confederated Tribes of the Umatilla Indian Reservation). 2020. Western freshwater mussel database. The Xerces Society for Invertebrate Conservation and the Confederated Tribes of the Umatilla Indian Reservation Mussel Project. <https://xerces.org/endangered-species/freshwater-mussels/database>
- Xerces Society. 2015. Native Freshwater Mussels in the Pacific Northwest. Stewardship & Environmental Education for Community-based Organizations. A Xerces Society Guide. Xerces Society, Portland, Oregon.
- Young, M. K., R. Smith, K. L. Pilgrim, D. J. Isaak, K. S. McKelvey, S. Parkes, J. Egge, and M. K. Schwartz. 2022. A molecular taxonomy of *Cottus* in western North America. *Western North American Naturalist* 82:307–345.
- Zhu, B., M. Huang, Y. Cheng, X. Xie, Y. Liu, G. Bisht, and X. Chen. 2021. Impact of vegetation physiology and phenology on watershed hydrology in a semiarid watershed in the Pacific Northwest in a changing climate. *Water Resources Research* 57:e2020WR028394. <https://doi.org/10.1029/2020WR028394>

REGULAR ARTICLE

CHARACTERIZATION OF THE NATIVE FRESHWATER MUSSEL ASSEMBLAGE ACROSS A REACH OF THE RAPPAHANNOCK RIVER, VIRGINIA

Traci P. DuBose^{1*}, Jason Rohweder¹, Catherine Gatenby^{2†}, Brian Watson³,
and Teresa J. Newton^{1†}

¹ U.S. Geological Survey, Upper Midwest Environmental Sciences Center, La Crosse, WI 54603 USA

² U.S. Fish and Wildlife Service, Lower Great Lakes Fish and Wildlife Conservation Office, Basom, NY 14013 USA

³ Virginia Department of Wildlife Resources, Forest, VA 24551 USA

ABSTRACT

Native freshwater mussels occur in rivers of all sizes, yet much of the literature focuses on small- to medium-sized rivers, likely because of logistic challenges of sampling deep, fast, and turbid large rivers. As large-river surveys become more common, the information resulting from a systematic sampling design can provide baseline data on population sizes to inform decision making by resource managers. We collected baseline data on mussel resources in the Rappahannock River, Virginia, in August 2024. This Atlantic coast river, which drains into the Chesapeake Bay, is ~320 km long, up to 1.6 km wide, and up to 35 m deep. We conducted a systematic quantitative survey across the 75-km reach of the river from Fredericksburg to Leedstown, Virginia. Divers obtained 187 whole substrate samples to estimate the species richness, density, population size, size distribution, community composition, and longitudinal spatial distribution of the native mussel assemblage. Six species were found, and the community was dominated by Eastern *Elliptio* (*Elliptio complanata*). Mean reach density (± 1 standard error) and population size was 1.25 (0.21) mussels m⁻² and 19.3 (3.3) million mussels, respectively. The presence of few fresh dead mussels indicated low to moderate natural mortality. Shell length ranged from 32 to 122 mm, and multiple size classes were observed in *E. complanata*. Species richness and density had similar longitudinal patterns, with the highest values in the middle of the sampled reach (river kilometers 60–95). These data provide resource managers with baseline information on the spatial distribution, density, length demography, diversity, and evenness of native freshwater mussels across this reach of the Rappahannock River. Identification of mussel occurrence, density, and evidence of reproduction can be used by managers to aid conservation and management decisions such as prioritizing areas for reintroductions and habitat restoration.

KEY WORDS: density, population size, native mussels, large rivers, diversity

INTRODUCTION

Native freshwater mussels (Family Unionidae; hereafter mussels) are benthic filter-feeding organisms that provide important ecological and cultural functions in rivers. Dense assemblages of mussels influence nutrient cycling and storage, create and modify habitats for other species, support aquatic food webs, and sequester contaminants (Vaughn and Hoellein 2018). In addition, mussels are a part of Native American tribes' traditional foodways and crafts (Strickland et al. 2016;

Wertz 2020). With more than 30% of mussel species in the USA federally listed as threatened or endangered (USFWS 2025), mussels are a substantial resource of concern to the Virginia Department of Wildlife Resources (VADWR) and the U.S. Fish and Wildlife Service (USFWS). The VADWR mission is to conserve wildlife populations and habitat for the benefit of present and future generations; the USFWS mission is to conserve, protect, and enhance fish, wildlife, plants, and their habitats (USFWS 2023; VADWR 2025a). A knowledge of extant populations in understudied rivers can inform the decision making that supports these missions (USFWS 2023; VADWR 2025a).

*Corresponding Author: tdubose@usgs.gov

†Retired.

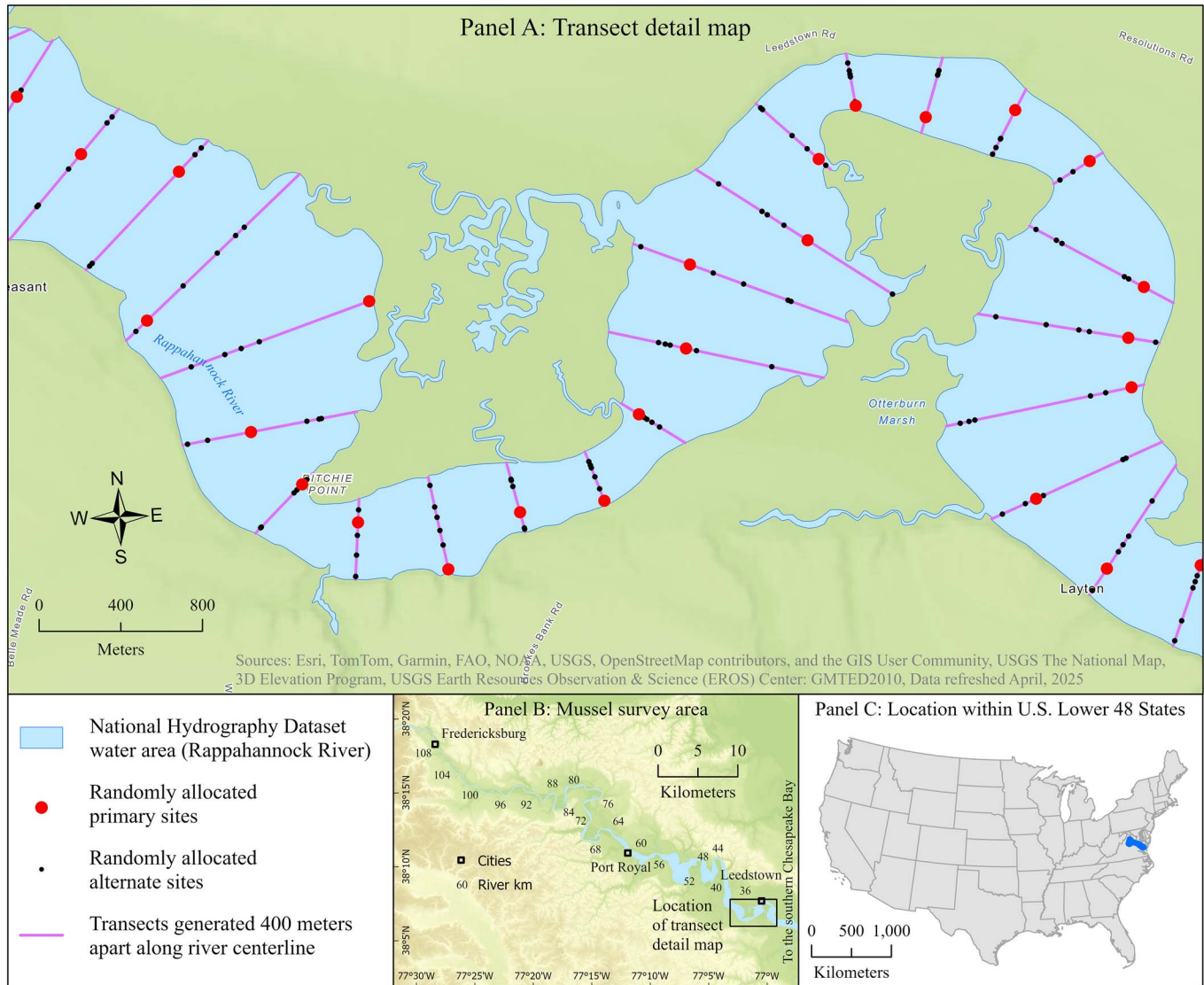


Figure 1. Map illustrating a systematic sampling design for native freshwater mussels across the 75-km reach of the Rappahannock River between Fredericksburg and Leedstown, Virginia (rkm 110 to 36) (Panel B) and the location of the Rappahannock River watershed in the conterminous United States (Panel C). Pink lines are transects generated 400 m apart along the river centerline, red circles are the randomly allocated primary sampling sites, and black circles are the randomly allocated alternate sites in cases where sampling the primary site is unsafe (Panel A).

Mussel assemblages in the lower Rappahannock River, Virginia, are understudied. The 314-km Rappahannock River flows into the southern Chesapeake Bay, and its river basin covers an area of 7,380 km² or about ~6% of the state of Virginia (Fig. 1) (Zhu et al. 2023). The river downstream of Fredericksburg, Virginia, at river kilometer (rkm) 108 (measuring from its confluence with the Chesapeake Bay) is tidal and transitions to brackish downstream of Port Royal, Virginia (rkm 60) (Fig. 1B). The river and its biota change abruptly in Fredericksburg at the fall line (where the river crosses from bedrock to soft sediments). Below Fredericksburg, the substrate is sand and silt, the water is turbid, and the fish assemblage comprises *Micropterus salmoides* (largemouth bass), catfish (order Siluriformes), and anadromous species (VADWR 2025b). In prior surveys, abundances of aquatic macroinvertebrates peaked between rkm 87 and 93

(Massmann et al. 1952). Thirteen mussel species have been documented in the Rappahannock River basin (Jones 2015; Black and Medlin 2020). Our objective was to quantitatively characterize the mussel assemblage in the lower Rappahannock River downstream of Falmouth, Virginia (rkm 110), just upstream of Fredericksburg, Virginia.

METHODS

We designed a systematic quantitative survey to sample mussels across a 75-km reach of the lower Rappahannock River (Fig. 1). First, we created transects every 400 m perpendicular to the centerline of the river based on the National Hydrography Dataset (Moore et al. 2019). Second, we clipped the transects by river area and randomly allocated sites along each transect. As an example, Figure 1A depicts a small segment of the total

study area and the clipped transects. A single (primary) site was sampled at each transect; alternate (secondary) site locations were provided in case the primary site was inaccessible or unsafe to sample. Divers surveyed 187 sites over the 75-km reach from rkm 110 to 36 (Fig. 1B).

The newly created USFWS Mussel Strike Team, consisting of divers and staff from eight states, conducted the survey August 19–22, 2024. At each site, divers placed two 0.25-m² quadrat frames on the river bottom about 0.5 m apart to increase the area sampled. They excavated the substrate within each quadrat to a depth of ~15 cm and placed all material into a 6-mm mesh bag (Newton et al. 2011). The contents in the bag were rinsed, and the bag was brought to the surface for processing. All mussels were categorized as alive or dead; dead shells were further categorized as fresh dead or weathered dead. Fresh dead mussels were defined by shiny nacre and an intact hinge, and weathered dead mussels were defined by chalky nacre (Newton et al. 2023b). The percent of fresh dead mussels has been used as a measure of mortality (Dunn et al. 2020). Individual mussels were identified to species, measured for shell length, and sexed if possible. Nomenclature follows the Integrated Taxonomic Information System (<https://www.itis.gov/>) and guidelines from the Freshwater Mollusk Conservation Society (https://www.molluskconservation.org/MServices_Names-Bivalves.html).

Divers recorded physical habitat conditions. Water depth was recorded to the nearest 0.1 m. Divers visually or tactilely assessed the substrate in each quadrat. Substrate composition was assessed inconsistently across sites. At 146 sites, divers assessed substrate composition as percentage across 10 substrate types (bedrock, boulder, cobble, gravel, sand, silt, clay, detritus, shells, and vegetation). At 37 sites, divers assessed substrate composition by recording the primary, secondary, and tertiary substrate type in the quadrat. At four sites, substrate data were not recorded. To reconcile substrate composition at the 37 sites with categorized substrate, we conservatively assigned 50% for the primary category, 35% for the secondary category, and 15% for the tertiary category.

We estimated species richness, total and species-specific density, reach-wide estimates of total and species-specific population size, and size distribution. Species richness was reported as the number of live species across all sites. Mussel data from the two quadrats at each site were summed for density and population estimation. We calculated total and species-specific density and population size using design-based statistical inference that incorporates spatial information (R package *spsurvey* ver. 5.5.1; Dumelle et al. 2023; Stevens and Olsen 2003). We calculated reach area by buffering site locations by 200 m in the NAD83 coordinate reference system, finding the rectangle that included all buffered site locations (i.e., the bounding box), cropping the polygon of the river from the National Hydrography Dataset Plus V2 (R package *nhdplusTools* ver. 1.2.1), and estimating the area of the resulting polygon (R package *sf* ver. 1.0-16) (Pebesma 2018; Moore et al. 2019; Blodgett and Johnson 2022). The river area was used to calculate inclusion probabilities of each site when estimating total and species-specific density.

Table 1. Life history (Haag 2012), reproductive (Watters et al. 2009; Hopper et al. 2023), and phylogenetic (Graf and Cummings 2021) guilds of the five species of native freshwater mussels found live during a systematic survey of the 75-km reach of the Rappahannock River between Fredericksburg and Leadstown, Virginia (rkm 110 to 36). Nomenclature follows the Integrated Taxonomic Information System (<https://www.itis.gov/>).

Species	Life history	Reproduction	Tribe
<i>Atlanticoncha ochracea</i>	Opportunistic	Long term	Lampsilini
<i>Elliptio complanata</i>	Equilibrium	Short term	Pleurobemini
<i>Pyganodon cataracta</i>	Opportunistic	Long term	Anodontini
<i>Utterbackia imbecillis</i>	Opportunistic	Long term	Anodontini
<i>Utterbackiana implicata</i>	Opportunistic	Long term	Anodontini

We depicted the size distribution of the two most abundant species separately using the frequency of individuals in 5-mm shell-length bins.

Community composition was assessed based on multiple metrics (Table 1). First, we categorized each species as an opportunistic, equilibrium, or periodic strategist following Haag (2012). Second, we categorized species based on the length of time glochidia are sustained in the marsupium as short-term or long-term brooders (Watters et al. 2009). Third, we classified the phylogenetic diversity of mussels into five tribes (Amblemini, Anodontini, Lampsilini, Pleurobemini, and Quadrilini) (Graf and Cummings 2021). Fourth, we estimated diversity (Simpson's D, Shannon's entropy H) and evenness (Pielou's J) indices (R package *vegan* ver. 2.6-6.1; Oksanen et al. 2024; R Core Team 2024).

The spatial distribution of mussels was portrayed based on species richness per river kilometer (in 5-km bins) and by plotting total mussel density across the longitudinal gradient of the river. We interpolated densities in areas not sampled using the *Topo to Raster* function in ArcGIS (ArcGIS Pro 3.3.1; Environmental Systems Research Institute, Redlands, California).

RESULTS

The 75-km reach of the lower Rappahannock River encompasses 3,079 ha of aquatic area; divers surveyed 187 sites from rkm 110 to 36. Although a larger survey was planned, the effort was terminated at rkm 36 because samples there comprised exclusively *Rangia cuneata*, an invasive clam that can tolerate salinities from 0 to 18 parts per thousand (Tang and Aldridge 2021). Across all sites, water depth averaged 4.1 m (range, 0.5–10 m) and the substrate was predominantly silt, followed by sand and gravel (Table 2).

Species Richness

Thirty-eight percent of the sites yielded a total of 117 live mussels and 34 dead mussels; 68% of the sites had no live mussels at all (Table 3). Of the dead mussels found, seven were fresh dead and 27 were weathered dead. There were five live species: *Atlanticoncha ochracea* (formerly *Leptodea*), *Elliptio complanata*, *Pyganodon cataracta*, *Utterbackia imbecillis* (formerly *Anodonta*), and *Utterbackiana implicata* (formerly

Table 2. Average water depth and substrate type (± 1 standard error) at sites sampled for native freshwater mussels across the 75-km reach of the Rappahannock River between Fredericksburg and Leedstown, Virginia (rkm 110 to 36).

Metric	Value (± 1 standard error)
Water depth (m)	4.1 $\pm$ 0.2
Bedrock (%)	1.4 $\pm$ 0.8
Boulder (%)	0.3 $\pm$ 0.3
Cobble (%)	2.0 $\pm$ 0.8
Gravel (%)	8.4 $\pm$ 1.5
Sand (%)	19.5 $\pm$ 2.5
Silt (%)	51.8 $\pm$ 3.1
Clay (%)	6.8 $\pm$ 1.4
Detritus (%)	5.0 $\pm$ 1.3
Shells (%)	0.5 $\pm$ 0.3
Vegetation (%)	1.7 $\pm$ 0.9

Anodonta). No additional species were found as fresh or weathered dead. *Elliptio complanata* had the highest relative abundance, comprising 55% of the live mussels found, followed by *U. implicata* at 33% (Table 4). Two of the five species, *A. ochracea* and *U. implicata*, are of high or moderate conservation need, respectively (VADWR 2025a).

Incidental to the systematic survey, we observed a 31-mm live *Lasmigona subviridis*, a species currently listed as in need of critical conservation in Virginia (<https://ecos.fws.gov/ecp/report>, accessed on February 20, 2026; VADWR 2025a) that is also proposed for federal listing as threatened. Found in an eddy behind a bridge pier at rkm 110, this individual was not included in statistical analyses.

Density and Population Size

Site density across the sampled reach averaged 1.25 mussels m^{-2} (95% confidence interval [CI], 0.83–1.67 mussels m^{-2}) and ranged from 0 to 36 mussels m^{-2} (Table 3). Across the 75-km reach, there were an estimated 19.3 million mussels (12.9–25.7 million; Table 3). Species density ranged from 0.03 mussels m^{-2} for *U. imbecillis* to 0.68 mussels m^{-2} for *E. complanata* (Table 4). Estimates of species-specific population sizes were approximately 11 million *E. complanata* (7.2–13.8 million), 6 million *U. implicata* (2.7–10.2 million), 1 million *A. ochracea* (0.3–1.6 million), 0.8 million *P. cataraacta* (0–1.7 million), and 0.5 million *U. imbecillis* (0–1.3 million).

Size Distribution

Shell lengths of the most abundant species, *E. complanata*, ranged from 32 to 122 mm (Fig. 2 and Table 4). However, about 81% of the individuals were 76–100 mm in length (Fig. 2). In contrast, shell lengths of *U. implicata* were negatively skewed, indicating that mostly larger individuals were found (Fig. 2 and Table 4). Specifically, 67% of the individuals were 108–124 mm in length (Fig. 2).

Table 3. Population and density estimates (± 1 standard error) and community characteristics of native freshwater mussels across the 75-km reach of the Rappahannock River between Fredericksburg and Leedstown, Virginia (rkm 110 to 36).

Parameter	Value
Number of sites sampled	187
Number of live mussels obtained	117
Number (%) of sites without live mussels	127 (68)
Total population estimate (millions)	19.3 $\pm$ 3.3
Total density (no. m^{-2})	1.25 $\pm$ 0.21
Range of densities (no. m^{-2})	0–36
Number of live species	5
Equilibrium strategists (%)	55
Opportunistic strategists (%)	45
Anodontini (%)	40
Lampsilini (%)	5
Pleurobemini (%)	55
Community diversity (Simpson's D)	0.41
Community diversity (Shannon's entropy H)	1.08
Community evenness (Pielou's J)	0.67

Diversity and Evenness

The mussel assemblage had moderate diversity and evenness. Simpson's D was 0.4 on a scale of 0 (not diverse) to 1 (infinitely diverse), and Shannon's entropy H was 1.08, where higher numbers indicate more diverse communities (Table 3). Similarly, evenness was 0.7 on a scale of 0 (uneven) to 1 (even) (Table 3). The assemblage was dominated by *E. complanata*, an equilibrium strategist with a short-term brooding strategy. The remaining four species were opportunistic strategists with long-term breeding strategies. Three of the five phylogenetic tribes in Unionidae were represented (Table 1). The only species that was sexually dimorphic was *A. ochracea*; of the six individuals found, two were females, one was male, and three were unknown.

Spatial Distribution

Species richness and mussel density peaked in the middle of the sampled reach. Species richness of live mussels showed few mussels in the most upstream (rkm 95–110) and most downstream (rkm 35–60) stretches, with greatest richness (three species) in the middle stretch (rkm 60–95) (Fig. 3). A similar pattern was observed for density (Fig. 4). In the middle stretch (rkm 60–95), there were 10 pockets of high mussel density (>8 mussels m^{-2}). Greatest densities were observed near rkm 73 (12 mussels m^{-2}), rkm 71 (14 mussels m^{-2}), and rkm 86 (36 mussels m^{-2}).

DISCUSSION

The lower Rappahannock River contained relatively low species richness and density, and many species were represented by older individuals (based on shell length); however, diversity and evenness were moderate, and natural mortality

Table 4. Species-specific characterization of the live native freshwater mussel assemblage (mean $\pm$ 1 standard error) across the 75-km reach of the Rappahannock River between Fredericksburg and Leedstown, Virginia (rkm 110 to 36). *Atlanticoncha ochracea* (formerly *Leptodea*) and *Utterbackiana implicata* (formerly *Anodonta*) are species of greatest conservation need in Virginia (VADWR 2025a).

Species	Number alive	Percent of total	Length (range, mm)	Density (no. m ⁻²)	Population estimate (millions)
<i>Elliptio complanata</i>	64	55	32–122	0.68 $\pm$ 0.11	10.54 $\pm$ 1.70
<i>Utterbackiana implicata</i>	39	33	50–138	0.42 $\pm$ 0.12	6.42 $\pm$ 1.91
<i>Atlanticoncha ochracea</i>	6	5	76–106	0.06 $\pm$ 0.02	0.99 $\pm$ 0.34
<i>Pyganodon cataracta</i>	5	4	96–122	0.05 $\pm$ 0.03	0.82 $\pm$ 0.50
<i>Utterbackia imbecillis</i>	3	3	58–67	0.03 $\pm$ 0.03	0.49 $\pm$ 0.43
Total	117	—	—	1.25 $\pm$ 0.21	19.26 $\pm$ 3.27

was low to moderate. Thus, mussels persist in this reach, but “evidence of them still existing in large numbers is rare” as in many Chesapeake Bay tributaries (Kreeger et al. 2018).

Seventy-seven species of native freshwater mussels have been recorded in Virginia, with 12 extant species reported in the Rappahannock River (Johnson 1970; Jones 2015; Black and Medlin 2020). The six species observed in this study (five in the systematic survey and one incidental observation) were reported

by Jones (2015), and the five species in the systematic survey are represented by museum records spanning 1973–2012 (Pfeiffer et al. 2024). Although *Alasmodonta undulata*, *Elliptio fisheriana*, *Elliptio lanceolata*, *Pyganodon grandis*, and *Strophitus undulatus* were reported in the Rappahannock River drainage, we did not find these species in the 2024 survey. Given the difficulty separating mussels in the anodontini phylogenetic tribe and the lack of *P. grandis* museum records located in the Rappahannock River (Pfeiffer et al. 2024), reports of *P. grandis* are dubious (B. Watson, VADWR, written communication, April 21, 2025). Of the five species not found in the current survey but reported by Pfeiffer et al. (2024), only one (*S. undulatus*) was found in the 75-km reach we sampled (it was last found in 1945). *Elliptio fisheriana* museum records include observations above and below our reach, and the remaining three species were found in upstream reaches and tributaries.

Quantitative sampling can underestimate the number of species present, thus, at sites where no mussels were found in quadrats, divers conducted 92 radial searches (5–10 min in an area about 3 m in diameter) and 18 timed searches (10–15 min) in areas of dense aggregations to increase search effort. Additional searches did not produce more species. Two recent surveys

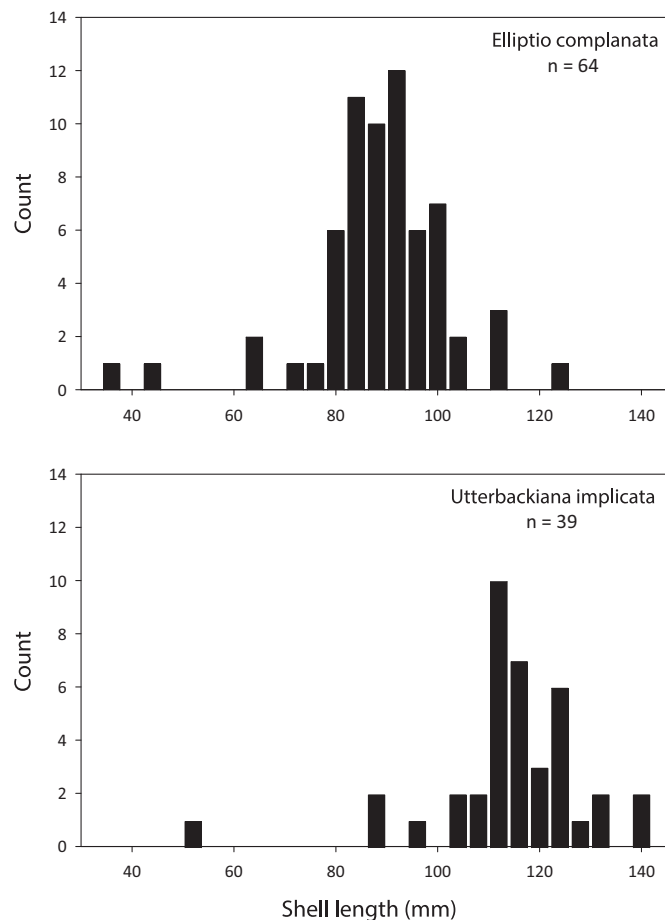


Figure 2. Size distribution of live individuals across the two most abundant species of native freshwater mussels across the 75-km reach of the Rappahannock River between Fredericksburg and Leedstown, Virginia (rkm 110 to 36). Bars represent (upper) bins of 4 mm.

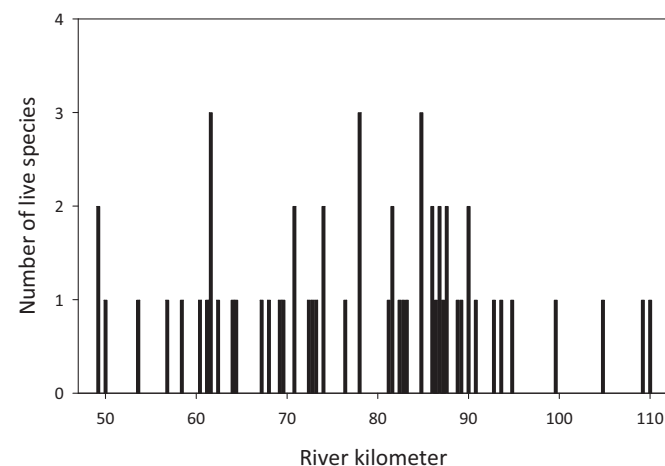


Figure 3. Species richness (number of live species) of native freshwater mussels across the 75-km reach of the Rappahannock River between Fredericksburg and Leedstown, Virginia (rkm 110 to 36). Note there were no live species between rkm 35 and rkm 49.

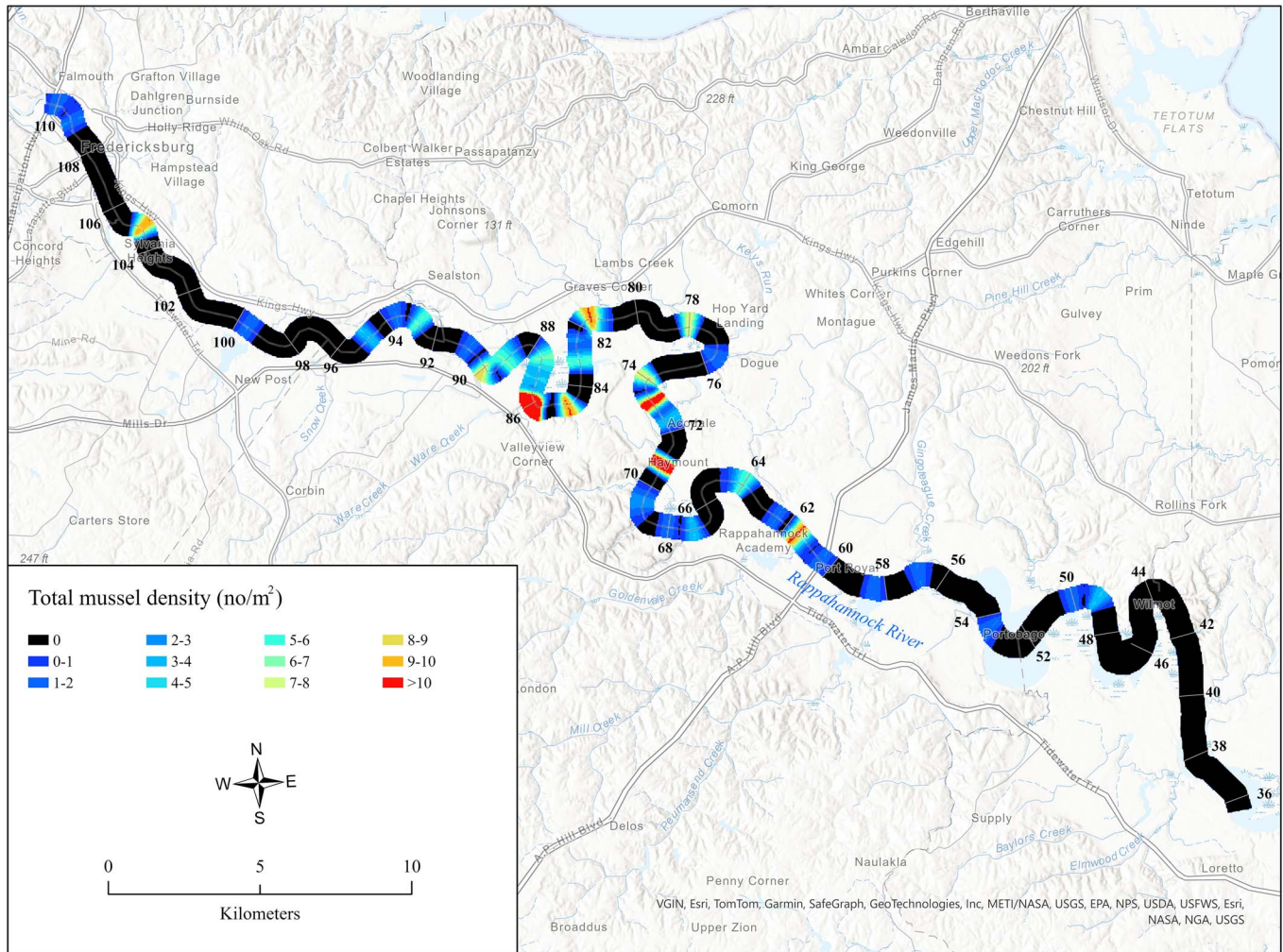


Figure 4. Spatial distribution of total density (mussels m⁻²) of native freshwater mussels across the 75-km reach of the Rappahannock River between Falmouth (rkm 110), just upstream of Fredericksburg, and Leedstown (about rkm 36), Virginia.

increased the species list of the lower Rappahannock River. Qualitative surveys by Neves and Henley (2019) near rkm 110 found the first record of *L. subviridis* downstream of the fall line. Black and Medlin (2020) found seven *E. fisheriana*, and a single, gravid *Lampsilis radiata*, the only record of this species in the river. In addition, since 2007, the Virginia Fisheries and Aquatic Wildlife Center has released between 3,000 and 62,000 mussels annually into Virginia rivers, including almost 38,000 *U. implicata* and 300 *L. subviridis* in the Rappahannock River (Kreeger et al. 2018; B. Watson, VADWR, written communication, April 22, 2025). Our quantitative survey highlights the rarity of *L. subviridis*, *E. fisheriana*, and *L. radiata* in the lower section of the river and produces robust population size and density estimates for the five species found during the systematic survey.

We chose a quantitative systematic survey design because it met our objective of providing robust estimates of population size and density in this reach (Strayer and Smith 2003). Other survey designs (e.g., adaptive sampling, stratified random sampling) are available, but each has advantages and disadvantages. We recognize that the design we chose can underestimate

species richness (Strayer and Smith 2003), but it does provide important baseline information. Our findings can be used to conduct additional complementary surveys to address different objectives. Mean total density in the Rappahannock River is low compared to systematic studies of mussels in other large rivers. For example, in the upper Hudson River (another large river in the Atlantic Slope drainage), mean density in strata that were unremediated or sampled before remediation for polychlorinated biphenyls was >15 mussels m⁻²; in contrast, strata that were sampled after remediation had densities <1 mussel m⁻² (Newton et al. 2023b). Similarly, mean density of mussels in the lower Hudson River prior to the invasion of *Dreissena polymorpha* was 8 mussels m⁻² (Strayer et al. 1994). The total population estimate in the Rappahannock River (19 million mussels) is low relative to other large rivers. In the upper Hudson River study, estimates of population size ranged from 0.1 million to 156 million across all strata, with population size estimates >10 million in 8 (of 17 total) nonremediated strata (Newton et al. 2023b).

The size distribution of live mussels and number of fresh dead mussels can reflect population age and mortality (Haag 2012;

Dunn et al. 2020). We predominantly found larger and thus older individuals. For example, *A. ochracea* shell lengths observed in our study (76–106 mm) are between the maximum shell lengths of *A. ochracea* reported in Maine and Massachusetts (Nedea et al. 2000; Massachusetts Division of Fisheries and Wildlife 2015). In all species, low recruitment could be related to reduced bottom dissolved oxygen associated with eutrophication in the lower Rappahannock River (Sullivan et al. 2024), but we cannot rule out other causes. In contrast, the seven fresh dead mussels observed indicate a mortality rate of ~4%, which in the upper Mississippi River is indicative of a mussel assemblage at the transition between “good” and “fair” condition (Dunn et al. 2020). Thus, our data indicate low recruitment and low to moderate natural mortality in this reach.

Assemblages in Atlantic Slope rivers are dominated by *E. complanata* (Haag 2012; Kreeger et al. 2018), and the assemblage observed in the lower Rappahannock River was no exception. This species is widely distributed across the Atlantic coast and can reach high densities (Nedea et al. 2000). Its widespread distribution could be due to its glochidia’s ability to complete transformation on many host species (Kneeland and Rhymer 2008; Lellis et al. 2013). The dominance of *E. complanata* could be attributed to its life history strategy: equilibrium strategists are often found in medium-sized and large rivers (Haag 2012). The remaining species are widespread across the Atlantic Slope region but are uncommon.

Even though the mussel assemblage was dominated by a single species (*E. complanata*), species diversity and evenness were moderate in the sampled reach. Diversity indices are forms of the same entropy equation but weigh abundant and rare species differently. Simpson’s D ignores rare species, Pielou’s evenness equally weighs rare and abundant species, and Shannon’s entropy acts intermediately to the two other indices (Hill 1973). The moderate relative abundance of *U. implicata* and low species richness offset the numerical dominance of *E. complanata* to contribute to a community with moderate diversity. Similar frequencies among the three rarer species contributed to moderate evenness. Diversity was comparable to that reported in two Midwest rivers and evenness was fourfold higher than the median of 0.19 reported for the Atlantic region (Haag 2012; Hornbach et al. 2018; McMurray et al. 2018).

In the lower Rappahannock River, we observed longitudinal patterns in species richness and total density of mussels, which aligns with patterns in fish and other invertebrate assemblages related to the fall line (Massmann et al. 1952; Maurakis et al. 1987). Some mussel species found in the survey use migratory fish as hosts for their glochidia (*E. complanata*, *U. implicata*, and *A. ochracea*) (Kneeland and Rhymer 2008; Lellis et al. 2013); the absence of barriers in this reach of the river (and lower portions of large rivers in general) contributes to the similar patterns between mussel and fish assemblages and reduces the chance of host-loss-induced extirpation. Concordance between mussel and other invertebrate assemblages could be due to similar habitat requirements or the ecosystem

engineering of freshwater mussels (hard-substrate, food, and flow-refugia generation; *sensu* DuBose et al. 2024). Although most of the lower Rappahannock River watershed has natural land use (Sullivan et al. 2024), high-density areas of mussels were often located near the Pettigrew Wildlife Management Area (rkm 86).

The quality of mussel assemblages is often based on a suite of biological metrics, including species richness, density, and demography (Dunn et al. 2020; Haag et al. 2024). The surveyed reach of the Rappahannock River supported low species richness, low density, low to moderate natural mortality, potential evidence of recruitment in *E. complanata* (based on size demography with individuals likely <10 years old), and moderate diversity and evenness. Systematic sampling designs and quantitative whole substrate samples, like those used on this reach of the Rappahannock River, can provide resource managers with robust information on mussel assemblages. Specifically, these data provide managers with baseline information on the spatial distribution, density, size demography, diversity, and evenness of mussels across a 75-km reach of the Rappahannock River. This information can be used to aid conservation and management efforts, such as identifying and prioritizing locations with rich and dense assemblages for reintroduction efforts (i.e., Cope and Waller 1995), and they are well suited as input data for models of mussel habitat (i.e., Zigler et al. 2012). Surveys of mussel assemblages in large rivers can inform management decisions in three ways. First, systematic longitudinal surveys detail mussel spatial distribution, including hot spots for biodiversity and density. Second, systematic surveys provide baseline abundance estimates that can be used in population viability or trend assessments. Third, by quantifying abundance and size, data from systematic surveys can be used to estimate the ecosystem services provided by mussels, which can be substantial in large rivers (Newton et al. 2011, 2023a). Importantly, resource managers can use baseline information on spatial distribution, size and age demography, density, and population size to assess how future management actions could affect this imperiled resource.

ACKNOWLEDGMENTS

We thank the Mussel Strike Team—folks from U.S. Fish and Wildlife Service offices in Arkansas, Florida, Maryland, New York, North Carolina, Tennessee, Virginia, and West Virginia—that assembled to share resources to map the abundance and diversity of mussels in the Rappahannock River. The Virginia Department of Wildlife Resources also shared resources including boats and expertise in mussel identification for this survey. We are especially grateful to Channing St. Aubin from the U.S. Fish and Wildlife Service Panama Fish and Wildlife Conservation Office for leading the mussel dive crews and training divers on advanced mussel survey methods. We thank Bill Crouch from the Rappahannock River National Wildlife Refuge for sharing his in-depth knowledge of the river and coordinating boat launches. We thank Jack Ryan, Department of Environmental

Services, Rappahannock Indian Tribe, for his assistance. This work was funded by the U.S. Fish and Wildlife Service and the U.S. Geological Survey Ecosystems Mission Area Species Management Research Program. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government. At the time of publication, these data have not been published by the U.S. Fish and Wildlife Service. Derived data are available at <https://doi.org/10.5066/P14MIULA> (DuBose et al. 2026).

LITERATURE CITED

- Black, T., and N. Medlin. 2020. Freshwater mussel (Unionidae) relocation report, Chatham Bridge on Route 3 over the Rappahannock River at the City of Fredericksburg. Virginia Department of Transportation, Fredericksburg, Virginia. 64 pp.
- Blodgett, D., and J. M. Johnson. 2022. nhdplusTools: Tools for accessing and working with the NHDPlus. Available at <https://doi.org/10.5066/P97AS8JD> (accessed April 3, 2025).
- Cope, W. G., and D. L. Waller. 1995. Evaluation of freshwater mussel relocation as a conservation and management strategy. *Regulated Rivers: Research & Management* 11:147–155.
- DuBose, T., T. Newton, and J. Rohweder. 2026. Freshwater mussel density and population estimates from a systematic survey in the Rappahannock River, Virginia, in 2024. U.S. Geological Survey data release. <https://doi.org/10.5066/P14MIULA>
- DuBose, T. P., C. C. Vaughn, G. W. Hopper, K. B. Gido, and T. B. Parr. 2024. Habitat engineering effects of freshwater mussels in rivers vary across spatial scales. *Hydrobiologia* 851:3897–3910.
- Dumelle, M., T. Kincaid, A. R. Olsen, and M. Weber. 2023. spsurvey: Spatial sampling design and analysis in R. *Journal of Statistical Software* 105:1–29.
- Dunn, H., S. Zigler, and T. Newton. 2020. Mussel community assessment tool for the upper Mississippi River system. *Freshwater Mollusk Biology and Conservation* 23:109–123.
- Graf, D. L., and K. S. Cummings. 2021. A ‘big data’ approach to global freshwater mussel diversity (Bivalvia: Unionoida), with an updated checklist of genera and species. *Journal of Molluscan Studies* 87:eyaa034.
- Haag, W. R. 2012. *North American Freshwater Mussels: Natural History, Ecology, and Conservation*. Cambridge University Press, New York. 538 pp.
- Haag, W. R., A. K. Burrow, T. P. DuBose, and S. J. Price. 2024. A broadly applicable, objective freshwater mussel assemblage health index (MAHI). *Freshwater Science* 43:150–168.
- Hill, M. O. 1973. Diversity and evenness: A unifying notation and its consequences. *Ecology* 54:427–432.
- Hopper, G. W., J. R. Bucholz, T. P. DuBose, K. J. Fogelman, S. M. Keogh, M. E. Kubala, M. B. Lodato, D. H. Nichols, I. S. González, J. M. Pfeiffer, J. A. Stoeckel, J. D. Lozier, and C. L. Atkinson. 2023. A trait dataset for freshwater mussels of the United States of America. *Scientific Data* 10. doi: 10.1038/s41597-023-02635-9
- Hornbach, D. J., D. C. Allen, M. C. Hove, and K. R. McGregor. 2018. Long-term decline of native freshwater mussel assemblages in a federally protected river. *Freshwater Biology* 63:243–263.
- Johnson, R. I. 1970. The systematics and zoogeography of the Unionidae (Mollusca: Bivalvia) of the Southern Atlantic Slope Region. *Bulletin of the Museum of Comparative Zoology*, Vol. 140, No. 6. Harvard University, Cambridge, Massachusetts.
- Jones, J. W. 2015. Freshwater mussels of Virginia (Bivalvia: Unionidae): An introduction to their life history, status and conservation. *Virginia Journal of Science* 66:309–331.
- Kneeland, S. C., and J. M. Rhymer. 2008. Determination of fish host use by wild populations of rare freshwater mussels using a molecular identification key to identify glochidia. *Journal of the North American Benthological Society* 27:150–160.
- Kreeger, D. A., C. M. Gatenby, and P. W. Bergstrom. 2018. Restoration potential of several native species of bivalve molluscs for water quality improvement in mid-Atlantic watersheds. *Journal of Shellfish Research* 37:1121–1157.
- Lellis, W. A., B. S. White, J. C. Cole, C. S. Johnson, E. V. S. Gray, H. S. Galbraith, and J. L. Devers. 2013. Newly documented host fishes for the Eastern Elliptio mussel *Elliptio complanata*. *Journal of Fish and Wildlife Management* 4:75–85.
- Massachusetts Division of Fisheries and Wildlife. 2015. Tidewater Mucket, *Leptodea ochracea*. Available at <https://www.mass.gov/files/documents/2016/08/no/leptodea-ochracea.pdf> (accessed April 3, 2025).
- Massmann, W. H., E. C. Ladd, and H. N. McCutcheon. 1952. A biological survey of the Rappahannock River, Virginia. Special scientific report. Virginia Institute of Marine Science, College of William and Mary. <https://doi.org/10.21220/V5759P>
- Maurakis, E. G., W. S. Wolcott, and R. E. Jenkins. 1987. Physiographic analyses of the longitudinal distribution of fishes in the Rappahannock River, Virginia. *Bulletin of the Association of Southeastern Biologists* 34:1–14.
- McMurray, S. E., J. T. Hundley, and J. S. Faiman. 2018. A survey of the freshwater mussel (Mollusca: Bivalvia: Unionoida) of the Niangua River basin, Missouri. *Freshwater Mollusk Biology and Conservation* 21:19–27.
- Moore, R. B., L. D. McKay, A. H. Rea, T. R. Bondelid, C. V. Price, T. G. Dewald, and C. M. Johnston. 2019. User’s guide for the national hydrography dataset plus (NHDPlus) high resolution. U.S. Geological Survey Open File Report 2019–1096. U.S. Geological Survey, Washington, DC.
- Neddeau, E. J., M. M. McCollough, and B. I. Swartz. 2000. The freshwater mussels of Maine. Maine Department of Inland Fisheries and Wildlife, Augusta, Maine.
- Neves, R. J., and W. F. Henley. 2019. A habitat assessment and abbreviated survey for freshwater mussels at the Route 3 Chatham Bridge crossing of the Rappahannock River at Fredericksburg, Virginia. Virginia Department of Transportation, Richmond, Virginia. 23 pp.
- Newton, T. J., D. A. Mayer, J. T. Rogala, and B. R. Gray. 2023a. Responses of native freshwater mussels to remedial dredging in the upper Hudson River. U.S. Geological Survey data release. <https://doi.org/10.5066/P97XDFZZ>
- Newton, T. J., D. A. Mayer, J. T. Rogala, S. S. Madden, and B. R. Gray. 2023b. Responses of native freshwater mussels to remediation to remove polychlorinated biphenyl-contaminated sediments in the upper Hudson River. *Aquatic Conservation: Marine and Freshwater Ecosystems* 33:1413–1430.
- Newton, T. J., S. J. Zigler, J. T. Rogala, B. R. Gray, and M. Davis. 2011. Population assessment and potential functional roles of native mussels in the Upper Mississippi River. *Aquatic Conservation: Marine and Freshwater Ecosystems* 21:122–131.
- Oksanen, J., G. Simpson, F. Blanchet, R. Kindt, P. Legendre, P. Minchin, R. O’Hara, P. Solymos, M. Stevens, E. Szoecs, H. Wagner, M. Barbour, M. Bedward, B. Bolker, D. Borcard, G. Carvalho, M. Chirico, M. De Caceres, S. Durand, H. Evangelista, R. FitzJohn, M. Friendly, B. Furneaux, G. Hannigan, M. Hill, L. Lahti, D. McGlinn, M. Ouellette, E. Ribeiro Cunha, T. Smith, A. Stier, C. Ter Braak, and J. Weedon. 2024. vegan: Community ecology package. R package version 2.6-6.1. Available at <https://CRAN.R-project.org/package=vegan> (accessed April 3, 2025).
- Pebesma, E. 2018. Simple features for R: Standardized support for spatial vector data. *R Journal* 10:439–446.
- Pfeiffer, J., T. P. DuBose, and S. M. Keogh. 2024. Synthesis of natural history collections data reveals patterns of US freshwater mussel diversity and decline. *Biological Conservation* 291:110462.
- R Core Team. 2024. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at <https://www.R-project.org> (accessed April 3, 2025).
- Stevens, D. L., and A. R. Olsen. 2003. Variance estimation for spatially balanced samples of environmental resources. *Environmetrics* 14:593–610.

- Strayer, D. L., D. C. Hunter, L. C. Smith, and C. K. Borg. 1994. Distribution, abundance, and roles of freshwater clams (*Bivalvia*, *Unionidae*) in the freshwater tidal Hudson River. *Freshwater Biology* 3:239–248.
- Strayer, D. L., and D. R. Smith. 2003. A Guide to Sampling Freshwater Mussel Populations. Monograph 8. American Fisheries Society, Bethesda, Maryland.
- Strickland, S. M., J. A. King, G. A. Richardson, M. McCartney, and V. Busby. 2016. Defining the Rappahannock indigenous cultural landscape. National Park Service Chesapeake Bay, Chesapeake Conservancy, and Rappahannock Tribe of Virginia. Available at <https://www.academia.edu/download/54731714/RappahannockReport-Final.pdf> (accessed November 18, 2025).
- Sullivan, B. M., K. Gootman, A. Gunnerson, S. Betts, C. Johnson, C. A. Mason, E. S. Perry, G. Bhatt, J. L. Keisman, J. S. Webber, J. Harcum, M. F. Lane, O. H. Devereux, Q. Zhang, R. Murphy, R. Karrh, T. Butler, V. Van Note, and A. Wei. 2024. Rappahannock tributary summary: A summary of trends in tidal water quality and associated factors, 1985–2022. Chesapeake Bay Program Partnership. Available at <https://d18lev1ok5leia.cloudfront.net/chesapeakebay/documents/Rappahannock-Tributary-Summary.pdf> (accessed May 5, 2025).
- Tang, F., and D. C. Aldridge. 2021. Using osmotic shock to control invasive aquatic species. *Journal of Environmental Management* 279:111604.
- USFWS (U.S. Fish and Wildlife Service). 2023. Freshwater mussels and fish: A timeless love affair. Available at <https://thefishingwire.com/freshwater-mussels-and-fish-a-timeless-love-affair/> (accessed March 4, 2025).
- USFWS (U.S. Fish and Wildlife Service). 2025. Environmental Conservation Online System. Available at <https://ecos.fws.gov/ecp/> (accessed April 23, 2025).
- VADWR (Virginia Department of Wildlife Resources). 2025a. Virginia's 2025 Wildlife Action Plan. Available at <https://dwr.virginia.gov/wp-content/uploads/media/Virginia-Wildlife-Action-Plan-2025.pdf> and <https://dwr.virginia.gov/wp-content/uploads/media/2025-SGCN-Aquatic-Mollusks.pdf> (accessed December 30, 2025).
- VADWR (Virginia Department of Wildlife Resources). 2025b. Upper Rappahannock River. Available at <https://dwr.virginia.gov/waterbody/rappahannock-river-upper/> (accessed April 3, 2025).
- Vaughn, C. C., and T. J. Hoellein. 2018. Bivalve impacts in freshwater and marine ecosystems. *Annual Review of Ecology, Evolution, and Systematics* 49:183–208.
- Watters, G. T., M. A. Hoggarth, and D. H. Stansbery. 2009. *The Freshwater Mussels of Ohio*. The Ohio State University Press, Columbus, Ohio.
- Wertz, G. T. W. 2020. Dwelling “Where the waters rise and fall”: The historical ecology of archaic period settlement in the Rappahannock River Valley. Doctoral dissertation, College of William & Mary, Virginia. Available at <https://scholarworks.wm.edu/server/api/core/bitstreams/39756325-0f61-4c77-b3b9-b5e8cb8753a2/content> (accessed May 5, 2025).
- Zhu, D., X. Cheng, W. Li, F. Niu, and M. N. Yazdi. 2023. Estimating the impact of temperature and streamflow change on river nitrogen pollution using the land-river integrated modeling system. *Journal of Hydrology* 626:130190.
- Zigler, S. J., T. J. Newton, M. Davis, and J. T. Rogala. 2012. Patterns in species richness and assemblage structure of native mussels in the Upper Mississippi River. *Aquatic Conservation: Marine and Freshwater Ecosystems* 22:577–587.

REGULAR ARTICLE

GENETIC VARIATION IN AN INVASIVE CLAM (FORM B: *CORBICULA LARGILLIERTII*) ACROSS ILLINOIS, USA

Brenden Mattfolk^{1§}, Julia W. Bondi^{1§}, Nicholas J. Iacarus¹, Jeremy Tiemann¹, Mark A. Davis¹, and Joel B. Corush^{1,2*}

¹ Illinois Natural History Survey, Prairie Research Institute, University of Illinois
Champaign-Urbana, Champaign, IL 61820 USA

² Illinois Institute of Technology, Department of Biology, Chicago, IL 60616 USA

ABSTRACT

Population genetics is an important tool for understanding the source, establishment, and subsequent spread of invasive species. It is particularly pertinent for invasive species that go unrecognized or are understudied due to their limited detection, cryptic diversity, and/or overall lack of interest. One genus of invasive clams, *Corbicula*, has been a particularly successful invader, both globally and in the USA, but has received less attention than other aquatic invaders (e.g., invasive carp or dreissenid mussels). Herein, we address the genetic diversity of one of the three invasive *Corbicula* species—Form B: *Corbicula largilliertii*—by using the 28S nuclear and cytochrome c oxidase subunit 1 (COI) mitochondrial gene in 74 individuals from six localities across multiple watersheds in Illinois, USA. We identified spatial structure in both genes and revealed an overall high level of genetic diversity and differentiation among localities. This information can be useful for addressing questions pertaining to propagule pressure, local spread, and overall genetic variation of this otherwise understudied invasive species.

KEY WORDS: invasive freshwater mollusk, basket clam, aquatic invasive species, biogeography, Mississippi River watershed, nonnative species

INTRODUCTION

Conventional wisdom holds that successful invaders establish and spread despite substantial genetic bottlenecks during founder events (i.e., founder effect) that result in low overall genetic diversity (Tsutsui et al. 2000). But this theoretical paradox quickly breaks down when empirical investigations reveal that successful invasions are, in fact, often the result of multiple introductions of numerous individuals (Roman and Darling 2007). Subsequently, high propagule pressure promotes high genetic diversity that in turn allows for species to thrive in a wider range of environmental and ecological conditions (Lockwood et al. 2005; Simberloff 2009). Once in a new environment, however, successful individuals may experience strong selection resulting in only a few genotypes persisting and propagating (Comeault et al. 2020). Understanding patterns of genetic diversity across a species' invaded range is pertinent to effective management of the species, because it provides information regarding the

evolutionary potential or the efficiency of potential biological control (Sakai et al. 2001).

Species of the bivalve genus *Corbicula*, commonly known as the basket clam, are native to freshwater environments in eastern Asia as well as to regions of Africa and Australia. However, historic invasions into nonindigenous watersheds worldwide, mainly facilitated by human transport (likely via ballast water exchange and purposeful transport as a food item), have rendered it a particularly prolific aquatic invasive species (AIS) for global freshwater ecosystems (Sousa et al. 2008a). Invasive *Corbicula* populations have been reported in North America for a century, first documented in the 1920s on Vancouver Island, British Columbia, Canada, as empty shells (McMahon 1982). Within a few decades, *Corbicula* spread along the Western Slope in Oregon, Washington, and California (Pennak 1953), USA, and, after secondary introductions, breached the Continental Divide by 1957 into the Tennessee (Sinclair and Isom 1961), Ohio (Stein 1962; Keup et al. 1963), and Pearl rivers, USA (Gunning and Suttikus 1966). Little is known regarding factors promoting the rapid North American expansion. Nevertheless,

*Corresponding Author: jcorush2@illinois.edu

§Cofirst authors equally contributed to this manuscript

through a combination of dispersal and continual introductions, *Corbicula* reached eastern North America watersheds in the 1950s (McMahon 1982). Additionally, *Corbicula* is hypothesized to have reached South America in the 1960s (Reyna et al. 2013) and was first observed in Western Europe in the 1980s (Mouthon 1981). Because basket clams frequently go undetected, a comprehensive understanding of their invasion history is nonexistent. Even recently, new occurrences have been identified in North America (Tiemann et al. 2017) and elsewhere, although it is likely they had long been established in those places (Kamburska et al. 2013; Reyna et al. 2013; Azevêdo et al. 2014; Beghelli et al. 2014; Tiemann et al. 2017). *Corbicula* has been particularly problematic because it is difficult to identify and can outcompete native bivalve species on many fronts, including rapid growth, early maturity, and high fecundity (Sousa et al. 2008a, 2008b; Haag et al. 2021).

Corbicula's asexual mode of reproduction may contribute to its successful establishment and spread. Although sexual reproduction occurs in native *Corbicula* populations, invasive populations seem to exclusively reproduce asexually, resulting in populations of androgenic clonal lineages that are genetically similar when examined via mitochondrial DNA (mtDNA), but different when nuclear DNA (nDNA) is compared, save any chance mutations (Pigneur et al. 2014; Tiemann et al. 2017; Haponski and Foighil 2019). Molecular and morphological analyses have identified three distinct species (formerly listed as forms) of *Corbicula*: *C. fluminea* (Form A) (Müller, 1774), *C. largillierii* (Form B) (Philippi, 1844), and *C. squallida* (Form D) (Deshayes, 1854) in the USA, including in Illinois (Tiemann et al. 2017; Haponski and Foighil 2019). Although true genetic distinction is unclear, these morphotypes do not necessarily correspond to named species and may be subject to mutations or different selective pressures in novel environments (Tiemann et al. 2017; Haponski and Foighil 2019).

The documented spread of *Corbicula* across North America has continued contemporarily (Siripattawan et al. 2000; Lee et al. 2005; Tiemann et al. 2017, 2018, 2024; Haponski and Foighil 2019; Modesto et al. 2023). Several studies have used molecular tools to address questions pertaining to taxonomy and phylogenetic relationships (Pigneur et al. 2011, 2014; Tiemann et al. 2017; Haponski and Foighil 2019), global biogeography (Lee et al. 2005; Pigneur et al. 2014), and detection (Blackman et al. 2022) of *Corbicula*. Fewer studies, however, have assessed population genetics and diversity on smaller scales, because the focus is typically on identifying source populations or large-scale levels of variation and less often on genetic variation between local populations.

In *C. largillierii* specifically, molecular tools seldom have been incorporated to study population genetics; thus, little is known about the genetic diversity, or lack thereof, within *C. largillierii*. Genetic analysis of other species of *Corbicula* suggest that invasive populations show a trend of low genetic diversity (Pigneur et al. 2014). These results can be attributed in part to the lack of sexual reproduction in these invasive populations in both North America and Europe (Hedtke et al.

Table 1. Sampling locations and collection codes of the six *Corbicula largillierii* (Form B) populations used in study. Specimens are organized in an upstream to downstream manner within the Mississippi River basin. Numbers after river names indicate multiple localities within the same county.

INHS catalog no.	Sample size (N)	Illinois county	River locality ID	Longitude	Latitude
87279	12	Will	DP1	41.64084	−88.0131
87195	12	Will	DP2	41.38366	−88.2405
86178	5	Kankakee	KR1	41.124	−87.8796
87281	16	Kankakee	KR2	41.19975	−87.9786
87245	14	LaSalle	IR	41.31511	−88.6909
45684	15	Randolph	MR	37.8808	−89.7837

INHS = Illinois Natural History Survey; DP = Des Plaines River; KR = Kankakee River; IR = Illinois River; MR = Mississippi River.

2008; Pigneur et al. 2011). This low genetic diversity also is replicated in North and South America: only four mitochondrial COI haplotypes were found from 212 individuals across 12 populations, each associated with one of the three Forms, with Form D containing 1 individual with a second haplotype (Lee et al. 2005). Herein, we assess aspects of the genetic diversity of *C. largillierii* across multiple watersheds in Illinois, a midwestern state that connects the Great Lakes watershed to the Mississippi River watershed. Specifically, we address the following questions: (1) Is there genetic variation across the invasive range of *C. largillierii* in Illinois? (2) If so, does it conform to any geographic pattern? and (3) Do mtDNA and nDNA recover similar spatial patterns? Addressing these questions will help clarify the invasion's history and potential future directionality of the invasion of *C. largillierii*.

METHODS

We used 74 specimens of *C. largillierii* from the Illinois Natural History Survey Mollusk Collections at the University of Illinois Champaign-Urbana. All specimens were collected between 2013 and 2017 from six sites within the upper Mississippi River basin (Table 1) and preserved in 95% ethanol. These sites represented multiple watersheds from the Des Plaines (DP1 and DP2) and Kankakee (KR1 and KR2) rivers, through their confluence at the Illinois River (IR), and downstream into the mainstem Mississippi River (MR) (Fig. 1).

We extracted and isolated DNA from all *Corbicula* samples by using a chloroform:isoamyl alcohol extraction method. After soft tissue samples were separated from the shells and soaked in 100% alcohol for at least 2 d, they were individually crushed and submerged in 400 µL tris-EDTA (TE) buffer. Samples were vortexed at 400 rpm for 1 hr with a stainless steel bead and then resubmerged in 400 µL of 60°C cetyltrimethylammonium bromide (CTAB) buffer (10 mL of CTAB solution, 0.2 g of polyvinyl pyrrolidone, and 20 µL of β-mercaptoethanol mixed with mechanical shaker at 3,000 rpm for 10 min) and 20 µL of Proteinase K and incubated overnight with mild

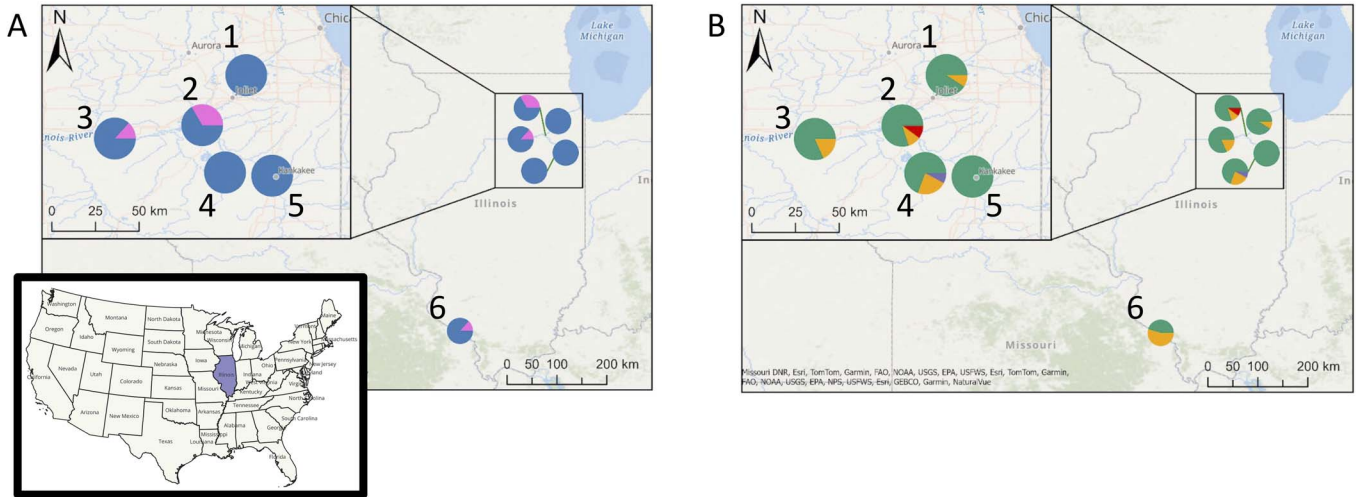


Figure 1. Sampling locations across the Mississippi and Illinois river basins, Illinois, USA. Inset in part A highlights the relative location of Illinois within the USA. Pie charts represent percentage of *Corbicula largillierti* (Form B) exhibiting each haplotype within the (A) mitochondrial and (B) nuclear gene. Mitochondrial haplotypes are separated into haplotypes A (Blue) and B (Pink). Nuclear haplotypes are separated and designated as haplotypes C (green), D (orange), E (red), and F (purple). Populations include 1 = Des Plaines River (DP)1, 2 = DP2, 3 = Illinois River (IR), 4 = Kankakee River (KR)2, 5 = KR1, and 6 = Mississippi River (MR).

mixing. Samples were extracted the next day with 400 μ L of chloroform: isoamyl alcohol (24:1) three times (removing the stainless steel bead after the first extraction), with a 5-min centrifugation at 12,000 rpm after each extraction. Afterward, the remaining pellet was washed with 800 μ L of 100% ethanol and 40 μ L of sodium acetate; centrifuged at 12,000 rpm for 10 min at 4°C; washed again with 200 μ L of 70% ethanol; centrifuged again at 12,000 rpm for 10 min; and dried in a vacufuge for 10 min (1,725 rpm at 43°C). Concentrated DNA was then rehydrated in 100 μ L of TE buffer and subsequently stored at -20°C.

We used PCR to amplify our targeted DNA. To amplify the mitochondrial COI gene, we used universal primers LCO1490 and HCO2198 (Folmer et al. 1994). Our PCR chemistry consisted of 10 μ L of GoTaq™ G2 Colorless MasterMix, 4.5 μ L of water, 1.5 μ L of LCO1490 primer, 1.5 μ L of HCO2198 primer, and 2.5 μ L of template DNA. Thermocycler settings for the PCR were set at 1 round of 95°C for 120 s, 35 rounds of 95°C for 60 s, 1 round of 40°C for 60 s, and 1 round of 72°C for 90 s, followed by a final extension round of 72°C for 420 s. For samples that had no amplification or contained multiple amplified bands, annealing temperature was increased to 41, 42, 43, 44, or 48°C to increase specificity of primer bonding. To amplify the 28S nuclear ribosomal gene, we used D23F and D6R primers (Park and O'Foighil 2000). Our PCR chemistry consisted of 10 μ L of MasterMix, 4.5 μ L of water, 1.5 μ L of D23F primer, 1.5 μ L of D6R primer, and 2.5 μ L of template DNA. Thermocycler settings were as follows: 1 round of 95°C for 240 s, 32 rounds of 95°C for 45 s, 1 round of 57°C for 45 s, and 1 round of 72°C for 105 s, followed by a final extension round of 72°C for 600 s. We used a 2% agarose gel to test amplification quality. If multiple nontarget bands were present, we conducted a cleanup with AMPure XP bead-based reagent

(Beckman Coulter, Brea, CA, USA) at a bead/sample ratio of 0.65 $\times$ that binds to the target fragments, allowing for removal of additional amplicons. We then used ExoSAP-IT™ Express Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and protocol to clean up all PCR products. Samples were sequenced at the Roy J. Carver Biotechnology Center at University of Illinois at Urbana-Champaign on an ABI 3730xl DNA analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

We used the program Geneious Prime v2019.0.4 (Kearse et al. 2012) to trim, align, compare, and produce consensus sequences of each gene from all samples. Sequences derived via the reverse primer were first reverse complemented within the program. Forward and reverse complemented sequences of each specimen were pairwise aligned using the MUSCLE Alignment option (Edgar 2004). Primer sequences were then removed. Finally, all sequences were then aligned using the Consensus Align option within Geneious to produce a consensus among all specimens' mtDNA and nDNA sequences.

We mapped haplotype frequencies across each of the six localities and manually created distance-based haplotype networks for each gene by using the minimum spanning tree as described in Kruskal (1956). In brief, this approach relies on minimizing the length of branches and restricts the number of branches to $n-1$ based on the number of nodes (n). Given that these networks exhibited limited complexity with only four haplotypes, they could readily be constructed manually. Pairwise fixation index (F_{ST}) values and Nei's genetic distance between each pair of localities were calculated using GenAlEx v6.5 (Peakall and Smouse 2006). Pairwise F_{ST} assesses the level of genetic differentiation between populations on a scale from 0 (identical) to 1 (complete differentiation). Additionally, we conducted an analysis of molecular variance (AMOVA) to assess genetic variation at three levels: (1)

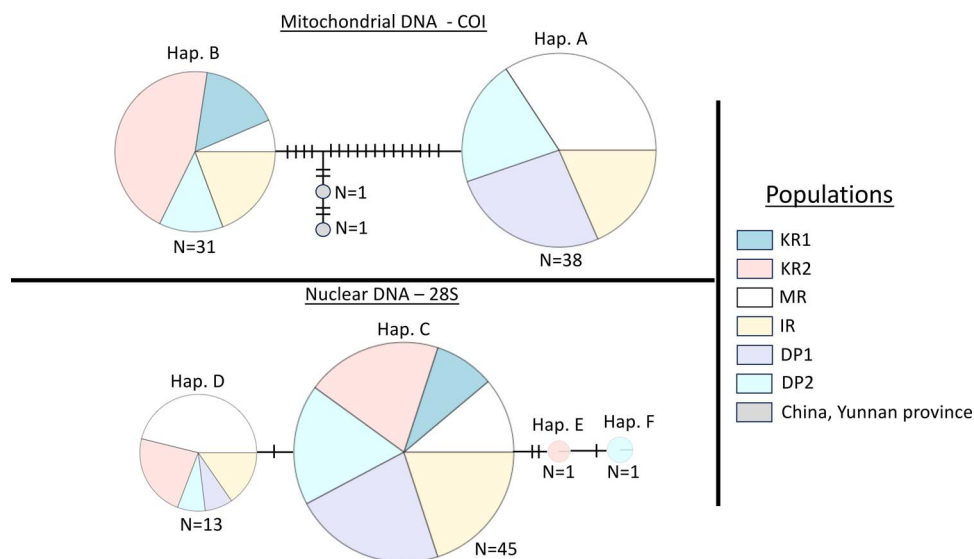


Figure 2. Haplotype network for mitochondrial DNA (top) and nuclear DNA (bottom) of *Corbicula largillierti* (Form B). Mitochondrial DNA network includes two samples of *C. largillierti* from China (GenBank KC211250.1 and KC211251.1). No additional samples for nuclear DNA were available. Size of pie charts is proportional to the number of individuals expressing each haplotype. Pie charts are divided based on localities. Each tick mark on the connecting lines represents a single single-nucleotide polymorphism (SNP) difference among haplotypes.

within-region variation (grouping KR1 and KR2 as well as DP1 and DP2), (2) between-location variation (KR1, KR2, DP1, DP2, IR, and MR), and (3) within-population variation.

RESULTS

We identified two distinct mtDNA haplotypes and four nDNA haplotypes (Fig. 2). For the COI region, we successfully sequenced 69 of 74 individuals. Both Kankakee River localities (KR1 and KR2) exhibited 100% haplotype A. One of the Des Plaines River localities (DP2) exhibited a ratio of 4:8, whereas the other (DP1) exhibited 100% haplotype B. The IR locality exhibited a ratio of 6:7, whereas the furthest downstream locality, the MR, exhibited a ratio of 2:13. Overall, we saw a haplotype distribution of 31:38 among all six localities. Both haplotypes exhibited single-nucleotide polymorphisms (SNPs) at 17 distinct locations across the 658-bp sequence (Table 2).

We successfully sequenced 60 of 74 specimens at the nuclear 28S gene. Of these 60, we were able to interpret only the reverse complement sequence of 48 specimens. For the other 12, we were able to interpret both the forward and

reverse complement sequences, producing a full alignment of these 12. Overall, we saw four haplotypes (labeled as C, D, E, and F). Each of the four haplotypes was observed in at least one individual with both forward and reverse complement sequences. In one Kankakee River locality (KR1), we saw a ratio of 4:0:0:0 (C:D:E:F), but in the other locality (KR2), we observed a haplotype ratio of 9:3:1:0. In one Des Plaines River locality (DP2), we saw a ratio of 8:1:0:1, whereas in the other locality (DP1), our haplotype ratio was 10:1:0:0. In the IR, our haplotype ratio was 9:2:0:0, and in the MR, we observed a haplotype ratio of 5:6:0:0. Across the 697-bp sequence, we observed four distinct locations where SNPs occur (Table 3).

The F_{ST} values ranged from 0.022 between Des Plaines River (DP2) and IR localities to 0.947 between Kankakee River (KR1) and Des Plaines (DP1) localities. Likewise, Nei's genetic distance ranged from 0.025 between Des Plaines River (DP2) and IR localities to 1.754 between Kankakee River (KR1) and Des Plaines (DP1) localities (Table 4). Kankakee River (KR1) and Des Plaines River (DP1) shared no mtDNA haplotypes, even though these sites are separated

Table 2. Sequence location (bp) of SNPs found in the COI mitochondrial gene between haplotypes A ($n = 31$ individuals) and B ($n = 38$) and the associated nucleotides in *Corbicula largillierti* (Form B) samples.

		SNP location															
	bp	bp	bp	bp	bp	bp	bp	bp	bp	bp	bp	bp	bp	bp	bp	bp	bp
	10	59	95	110	163	175	241	289	298	349	397	478	484	490	556	580	625
A	T	G	T	C	G	A	G	G	A	C	C	T	A	C	A	A	C
B	C	A	C	T	A	G	A	A	G	T	T	G	G	T	G	G	T

SNP = single-nucleotide polymorphism; COI = cytochrome c oxidase subunit.

Table 3. Sequence location (bp) of SNPs found in the nuclear 28S gene between haplotypes C, D, E, and F and the associated nucleotides in *Corbicula largillierti* (Form B) samples.

Haplotype	bp 86	bp 87	bp 261	bp 328
C (N = 45)	C	A	A	T
D (N = 13)	*	*	*	C
E (N = 1)	G	C	*	*
F (N = 1)	G	C	T	*

SNP = single-nucleotide polymorphism.

Asterisk (*) indicates the same base as haplotype C.

by <100 km of river. All F_{ST} values above 0.36 included Kankakee River localities, which only contained individuals with haplotype B. This result is exemplified by the high level of differentiation compared with the Des Plaines River (DP1) location, for which all individuals were haplotype A (Table 4). Results from the AMOVA show that most of the variation (58%) occurs within populations. Following that, 37% of the variation occurs between among regions and only 5% is explained by among-population variation (Table 5).

DISCUSSION

There was greater genetic variation within *C. largillierti* across Illinois than in the initial analysis that found only one mtDNA haplotype and two nDNA haplotypes (Tiemann et al. 2017). We found two distinct mtDNA haplotypes distinguished by 17 SNPs as well as four distinct nuclear DNA haplotypes distinguished by 4 SNPs. Individuals containing both mtDNA haplotypes A and B contained both nuclear haplotypes C and D (i.e., A-C, A-D, B-C, and B-D individuals). The high amount of variation between the two mtDNA haplotypes, coupled with no intermediate haplotypes, suggests that these individuals may have come from different sources, although low sample sizes decreased the probability of capturing intermediate genotypes. High pairwise F_{ST} and Nei's genetic distance values were the result of spatial variation in mtDNA haplotypes driven by the lack of haplotype A in the

Table 4. Nei's genetic distance (below diagonal) and pairwise F_{ST} values (above diagonal) for combined COI and 28S gene SNPs for the six *Corbicula largillierti* localities used in the study. Highlighted values represent within-river variation.

	DP1	DP2	KR1	KR2	IR	MR
DP1		0.091	1.677	1.754	0.213	0.021
DP2	0.169		0.568	0.586	0.025	0.055
KR1	0.947	0.415		0.003	0.319	1.182
KR2	0.856	0.409	0.070		0.329	1.190
IR	0.284	0.022	0.354	0.317		0.135
MR	0.081	0.064	0.743	0.659	0.130	

F_{ST} = fixation index; SNP = single-nucleotide polymorphism; COI = cytochrome c oxidase subunit; DR = Des Plaines; KR = Kankakee; IR = Illinois River; MR = Mississippi River.

Table 5. Analysis of molecular variance among *Corbicula largillierti* localities.

Source of variation	df	Sum of squares	Estimated variance	% variation
Among regions	3	285.570	2.269	37
Among populations	2	20.160	0.331	5
Within populations	142	509.217	3.586	58
Total	147	814.946	6.186	100

$F_{ST} = 0.4203$ ($P = 0.001$).

two Kankakee River localities and, to a lesser extent, the lack of nuclear haplotype D at KR1. This high level of genetic variation was unexpected based on previous studies that showed less variation on smaller spatial scales (Lee et al. 2005; Tiemann et al. 2017; Haponski and Foighil 2019). Within the IR, some studies on bivalves show little genetic variation across the watershed (Sanfilippo et al. 2025), whereas other studies show levels of population structure in bivalves (Mathias et al. 2018) and fish (Corush et al. 2025). Despite the observed biogeographical variation, the AMOVA suggests that most of the genetic variation occurs within populations, but a large amount of variation also occurs at the watershed level.

The haplotype distribution patterns we observed might result from a wide variety of past and ongoing postinvasion effects driven by the interplay of introduction bottlenecks, multiple founder events, gene flow, and genetic drift (Jaspers et al. 2021). Multiple founder effects at a broad spatial scale can result in distinct haplotypes being differentially observed across the species' range (Zalewski et al. 2010; Jaspers et al. 2021). Our observations might be the result of separate introductions in both the Mississippi and Kankakee rivers, each with subsequent dispersal into the IR and its tributaries. Additionally, selection of specific genotypes in different rivers can increase differentiation between sites and lower genetic diversity within sites (Schulte et al. 2013), although these patterns can be difficult to detect in invasive species (Fitzpatrick et al. 2012). A hypothesis based on collections and occurrence data suggests that the species is moving upstream from the southern USA (Tiemann et al. 2022). Although this hypothesized upstream directionality has been observed in the IR in multiple species (Silver Carp, *Hypophthalmichthys molitrix*; Bighead Carp, *Hypophthalmichthys nobilis*; and Black Carp, *Mylopharyngodon piceus*; Chick and Pegg 2001; Kroboth et al. 2019), downstream invasions are also common. Examples include the Round Goby, *Neogobius melanostomus*; Eastern Banded Killifish, *Fundulus diaphanus*; Quagga Mussel, *Dreissena bugensis*; and Zebra Mussel, *Dreissena polymorpha*, all of which first invaded the Great Lakes and dispersed downstream (Willink et al. 2018; Tiemann et al. 2022, Harris et al. 2024; Hartman et al. 2024). The numerous examples of invasion in both directions removes assumptions based on generalities.

Although no definitive point of introduction or patterns of spread for either haplotype can be deduced from our data, there are some trends worth noting. MR is the only location to be comprised of primarily haplotype D individuals, whereas the northern locations are comprised primarily of haplotype C individuals. Single instances of haplotype E and F found in KR2 and DP2, respectively, suggest that increased sampling may reveal additional haplotypes. Both passive downstream and, albeit slow, active upstream dispersal (via humans, fish and bird digestive tracts, or actively moving pediveligers) are possible in this species (Prezant and Chalmers 1984; Isom 1986; Voelz et al. 1998; Beran 2006; Gatlin et al. 2013; Rosa et al. 2014; Pernecker et al. 2021). However, unlike unionid bivalves, *Corbicula* species do not have glochidia (Hoggarth and Gaunt 1988).

Multiple introduction events may explain the presence of multiple haplotypes (barring the possibility of postinvasion mutations), but reasons for current distributions are less clear. The idea of location-specific haplotype competition has been hypothesized such as “boom-and-bust” in which one haplotype may temporarily, substantially outcompete others in one area before crashing, with concomitant opportunity for subsequent diversification (Doebeli et al. 2021). A boom-and-bust dynamic may explain higher or lower rates of certain haplotypes than we would expect to normally find in an area, as abrupt, temporary proliferation of one haplotype may persist or disappear over time. Mixing of gametes to create crossed, sexual lineages also may explain the discordance between COI patterns and 28S patterns, although this explanation contradicts previous studies of invasive *Corbicula* (Pigneur et al. 2014).

Further exploration of population structure of *C. largillierii* as well as other *Corbicula* lineages is warranted based on the occurrence of rare haplotypes and the patterns of fixation in mtDNA haplotypes at such a fine geographic scale. Specifically, expansion of the geographic scope with particular emphasis on sampling between current locations is imperative to gain a finer-grained perspective, particularly with respect to the differential haplotype composition among disparate sampling localities. Further exploration will facilitate a more nuanced understanding of how haplotype proportions are changing at a watershed scale and facilitate testing hypotheses related to passive and active dispersal, propagule pressure, and other factors related to establishment and spread of this invasive species. Combining a finer-grained perspective with biological and ecological covariates should yield an opportunity to understand how patterns of genetic diversity may be shaped via selection and/or are driving local adaptation, ultimately shaping our understanding of the evolution of this invasive species in novel waters. From a more technical standpoint, leveraging modern population genomic approaches (e.g., restriction site-associated DNA sequencing) and next-generation sequencing would provide a much more fulsome and nuanced perspective on invasion genetics by increasing the number of variable loci by orders of magnitude. On balance, these research lines may provide insight into the spread of this AIS and inform management strategies aimed at more efficient and effective mitigation.

AUTHOR CONTRIBUTION

BM, JBW, NJI, and JBC designed the study with input from all authors. BM, JBW, NJI, and JBC conducted the molecular lab work and data analysis. BM, JBW, and JBC wrote the original draft. All authors contributed to the final draft of the manuscript.

ACKNOWLEDGMENTS

We thank Alison Stodola and Rachel Vinsel of the Illinois Natural History Survey Mollusk Collection for providing specimens. Funding was provided by the U.S. Geological Survey Water Resources Research Act Program-104b to JBC.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY

All sequence data is available via GenBank (accession numbers PZ228526–PZ228594 and PZ229305–PZ229363).

LITERATURE CITED

- Azevêdo, E. D. L., J. E. D. L. Barbosa, T. H. Vidigal, M. Callisto, and J. Molozzi. 2014. First record of *Corbicula largillierii* (Philippi 1844) in the Paraíba River Basin and potential implications from water diversion of the São Francisco River. *Biota Neotropica* 14:e20140036.
- Beghelli, F. G. D. S., M. L. M. Pompêo, and V. M. Carlos. 2014. First occurrence of the exotic Asian clam *Corbicula fluminea* (Müller, 1774) in the Jundiá-Mirim River Basin, SP, Brazil. *Revista Ambiente e Água* 9:402–408.
- Beran, L. 2006. Spreading expansion of *Corbicula fluminea* (Mollusca: Bivalvia) in the Czech Republic. *Heldia* 6:187–192.
- Blackman, R. C., J. Brantschen, J. C. Walser, R. Wüthrich, and F. Altermatt. 2022. Monitoring invasive alien macroinvertebrate species with environmental DNA. *River Research and Applications* 38:1400–1412.
- Chick, J. H., and M. A. Pegg. 2001. Invasive carp in the Mississippi River Basin. *Science* 292:2250–2251.
- Comeault, A. A., J. Wang, S. Tittes, K. Isbell, S. Ingley, A. H. Hurlbert, and D. R. Matute. 2020. Genetic diversity and thermal performance in invasive and native populations of African fig flies. *Molecular Biology and Evolution* 37:1893–1906.
- Corush, J. B., R. V. Cucalón, B. A. Metzke, M. Tan, and M. A. Davis. 2025. Pleistocene glaciation and Anthropocene fragmentation influence genetic variation in the Illinois state-listed mottled sculpin (*Cottus bairdii*). *Environmental Biology of Fishes* 108:905–922.
- Doebeli, M., E. C. Jaque, and Y. Ispolatov. 2021. Boom-bust population dynamics increase diversity in evolving competitive communities. *Communications Biology* 4:502.
- Edgar, R. C. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32:1792–1797.
- Fitzpatrick, B. M., J. A. Fordyce, M. L. Niemiller, and R. G. Reynolds. 2012. What can DNA tell us about biological invasions? *Biological Invasions* 14:245–253.
- Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3:294–299.
- Gatlin, M. R., Shoup, D. E., and J. M. Long. 2013. Invasive zebra mussels (*Dreissena polymorpha*) and Asian clams (*Corbicula fluminea*) survive gut passage of migratory fish species: Implications for dispersal. *Biological Invasions* 15:1195–1200.

- Gunning, G. E., and R. D. Suttks. 1966. Occurrence and distribution of the Asiatic clam, *Corbicula leana*, in the Pearl River, Louisiana. *Nautilus* 79:113–116.
- Haag, W. R., J. Culp, A. N. Drayer, M. A. McGregor, D. E. White, and S. J. Price. 2021. Abundance of an invasive bivalve, *Corbicula fluminea*, is negatively related to growth of freshwater mussels in the wild. *Freshwater Biology* 66:447–457.
- Haponski, A. E., and D. Ó. Foighil. 2019. Phylogenomic analyses confirm a novel invasive North American *Corbicula* (Bivalvia: Cyrenidae) lineage. *PeerJ* 7:e7484.
- Harris, B. S., M. J. Spear, A. L. Whitten, E. C. Hine, A. W. Lenaerts, A. T. Mathis, K. Maxson, M. Myers, M. Oubre, S. J. Schaick, L. E. Solomon, A. T. Wieland, J. A. Williams, and J. T. Lamer. 2024. Invasive round goby *Neogobius melanostomus* distribution, relative abundance, and establishment in pools of the Illinois Waterway following 30 years of invasion. *Journal of Freshwater Ecology* 39:2301096.
- Hartman, J. H., J. Corush, E. R. Larson, J. S. Tiemann, P. W. Willink, and M. A. Davis. 2024. Niche conservatism and spread explain introgression between native and invasive fish. *Molecular Ecology* 33(11):e17363.
- Hedtke, S. M., K. Stanger-Hall, R. J. Baker, and D. M. Hillis. 2008. All-male asexuality: Origin and maintenance of androgenesis in the Asian clam *Corbicula*. *Evolution* 62:1119–1136.
- Hoggarth, M. A., and A. S. Gaunt. 1988. Mechanics of glochidial attachment (Mollusca: Bivalvia: Unionidae). *Journal of Morphology* 198:71–81.
- Isom, B. G. 1986. Historical review of Asiatic clam (*Corbicula*) invasion and biofouling of waters and industries in the Americas. *American Malacological Bulletin*. Special issue 2:1–5.
- Jaspers, C., M. Ehrlich, J. M. Pujolar, S. Künzel, T. Bayer, M. T. Limborg, F. Lombard, and T. B. Reusch. 2021. Invasion genomics uncover contrasting scenarios of genetic diversity in a widespread marine invader. *Proceedings of the National Academy of Sciences of the United States of America* 118:e2116211118.
- Kamburska, L., R. Lauceri, M. Beltrami, A. Boggero, A. Cardeccia, I. Guarneri, M. Manca, and N. Riccardi. 2013. Establishment of *Corbicula fluminea* (OF Müller, 1774) in Lake Maggiore: A spatial approach to trace the invasion dynamics. *BioInvasions Records* 2:105–117.
- Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A. Cooper, S. Markowitz, C. Duran, T. Thierer, B. Ashton, P. Meintjes, and A. Drummond. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28:1647–1649.
- Keup, L., W. B. Horning, and W. M. Ingram. 1963. Extension of range of Asiatic clam to Cincinnati reach of the Ohio River. *Nautilus* 77:18–29.
- Kroboth, P., C. Cox, D. C. Chapman, and G.W. Whitedge. 2019. Black carp in North America: A description of range, habitats, time of year, and methods of reported captures. *North American Journal of Fisheries Management* 39:1046–1055.
- Kruskal, J. B. 1956. On the shortest spanning subtree of a graph and the traveling salesman problem. *Proceedings of the American Mathematical Society* 7:48–50.
- Lee, T., S. Siripattawan, C. F. Ituarte, and D. Ó. Foighil. 2005. Invasion of the clonal clams: *Corbicula* lineages in the New World. *American Malacological Bulletin* 20:113–122.
- Lockwood, J. L., P. Cassey, and T. Blackburn. 2005. The role of propagule pressure in explaining species invasions. *Trends in Ecology and Evolution* 20:223–228.
- Mathias, P. T., J. R. Hoffman, C. C. Wilson, and D. T. Zanatta. 2018. Signature of postglacial colonization on contemporary genetic structure and diversity of *Quadrula* (Bivalvia: Unionidae). *Hydrobiologia* 810:207–225.
- McMahon, R. F. 1982. The occurrence and spread of the introduced Asiatic freshwater clam, *Corbicula fluminea* (Müller), in North America 1924–1982. *Nautilus* 96:134–141.
- Modesto, V., M. Ilarri, A. M. Labecka, N. Ferreira-Rodríguez, N. E. Coughlan, X. Liu, and R. Sousa. 2023. What we know and do not know about the invasive Asian clam *Corbicula fluminea*. *Hydrobiologia* 852:1183–1214.
- Mouthon, J. 1981. Sur la présence en France et au Portugal de *Corbicula* (Bivalvia, Corbiculidae) originaire d'Asie. *Basteria* 45:109–116.
- Park, J. K., and D. Ó. Foighil. 2000. Sphaeriid and corbiculid clams represent separate heterodont bivalve radiations into freshwater environments. *Molecular Phylogenetics and Evolution* 14:75–88.
- Peakall, R., and P. E. Smouse. 2006. GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6:288–295.
- Pennak, R. W. 1953. *Fresh-water Invertebrates of the United States*. Ronald Press, New York. 769 pp.
- Perneck, B., A. Czirok, P. Mauchart, P. Boda, A. Móra, and Z. Csabai. 2021. No experimental evidence for vector-free, long-range, upstream dispersal of adult Asian clams [*Corbicula fluminea* (Müller, 1774)]. *Biological Invasions* 23:1393–1404.
- Pigneur, L.-M., J. Marescaux, K. Roland, E. Etoundi, J.-P. Descy, and K. Van Doninck. 2011. Phylogeny and androgenesis in the invasive *Corbicula* clams (Bivalvia, Corbiculidae) in Western Europe. *BMC Evolutionary Biology* 11:147.
- Pigneur, L., E. Etoundi, D. C. Aldridge, J. Marescaux, N. Yasuda, and K. Van Doninck. 2014. Genetic uniformity and long-distance clonal dispersal in the invasive androgenetic *Corbicula* clams. *Molecular Ecology* 23:5102–5116.
- Prezant, R. S., and K. Chalermwat. 1984. Flotation of the bivalve *Corbicula fluminea* as a means of dispersal. *Science* 225:1491–1493.
- Reyna, P. B., A. G. Morán, and M. Tatián. 2013. Taxonomy, distribution and population structure of invasive Corbiculidae (Mollusca, Bivalvia) in the Suquia River basin, Córdoba, Argentina. *Iheringia. Série Zoologia* 103:77–84.
- Roman, J., and J. A. Darling. 2007. Paradox lost: Genetic diversity and the success of aquatic invasions. *Trends in Ecology and Evolution* 22:454–464.
- Rosa, I. C., J. L. Pereira, R. Costa, J. Gomes, M. de Lourdes Pereira, and F. Gonçalves. 2014. Dispersal of *Corbicula fluminea*: Factors influencing the invasive clam's drifting behavior. *Annales de Limnologie* 50:37–47.
- Sakai, A. K., F. W. Allendorf, J. S. Holt, D. M. Lodge, J. Molofsky, K. A. With, S. Baughman, R. J. Cabin, J. E. Cohen, N. C. Ellstrand, D. E. McCauley, P. O'Neil, I. M. Parker, J. N. Thompson, and S. G. Weller. 2001. The population biology of invasive species. *Annual Review of Ecology and Systematics* 32:305–332.
- Sanfilippo, G. E., K. Inoue, and D.T. Zanatta. 2025. Patterns of genetic diversity and structure in *Pressodonta viridis*, Slippershell Mussel, in streams of the US Midwest. *Heredity* 134:680–694.
- Schulte, U., M. Veith, V. Mingo, C. Modica, and A. Hochkirch. 2013. Strong genetic differentiation due to multiple founder events during a recent range expansion of an introduced wall lizard population. *Biological Invasions* 15:2639–2649.
- Simberloff, D. 2009. The role of propagule pressure in biological invasions. *Annual Review of Ecology, Evolution, and Systematics* 40:81–102.
- Sinclair, R. M., and B. G. Isom. 1961. A preliminary report on the introduced Asiatic clam *Corbicula* in Tennessee. Tennessee Stream Pollution Control Board and Tennessee Department of Public Health. 33 pp.
- Siripattawan, S., J.-K. Park, and D. Ó. Foighil. 2000. Two lineages of the introduced Asian freshwater clam *Corbicula* occur in North America. *Journal of Molluscan Studies* 66:423–429.
- Sousa, R., C. Antunes and L. Guilhermino. 2008a. Ecology of the invasive Asian Clam *Corbicula fluminea* (Müller, 1774) in aquatic ecosystems: An overview. *Annales de Limnologie* 44:85–94.
- Sousa, R., A. J. A. Nogueira, M. B. Gaspar, C. Antunes, and L. Guilhermino. 2008b. Growth and extremely high production of the non-indigenous invasive species *Corbicula fluminea* (Müller, 1774): Possible implications for ecosystem functioning. *Estuarine, Coastal and Shelf Science* 80:289–295.

- Stein, C. B. 1962. An extension of the known range of the Asiatic clam *Corbicula fluminea* (Müller) in the Ohio and Mississippi rivers. *Ohio Journal of Science* 62:326–327.
- Tiemann, J., A. Haponski, S. Douglass, T. Lee, K. Cummings, M. Davis, and D. Ó. Foighil. 2017. First record of a putative novel invasive *Corbicula* lineage discovered in the Illinois River, Illinois, USA. *BioInvasions Records* 6:159–166.
- Tiemann, J., C. Lawlis, and S. Douglass. 2018. First occurrence of a novel *Corbicula* (Bivalvia: Corbiculidae) Form D lineage in the Ohio River, USA. *Nautilus* 132:30–32.
- Tiemann, J. S., A. P. Stodola, S. A. Douglass, R. M. Vinsel, and K. S. Cummings. 2022. Nonindigenous aquatic mollusks in Illinois. *Illinois Natural History Survey Bulletin* 43. <https://doi.org/10.21900/j.inhs.v43.862>
- Tiemann, J., K. S. Cummings, E. Barba-Macías, and C. R. Randklev. 2024. Distribution of nonindigenous Basket clams (*Corbicula* spp.) in Mexico. *Revista Mexicana de Biodiversidad* 95:e955284–e955284.
- Tsutsui, N. D., A. V. Suarez, D. A. Holway, and T. J. Case. 2000. Reduced genetic variation and the success of an invasive species. *Proceedings of the National Academy of Sciences of the United States of America* 97: 5948–5953.
- Voelz, N. J., J. V. McArthur, and R. B. Rader. 1998. Upstream mobility of the Asiatic Clam *Corbicula fluminea*: Identifying potential dispersal agents. *Journal of Freshwater Ecology* 13:39–45.
- Willink, P. W., T. A. Widloe, V. J. Santucci, D. Makauskas, J. S. Tiemann, S. D. Hertel, J. T. Lamer, and J. L. Sherwood. 2018. Rapid expansion of banded killifish *Fundulus diaphanus* across northern Illinois: Dramatic recovery or invasive species? *American Midland Naturalist* 179: 179–190.
- Zalewski, A., A. Michalska-Parda, M. Bartoszewicz, M. Kozakiewicz, and M. Brzeziński. 2010. Multiple introductions determine the genetic structure of an invasive species population: American mink *Neovison vison* in Poland. *Biological Conservation* 143:1355–1363.

REGULAR ARTICLE

METHOD DEVELOPMENT FOR RAPID STREAMSIDE ASSESSMENT OF FRESHWATER MUSSEL (ORDER UNIONIDA) HEMOCYTE CONCENTRATION AND MORPHOMETRICS

Madison E. Mueller^{*†}, W. Gregory Cope, Christopher B. Eads, Loretta M. Lutackas, and Dorian J. Hayes

Department of Applied Ecology, Campus Box 7617, North Carolina State University, Raleigh, NC 27695 USA

ABSTRACT

Contemporary freshwater mussel population declines often lack discriminant explanations, and diagnoses are commonly post hoc, subjective, expensive, and/or lethal. To avoid further compromising imperiled populations, we need sublethal metrics and biomarkers that identify early warning signs of infection, stress responses, and deviations from physiological homeostasis or potential resilience to environmental pressures. Immune modulators can be differentiated by quantifying multiple physiological endpoints based on hemocyte form and function. Spontaneous hemocyte aggregation, cell instability, travel times from mussel collection sites, and a lack of access to costly instrumentation currently limit immune status assessments. Widespread capacity to understand immune responses to disease, toxicants, and other stressors relies on effective hemolymph handling and preservation techniques. In this study we optimized methods to measure total hemocyte concentration and stability across five antiaggregants (Alsever's Solution, K₂EDTA and sodium citrate Microtainers, Isoton II, and Leibovitz's L15) over time using a Beckman Coulter Multisizer 4e (a laboratory benchtop unit) to validate results from a Millipore Scepter (a portable, field-ready hand-held unit). K₂EDTA Microtainers held on ice for up to 6 h preserved hemolymph most effectively based on consistency in total hemocyte concentration and morphometrics over time. There was a highly significant agreement ($R^2 = 0.9156$, $P < 0.0001$) in total hemocyte concentration between the Multisizer 4e and Millipore Scepter. Portable and compact cell counters provide a less expensive, advantageous alternative to benchtop instruments for instantaneous evaluation at mussel collection sites when timely quantification would otherwise require extensive logistical planning. Our results lay the foundation for accessible streamside assessment, which can expedite the establishment of reference ranges among species and populations and identify how anticipated stressors such as infection and starvation alter the immune response. This approach also may be useful for quantifying freshwater mussel health among propagation facilities and in stable or declining ecosystems.

KEY WORDS: hemolymph, immune biomarkers, conservation, *Lampsilis radiata*

INTRODUCTION

Unionids (Class Bivalvia) are a highly imperiled, species-rich, environmentally important family of freshwater mussels facing enigmatic die-offs and widespread population declines (Graf and Cummings 2007; Haag 2012; Haag and Williams

2014; Vaughn 2018). Historically, these declines followed conspicuous impacts of habitat destruction, pollution, invasive species, and stream impoundments (Haag 2019). In contrast, contemporary mussel die-offs often lack such discriminant explanations, develop rapidly, and do not always affect other sympatric freshwater mussel species or aquatic organisms, thus prompting questions about the role of disease (Waller and Cope 2019). Though historic declines and associated factors have been documented since the 1950s, research considering

^{*}Corresponding Author: Madison_Mueller@fws.gov

[†]Current address: Tennessee Ecological Services Field Office, U.S. Fish and Wildlife Service, 446 Neal Street, Cookeville, TN 38501 USA.

freshwater mussel health status and infectious diseases has not progressed, nor are tools readily available or optimized to investigate immune status (Grizzle and Brunner 2009; Carella et al. 2016). Conservationists and mussel propagation biologists have expressed the need to move beyond post-hoc study of the presence of disease to include individual and population competence and resilience against infection, xenobiotics, and other exogenous stressors with added resources and approaches (Mueller et al. 2025).

Mollusks rely on a primitive but robust innate immune system consisting of a physical defense barrier (shell, epithelium, and mucus secretion) and internal cellular and humoral mechanisms (Song et al. 2010; Song 2016). Hemocytes are multifunctional hemolymphatic cells distributed in hemolymph, a fluid analogous to vertebrate blood, and moved through an open circulatory system transporting metabolites and oxygen (Auffret 1988). Hemocytes are the base of the innate immune response. They also serve a biomechanical purpose in biomineralization processes, shell formation, neuroendocrine regulation, nutrient digestion and transport, tissue repair, and general homeostatic maintenance (Zannella et al. 2017). Hemocytes work in tandem with humoral factors dissolved in hemolymph, including antimicrobial peptides, lytic enzymes, and cytokines, all of which serve in defense and signaling (Chu 1988).

Bivalve cellular and humoral defenses efficiently control infections despite lacking adaptive components (van Niekerk et al. 2016). Hemocytes engage in non-self-recognition defense against invaders through aggregation, phagocytosis, encapsulation, and cytotoxic reactions that produce reactive oxygen or nitrogen compounds and lysosomal enzymes (Canesi et al. 2002; Farcy et al. 2011). Hemocyte-bound protein-protein recognition factors trigger the production of cytokines, antimicrobial peptides, and chemokines; recruit additional hemocytes; and increase phagocytosis, autophagy, and apoptosis (Allam and Raftos 2015).

Major cell types are generally differentiated by morphology, physiological activities, biological functions, and enzymatic cytochemistry; see reviews in Anisimova (2013) and de la Ballina et al. (2022). Currently, the two accepted main hemocyte types are granulocytes and agranulocytes (hyalinocytes). Subtypes may be further differentiated by carbon:nitrogen morphometrics or organelle content and characterized as precursors to other hemocytes (Giamberini et al. 1996; Burkhard et al. 2009; Evariste et al. 2016).

Hemolymph morphological and functional biomarkers are integral to understanding organism health, disease, and ecological risks integrated over time (King et al. 2020; Kataoka and Kashiwada 2021). Perturbations to the immune system and other associated responses are likely to manifest in hemolymph, but hematological and immunological utility as biomarkers is typically limited due to their nonspecific responses to multiple stressors. However, these biomarkers are sensitive, reliable, reproducible, and closely related to other physiological systems, and they can be measured rapidly and

relatively inexpensively. Hemolymph can be sampled nonlethally with no significant difference in mortality after hemolymph extraction in mussels greater than 40 mm in length after 3 yr of observation (Gustafson et al. 2005a, 2005b; Fritts 2015a, 2015b; Richling and Krause 2022). Further, some biomarkers, such as heat shock proteins or lysozymes, may be conserved across species. Thus, it is possible to identify additional universal biomarkers of health in surrogates for threatened and endangered species or to use full communities as environmental sentinels (Van der Oost et al. 2003; Anisimova 2013).

Potential cytotoxic or immunotoxic biomarkers include the hemogram (cell concentration, relative percentage of hemocyte morphotypes) and hemocyte morphology (volume, diameter). Hemograms operate under the same principles as differential cell counts in vertebrates (Levine et al. 2022). Changes in hemocyte counts are considered a general, early warning sign of stress and activation of the first-line defense against invaders (Auffret et al. 2006).

Despite the health-assessment advantages of using hemolymph, wide validation and adoption of optimal conditions to withdraw and isolate viable unionid hemocytes severely lag behind marine mollusk models. Particle-size analysis is a valuable high-throughput tool that can replace or supplement microscopy in molluscan immunology (Nguyen and Alfaro 2019). However, limited progress has been made to experimentally validate particle-size analysis of multiple immunological biomarkers in freshwater bivalves. A single protocol may not optimize accurate measurement of all immune parameters, necessitating multiple assays for a holistic description of immune status (Burkhard et al. 2009). Additionally, current methods require expensive instrumentation and extensive preparation protocols that are not feasible for streamside use.

Cell aggregation is one primary challenge of total and differential cell counts (Chakraborty et al. 2021). Aggregation and adhesion are physiological cell behaviors that occur spontaneously when hemolymph is removed (Chen and Bayne 1995). Antiaggregants are prone to interference with the matrix. Some common antiaggregant solutions used during hemolymph extraction and preparation include heparin, EDTA, Alsever's solution, Leibovitz's L15-medium, Coulter Isoton II diluent, and N-ethylmaleimide (Evariste et al. 2016; Grandiosa et al. 2018; Matozzo et al. 2018) held either at room temperature or on ice (Gagnaire et al. 2004). Accurate measurement of other immunopathological and functional parameters relies on combating the potential toxicity of preservatives, reducing holding times, and ensuring access to affordable instrumentation. Therefore, our study aimed to optimize methods for measuring total hemocyte concentration and stability across five antiaggregant solutions and to compare a handheld cell counter with the standardized automated benchtop cell counter.

METHODS

Mussel Care and Hemolymph Sampling

A total of 125 adult Eastern Lampmussels (*Lampsilis radiata*) with an average total shell length of 63.7 ± 3.04 mm were obtained from the U.S. Fish and Wildlife Service's Harrison Lake National Fish Hatchery (Charles City, Virginia) and transported in coolers containing aerated hatchery pond water in a three-hour trip to North Carolina State University's Yates Mill Aquatic Conservation Center in Raleigh, North Carolina. Mussels were acclimated for 5 d to a temperature of 22.0°C in a 530-L Living Stream System water tank (Frigid Units, Toledo, OH, USA) using pond water filtered to 25.0 µm. Water changes were conducted daily prior to feeding with a commercial feed mixture of *Nannochloropsis* sp., *Isochrysis*, *Pavlova*, *Tetraselmis*, *Thalassiosira pseudonana*, and *Thalassiosira weissflogi* (Reed Mariculture Nanno 3600 and Shellfish Diet 1800). Mussels were isolated in pairs in 9.5-L pulse flow through tanks containing sand substrate (500–1000 µm grain size) with daily water turnover of 25.0 µm pond water; following hemolymph withdrawals, they were monitored for behavioral changes (e.g., decreased filtering, burrowing) and mortality for at least 2 wk.

Experimental Design and Sampling

Ten experiments were conducted to assess (1) the effect of multiple antiaggregants and holding temperature on total hemocyte concentration (THC), volume, size, and stability over time and (2) the reliability of a handheld automated cell counter (Scepter™ 3.0 Automated Cell Counter, Millipore, Miami, FL, USA; hereafter "Scepter") compared to a bench-top particle counter/size analyzer (Multisizer™ 4e Coulter Counter, Beckman Coulter, Brea, CA, USA; hereafter "Multisizer"). Hemolymph (0.5–1.0 mL) was withdrawn from the anterior adductor muscle with a pre-chilled 27.0-gauge, 12.7-mm needle attached to a 1.0-mL sterile Leur Slip syringe and immediately dispensed into a 6.0-mL Fisherbrand™ borosilicate glass culture tube with prechilled antiaggregant. Hemolymph withdrawal practices included removing the needle and vacutainer lids before dispensing out of the syringe to maintain cell integrity. To assess the effects of antiaggregant and holding time, hemolymph was withdrawn from six randomly selected *L. radiata* and held in antiaggregants on ice and from three randomly selected *L. radiata* and held in antiaggregants at room temperature (untreated hemolymph, K₂EDTA Vacutainers, and Isoton II diluent) for the duration of the experiment and assessed after 30 min and 1, 2, and 3 h postremoval. Treatments included the following:

- Untreated hemolymph, osmolarity 300 mOsm/kg
- 2.0 mL K₂EDTA Vacutainer, pH 7.2, osmolarity 300 mOsm/kg
- 3.0 mL 3.2% sodium citrate Vacutainer, pH 7.1, osmolarity 300 mOsm/kg
- Isoton II diluent, pH 7.2, osmolarity 300 mOsm/kg (1:1 dilution v:v)

Alsever's solution, pH 6.9, osmolarity 300 mOsm/kg (1:1 dilution v:v)

Leibovitz's L15, pH 7.2, osmolarity 300 mOsm/kg (1:1 dilution v:v)

To validate the instrumentation, hemolymph was withdrawn from 72 randomly selected *L. radiata*, and paired samples were immediately analyzed using both the Scepter and the Multisizer.

Hemocyte Size Frequency Distribution

We determined total hemocyte concentration, size frequency distribution, and hemocyte volume by dispensing a 20-µL aliquot of hemolymph-antiaggregant (1:1 dilution v:v) solution into a sterile 1.5-mL microcentrifuge tube (Basix™, Fisher Scientific, Waltham, MA) and aspirating into the Scepter equipped with a 60-µm sensor. A separate 100-µL aliquot of the hemolymph solution was added to 5.0 mL of Isoton II diluent in individual Accuvette™ ST sampling vials (Beckman Coulter) for Multisizer analysis. The Multisizer was equipped with a 100-µm aperture set to an aperture current of 800 µA, and a gain setting of two; cell concentration was determined by volumetric analysis. Duplicate 100-µL aliquots were passed into the Multisizer and cells between 5.0 µm and 30.0 µm were counted.

Quality Assurance

A thorough quality assurance protocol was followed through analysis of blanks, duplicates, and standard reference materials. Before each experiment, the Scepter was calibrated with 11-µm PHCC3 beads (Millipore), and the Multisizer was calibrated using certified NIST CC size standard L20 20-µm latex beads (Lot BR0072.2312; Beckman Coulter). Experiments began by blanking the instruments with each antiaggregant in Isoton II. The aperture was increased from 50 µm to 100 µm after preliminary analysis to decrease the rate of aperture blockage, monitored for further blockages, and flushed after every 10 samples. Samples were visually inspected for debris before transferring to Isoton II in accuvettes and mixed well by gently rolling the accuvette and removing air bubbles. Duplicate measurements were taken on each sample with an average coefficient of variance of $3.94\% \pm 3.69\%$ (range 0.00–16.9%; $n = 108$). Samples were removed and repeated if an individual produced significantly apparent discrepancies. Particles less than 5 µm were gated out to avoid cellular debris and improve consistency between instruments based on the manufacturer's specifications.

Statistical Analysis

We exported data from the Beckman Coulter Particle Characterization software (version 4.03) and conducted statistical analyses in GraphPad Prism 10.3.1 (GraphPad Software, Boston, MA). Prior to analysis, we checked data for normality using the Shapiro–Wilk test. Mixed-effects models and Tukey's post-hoc tests were used to compare differences in mean THC, cell volume, and cell size standard deviation over

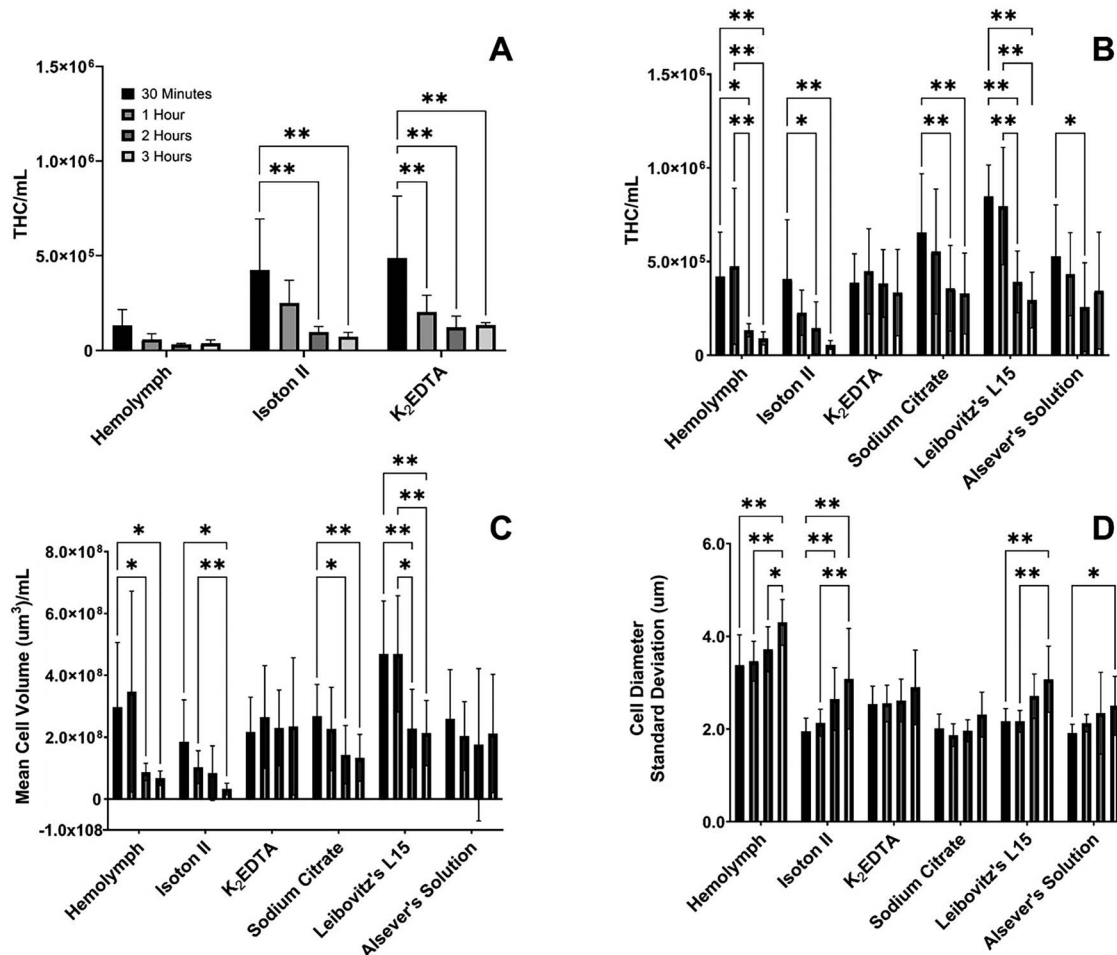


Figure 1. Changes in total hemocyte concentration (THC) in (A) treatments held at room temperature and (B) treatments held on ice. (C) changes in mean cell volume and (D) standard deviation of cell diameter. Error bars represent standard deviation. Asterisks over bars represent statistical significance at $*P < 0.05$ and $**P < 0.01$.

time. Kruskal–Wallis tests were used when data were not normally distributed (only in the case of K₂EDTA held after 6 h). We used linear regression to compare THC between the Multisizer and Scepter. Statistical significance was indicated at $\alpha = 0.05$; P values of ≤ 0.05 and ≤ 0.01 were both presented to indicate relative strength of relations.

RESULTS

Hemogram

Baseline THC (after 30 min of incubation) in all treatments held on ice averaged 5.37×10^5 cells/mL (95% CI, 4.69×10^5 to 6.05×10^5 ; $n = 70$). THC changed over time in all treatments except K₂EDTA Microtainers when held on ice (Fig. 1A, B and Table 1). The greatest differences were observed between untreated hemolymph and hemolymph treated with Isoton II. Hemocyte viability immediately decreased when untreated and incubated at room temperature. THC appeared roughly stable in Alsever's solution, but the highest biological variation was observed in those samples,

and additional individuals had to be sampled for cells to be counted. THC remained stable for up to six hours in K₂EDTA incubated on ice (Fig. 2). Hemocyte size frequency produced a typical bimodal distribution with fewer cells between 6 μm and 10 μm and more between 12 μm and 18 μm , characteristic of hyalinocytes and granulocytes (Fig. 3). Cell volume decreased in most treatments except K₂EDTA and Alsever's solution (Table 2). However, Alsever's solution again produced the most variation between individuals (Fig. 1C), while variation in cell diameter increased in untreated hemolymph and hemolymph treated with all antiaggregants except K₂EDTA and sodium citrate (Fig. 1D and Table 3).

Instrument Validation

There was significant agreement in total hemocyte concentration between the Multisizer and Scepter ($R^2 = 0.9156$ [$F_{(1,70)} = 759.6$, $P < 0.0001$]; $n = 72$; Fig. 4). The Scepter slightly underestimated total particle concentration compared to the Multisizer; the 60- μm Scepter sensor is designed to count particles between 6 μm and 36 μm and cells between 8

Table 1. Percent change of total hemocyte counts in each antiaggregant and holding temperature compared to baseline concentrations. Boldface type represents statistical significance at $P < 0.05$.

Treatment		After 1 h	After 2 h	After 3 h
Held on ice				
	Compared to 30 min at room temperature			
Hemolymph	−68.5%	12.9%	−68.3%	−78.6%
Isoton II	4.73%	−44.2%	−64.2%	−86.6%
K ₂ EDTA	26.2%	15.8%	−0.95%	−13.5%
Sodium citrate	—	−15.6%	−45.5%	−49.8%
Leibovitz L15	—	−6.06%	−53.8%	−65.2%
Alsever's solution	—	−18.2%	−51.4%	−34.8%
Held at room temperature				
Hemolymph		−55.9%	−75.9%	−70.5%
Isoton II		−41.0%	−77.0%	−82.8%
K ₂ EDTA		−58.3%	−74.9%	−72.5%

and 25 μm , whereas the Multisizer 100- μm sensor has a much greater range depending on user settings. Additionally, inherent biological variability of the hemolymph matrix generated minor differences between technical replicates measured on

the Multisizer ($R^2 = 0.9698$ [$F_{(1,106)} = 3401$], $P < 0.0001$; $\text{CV} = 3.94\% \pm 3.69\%$; range 0.00–16.9%; 95% CI = 3.23–4.64%; $n = 108$).

DISCUSSION

We optimized methods to preserve freshwater mussel hemolymph for rapid, automated quantification of multiple parameters reflective of instantaneous immune status. THC recorded by the Scepter was significantly concordant with the Multisizer measurements. Regardless of the antiaggregant used, we concurrently demonstrated that hemolymph must be held on ice as hemocytes lost integrity immediately after withdrawal at room temperature. Antiaggregants that chelate calcium ions, such as sodium citrate and EDTA, performed better than other antiaggregants we investigated, particularly when potassium ions were added to further decrease free calcium, as demonstrated in *in vitro* studies (Grandiosa et al. 2018). The critical role of calcium in regulating hemocyte aggregation offers a potential strategy for managing hemocyte behavior for biomarker identification, quantification, and monitoring, as well as for preserving hemocytes for hemogram characterization.

The invertebrate hemogram has informed diagnoses of stress, disease, contaminant exposure, and starvation, but determination conventionally relies on microscopy (Hinzmann et al. 2017; Steinagel et al. 2018). Cell identification and quantification, while useful, can be less practical without extensive training or significant dedicated time resources. In contrast, flow cytometry quantifies morphological, chemical, and functional endpoints (Donaghy and Volety 2011; Nguyen and Alfaro 2019; Barjhoux et al. 2020). Yet flow cytometry requires costly instrumentation and extensive preparation protocols unsuitable for field use. Coulter counters balance some of these limitations and are commonly available in mussel propagation facilities as instruments to monitor food availability and filtration rates (Carter et al. 1985; Mueller et al. 2025).

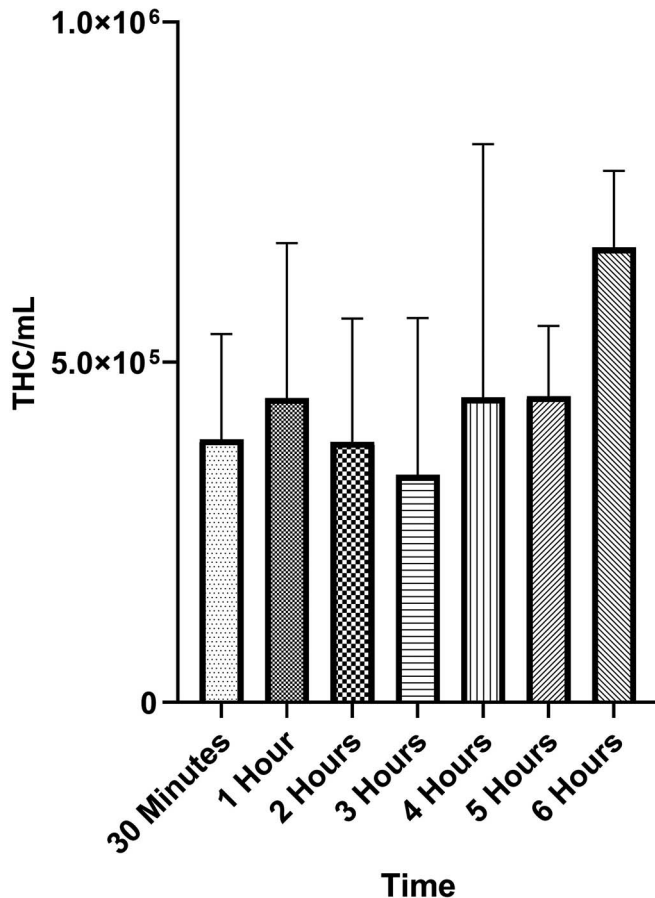


Figure 2. Changes in total hemocyte concentration (THC) in K₂EDTA held on ice for 6 h. No significant differences were recorded at $P < 0.05$ by Kruskal–Wallis test $H(6) = 11.10$, $P = 0.0852$.

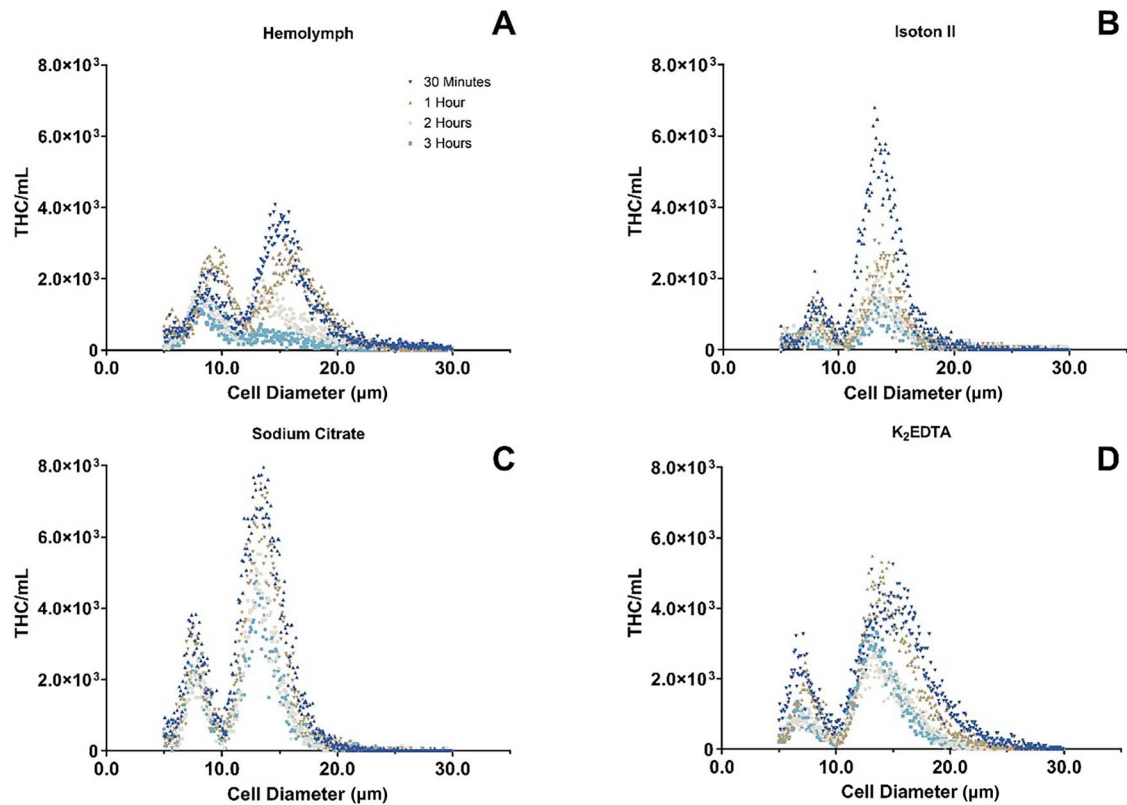


Figure 3. Total hemocyte concentration (THC) distributions averaged among six individuals reported on the Multisizer 4e.

Antiaggregant Effectiveness

Hemocyte stability varied widely across treatments. We found that THC, volume, and cell diameter variation changed significantly in hemolymph alone with no treatment. Aggregation can be attributed to a combination of factors as hemocytes adapt to new conditions outside the circulatory system.

Spontaneous hemocyte aggregation occurs as a part of the innate immune response activated in part by calcium serving

as an intracellular signaling molecule in transduction pathways involved in cell adhesion (Cheng et al. 2006). Specifically, calcium ions are essential for the activation of adhesion molecules, such as cadherins and integrins, that mediate cell–cell and cell–substrate interactions (Shozawa and Suto 1990; Alberts et al. 2002). This cascade of events begins with the activation of phospholipase C, leading to the production of inositol trisphosphate, which triggers the release of calcium ions from intracellular stores into the cytoplasm (Putney and

Table 2. Percent change of hemocyte volume in each antiaggregant and holding temperature compared to baseline concentrations. Boldface type represents statistical significance at *P* < 0.05.

Treatment		After 1 h	After 2 h	After 3 h
Held on ice				
Compared to 30 min at room temperature				
Hemolymph	−61.7%	16.5%	−70.7%	−77.5%
Isoton II	17.9%	−44.0%	−54.7%	−82.4%
K ₂ EDTA	70.6%	22.1%	5.88%	8.0%
Sodium citrate	—	−15.5%	−46.7%	−50.4%
Leibovitz L15	—	0.04%	−51.5%	−54.6%
Alsever’s solution	—	−21.5%	−32.3%	−18.3%
Held at room temperature				
Hemolymph		−66.9%	−78.9%	−78.5%
Isoton II		−27.8%	−69.4%	−80.1%
K ₂ EDTA		−38.3%	−64.4%	−61.2%

Table 3. Percent change of hemocyte diameter standard deviation in each antiaggregant and holding temperature compared to baseline concentrations. Boldface type represents statistical significance at $P < 0.05$.

Treatment		After 1 h	After 2 h	After 3 h
Held on ice	Compared to 30 min at room temperature			
Hemolymph	5.0%	2.5%	10.1%	27.2%
Isoton II	−2.5%	9.5%	35.7%	58.1%
K ₂ EDTA	21.8%	0.7%	3.19%	14.3%
Sodium citrate	—	−7.3%	−2.7%	14.7%
Leibovitz L15	—	−0.18%	24.9%	41.8%
Alsever's solution	—	11.4%	22.6%	30.9%
Held at room temperature				
Hemolymph		−2.8%	13.1%	4.6%
Isoton II		27.0%	70.0%	81.5%
K ₂ EDTA		37.7%	46.1%	34.1%

Tomita 2012). Elevated calcium levels then activate protein kinases and other signaling molecules, enhancing the affinity of adhesion molecules and further promoting hemocyte aggregation (Ramos Martínez et al. 2012; Meier et al. 2024).

Hemocyte aggregation mediates phagocytosis, one of the primary functions of hemocytes to defend against foreign pathogens and eliminate cellular debris across multiple taxa (Liu et al. 2020; de la Ballina et al. 2022). Hemocytes also aggregate to facilitate wound healing and tissue repair by releasing signaling molecules such as cytokines to promote healing (Chakraborty et al. 2021). Once removed from circulation, hemocytes are activated from a resting state without inhibitory signals (Chen and Bayne 1995). Similarly, while demonstrated to be nonlethal, handling and hemolymph withdrawal may stimulate a rapid release of cytokines and chemokines (Gustafson et al. 2005a). Environmental effects, such as changes in temperature or oxygen levels, can also promote an emergency response (Ericson et al. 2024).

Colder temperatures slow aggregation, cell metabolism, and cell death. They also reduce metabolic rates and stabilize cell counts for longer-term holding (Chen and Bayne 1995; Lapointe et al. 2012). Hemocytes require consistent osmotic balance and nutrient supply for physiological equilibrium, or they may experience rapid apoptosis or aggregation (Hinzmann 2016). Like hemolymph, Isoton II, a salt solution, maintains osmolarity, but in our study it did not actively maintain cell integrity or prevent aggregation. Alsever's solution and Leibovitz's L15 are routinely used to preserve red blood cells in vertebrates by maintaining a balanced ionic environment and providing nutrients, but they are less effective in invertebrates (Tang et al. 2022). Calcium buffering effectively decreases cell adhesion, which has important implications for maintaining more regulated, automated cell differentiation (Eisner et al. 2023).

Hemogram Biomarkers

The Multisizer provided simultaneous quantification of multiple immunological biomarkers that can expand interpretations beyond simple cell counting. Baseline THC levels were lower than what might be expected from previous research, but because mussels were collected during winter, they may have had depressed hemocyte production and lowered metabolic rates (Blaise et al. 2002; Salimi et al. 2009; Gillis 2012; Yao and Somero 2012; Evariste et al. 2016; Lubawy and Słocińska 2020).

Future studies are needed to quantify the hemogram across different seasons. For example, an average of 5.86×10^6 cells/mL ($n = 4$), an order of magnitude higher than this study, was found while determining that hemolymph withdrawal can be a nonlethal technique in *Elliptio complanata* (Gustafson et al. 2005a). Observed changes in cell concentration from baseline could be the result of either aggregation or cell lysis. Expected bimodal cell size distribution reflects the two main cell populations, hyalinocytes and granulocytes, but

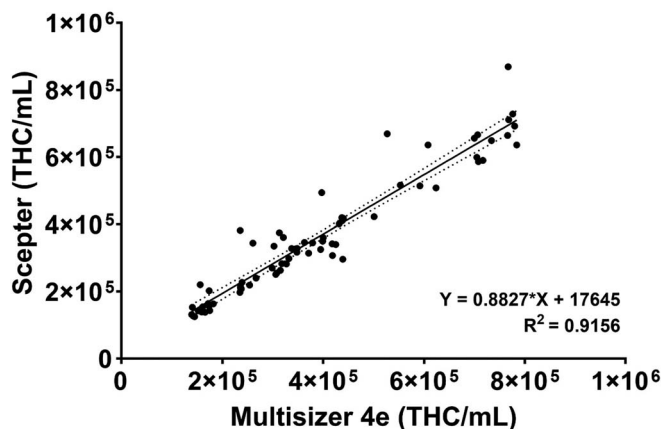


Figure 4. Relationship between total hemocyte concentration (THC) simultaneously measured on both the Beckman Coulter Multisizer 4e and Millipore Scepter ($P < 0.001$).

intermediate cell types also have been described in bivalves—qualities that are not easily discernible by the Multisizer compared to a flow cytometer (Burkhard et al. 2009; Steinagel et al. 2018).

Changes to cell volume can reflect allometric scaling, the biological process where cell volume decreases as diameter increases, indicative of cell-shape changes and effects of osmotic pressure. The decrease in mean cell volume across multiple antiaggregants suggests either changes to the overall differential cell populations, cell lysis, atrophy, or compensatory mechanisms as cells reduce their volume to optimize metabolic efficiency (Fig. 1C). Similarly, increased heterogeneity of cell diameter indicates loss of homeostasis as cell types respond differentially to stress (Fig. 1D). Resiliency to stress among cell types varies, disrupting the expected distribution.

Instrument Validation

Freshwater mussel conservation relies heavily on in-stream, on-site surveys to monitor population level stability, collect healthy broodstock for propagation, and augment populations with propagated individuals. Many sites are relatively remote with long travel times to laboratory or facility resources. Surveys can benefit from additional tools to assess individual and population health, especially in the event of active die-offs or declines. The Scepter has been used in other invertebrate investigations to determine THC, hemocyte diameter, and hemocyte volume in the crab *Carcinus aestuarii* following exposure to bisphenols, and to observe increased hemocyte counts in *Mytilus galloprovincialis* exposed to a mixture of glyphosate and aminomethylphosphonic acid and *Crassostrea gigas* exposed to higher temperatures and *Vibrio* infection (Wendling and Wegner 2015; Matozzo et al. 2018; Fabrello et al. 2024).

Total hemocyte concentration compared between the Scepter and Multisizer was within the expected variation as measured by duplicate samples processed by the Multisizer. Dilution during sample preparation produces inherent sample heterogeneity. Further investigations should facilitate generating reference ranges across species and environmental conditions to understand if biotic and abiotic influences transcend observed instrumental variation and provide relevant diagnostic or prognostic tools. Additionally, more refined differentiation between hemocyte subpopulations' responses to immune stressors would further validate the use of instruments such as the Scepter.

Ecological Relevance and Implications for Conservation

Our study supports the use of hemolymph to assess the health status of freshwater mussels, and it offers a practical tool and solution for population conservation efforts. Rapid generation and deployment of hemogram reference ranges can expand the potential for diagnosing and predicting declines in health, support mussel-care techniques to maximize health and resilience, and connect environmental stressors to physiological conditions.

ACKNOWLEDGMENTS

This research was funded by the North Carolina Department of Transportation through Research Project No. 2022-25 to W. Gregory Cope. The participation of W. Gregory Cope in this study was made possible by the Research Capacity Fund (Hatch) project award no. 7007359 from the U.S. Department of Agriculture's National Institute of Food and Agriculture. We thank Brian Watson of the Virginia Department of Wildlife Resources, and Rachel Mair, Jaclyn Zelko, and the staff of Harrison Lake National Fish Hatchery for providing the freshwater mussels used in this study. We also thank Jared Gray and Dr. Jordan Richard for reviewing an earlier draft of the manuscript.

LITERATURE CITED

- Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter. 2002. Cell-cell adhesion, pages 1035–1090. *Molecular Biology of the Cell*, 4th ed. Garland Science, New York.
- Allam, B., and D. Raftos. 2015. Immune responses to infectious diseases in bivalves. *Journal of Invertebrate Pathology* 131:121–136. doi: 10.1016/J.JIP.2015.05.005
- Anisimova, A. A. 2013. Morphofunctional parameters of hemocytes in the assessment of the physiological status of bivalves. *Russian Journal of Marine Biology* 39:381–391. doi: 10.1134/S1063074013060023
- Auffret, M. 1988. Bivalve hemocyte morphology. *American Fisheries Society Special Publications* 18:169–177.
- Auffret, M., S. Rousseau, I. Boutet, A. Tanguy, J. Baron, D. Moraga, and M. Duchemin. 2006. A multiparametric approach for monitoring immunotoxic responses in mussels from contaminated sites in Western Mediterranean. *Ecotoxicology and Environmental Safety* 63:393–405. doi: 10.1016/J.ECOENV.2005.10.016
- Barjhoux, I., D. Rioult, A. Geffard, and M. Palos Ladeiro. 2020. A new protocol for the simultaneous flow cytometric analysis of cytotoxicity and immunotoxicity on zebra mussel (*Dreissena polymorpha*) hemocytes. *Fish & Shellfish Immunology* 98:224–235. doi: 10.1016/J.FSI.2019.12.092
- Blaise, C., S. Trottier, F. Gagné, C. Lallement, and P. D. Hansen. 2002. Immunocompetence of bivalve hemocytes as evaluated by miniaturized phagocytosis assay. *Environmental Toxicology* 17:160–169. doi: 10.1002/TOX.10047
- Burkhard, M. J., S. Leavell, R. B. Weiss, K. Kuehn, H. Valentine, G. T. Watters, and B. A. Wolfe. 2009. Analysis and cytologic characterization of hemocytes from freshwater mussels (*Quadrula* sp.). *Veterinary Clinical Pathology* 38:426–436. doi: 10.1111/J.1939-165X.2009.00148.X
- Canesi, L., G. Gallo, M. Gavioli, and C. Pruzzo. 2002. Bacteria-hemocyte interactions and phagocytosis in marine bivalves. *Microscopy Research and Technique* 57:469–476. doi: 10.1002/JEMT.10100
- Carella, F., G. Villari, N. Maio, and G. D. De Vico. 2016. Disease and disorders of freshwater unionid mussels: A brief overview of recent studies. *Frontiers in Physiology* 7:489. doi: 10.3389/FPHYS.2016.00489
- Carter, J. H., S. L. Ledis, and H. R. Crews. 1985. Multi-purpose blood diluent and lysing agent for differential determination of lymphoid-myeloid population of leukocytes. Patent US4521518A.
- Chakraborty, S., M. Ray, and S. Ray. 2021. Bivalve haemocyte adhesion, aggregation and phagocytosis: A tool to reckon arsenic induced threats to freshwater ecosystem. *Fish & Shellfish Immunology* 114:229–237. doi: 10.1016/J.FSI.2021.05.008
- Chen, J. H., and C. J. Bayne. 1995. Bivalve mollusc hemocyte behaviors: Characterization of hemocyte aggregation and adhesion and their inhibition in the California mussel (*Mytilus californianus*). *Biological Bulletin* 188:255–266. doi: 10.2307/1542303

- Cheng, H., S. Wei, L. Wei, and A. Verkhatsky. 2006. Calcium signaling in physiology and pathophysiology. *Acta Pharmacologica Sinica* 27:767–772. doi: 10.1111/j.1745-7254.2006.00399.x
- Chu, F. L. E. 1988. Humoral defense factors in marine bivalves. *American Fisheries Society Special Publications* 18:178–188. <https://scholarworks.wm.edu/vimsbooks/121>
- de la Ballina, N. R., F. Maresca, A. Cao, and A. Villalba. 2022. Bivalve haemocyte subpopulations: A review. *Frontiers in Immunology* 13:1069. doi: 10.3389/FIMMU.2022.826255/BIBTEX
- Donaghy, L., and A. K. Volety. 2011. Functional and metabolic characterization of hemocytes of the green mussel, *Perna viridis*: In vitro impacts of temperature. *Fish & Shellfish Immunology* 31:808–814. doi: 10.1016/J.FSI.2011.07.018
- Eisner, D., E. Neher, H. Taschenberger, and G. Smith. 2023. Physiology of intracellular calcium buffering. *Physiological Reviews* 103:2767–2845. doi: 10.1152/physrev.00042.2022
- Ericson, J. A., O. Laroche, L. Biessy, N. J. Delorme, X. Pochon, J. Thomson-Laing, N. L. C. Ragg, and K. F. Smith. 2024. Differential responses of selectively bred mussels (*Perna canaliculus*) to heat stress—survival, immunology, gene expression and microbiome diversity. *Frontiers in Physiology* 14:1265879. doi: 10.3389/fphys.2023.1265879
- Evariste, L., M. Auffret, S. Audonnet, A. Geffard, E. David, P. Brousseau, M. Fournier, and S. Betoulle. 2016. Functional features of hemocyte subpopulations of the invasive mollusk species *Dreissena polymorpha*. *Fish & Shellfish Immunology* 56:144–154. doi: 10.1016/j.fsi.2016.06.054
- Fabrello, J., E. Tarussio, A. Romanello, A. Schiavon, V. R. Damoli, F. Luisi, M. Roverso, S. Bogianni, and V. Matozzo. 2024. A multibiomarker approach to assess the effects of a BPA analogue-contaminated diet in the crab *Carcinus aestuarii*. *Aquatic Toxicology* 276:107084. doi: 10.1016/j.aquatox.2024.107084
- Farcy, E., F. Gagné, L. Martel, M. Fortier, S. Trépanier, P. Brousseau, and M. Fournier. 2011. Short-term physiological effects of a xenobiotic mixture on the freshwater mussel *Elliptio complanata* exposed to municipal effluents. *Environmental Research* 111:1096–1106. doi: 10.1016/J.ENVRES.2011.07.009
- Fritts, A. K., J. T. Peterson, P. D. Hazelton, and R. B. Bringolf. 2015a. Evaluation of methods for assessing physiological biomarkers of stress in freshwater mussels. *Canadian Journal of Fisheries and Aquatic Sciences* 72:1450–1459. doi: 10.1139/CJFAS-2014-0564
- Fritts, A. K., J. T. Peterson, J. M. Wisniewski, and R. B. Bringolf. 2015b. Nonlethal assessment of freshwater mussel physiological response to changes in environmental factors. *Canadian Journal of Fisheries and Aquatic Sciences* 72:1460–1468. doi: 10.1139/CJFAS-2014-0565
- Gagnaire, B., H. Thomas-Guyon, and T. Renault. 2004. In vitro effects of cadmium and mercury on Pacific oyster, *Crassostrea gigas* (Thunberg), haemocytes. *Fish & Shellfish Immunology* 16:501–512. doi: 10.1016/j.fsi.2003.08.007
- Giamberini, L., M. Auffret, and J. C. Pihan. 1996. Haemocytes of the freshwater mussel, *Dreissena polymorpha* Pallas: cytology, cytochemistry, and X-ray microanalysis. *Journal of Molluscan Studies* 62:367–379. doi: 10.1093/mollus/62.3.367
- Gillis, P. L. 2012. Cumulative impacts of urban runoff and municipal wastewater effluents on wild freshwater mussels (*Lasmigona costata*). *Science of the Total Environment* 431:348–356. doi: 10.1016/j.scitotenv.2012.05.061
- Graf, D. L., and K. S. Cummings. 2007. Review of the systematics and global diversity of freshwater mussel species (Bivalvia: Unionoida). *Journal of Molluscan Studies* 73:291–314. doi: 10.1093/MOLLUS/EYM029
- Grandiosa, R., M. L. Bouwman, T. Young, F. Mérien, and A. C. Alfaro. 2018. Effect of antiaggregants on the in vitro viability, cell count and stability of abalone (*Haliotis iris*) haemocytes. *Fish & Shellfish Immunology* 78:131–139. doi: 10.1016/J.FSI.2018.04.038
- Grizzle, J. M., and C. J. Brunner. 2009. Infectious diseases of freshwater mussels and other freshwater bivalve mollusks. *Reviews in Fisheries Science* 17:425–467. doi: 10.1080/10641260902879000
- Gustafson, L. L., M. K. Stoskopf, A. E. Bogan, W. Showers, T. J. Kwak, S. Hanlon, and J. F. Levine. 2005a. Evaluation of a nonlethal technique for hemolymph collection in *Elliptio complanata*, a freshwater bivalve (Mollusca: Unionidae). *Diseases of Aquatic Organisms* 65:159–165. doi: 10.3354/DAO065159
- Gustafson, L. L., M. K. Stoskopf, W. Showers, G. Cope, C. Eads, R. Linnehan, T. J. Kwak, B. Andersen, and J. F. Levine. 2005b. Reference ranges for hemolymph chemistries from *Elliptio complanata* of North Carolina. *Diseases of Aquatic Organisms* 65:167–176. doi: 10.3354/DAO065167
- Haag, W. R. 2012. *North American Freshwater Mussels: Natural History, Ecology, and Conservation*. Cambridge University Press, Cambridge, England. 505 pp.
- Haag, W. R. 2019. Reassessing enigmatic mussel declines in the United States. *Freshwater Mollusk Biology and Conservation* 22:43–60. doi: 10.31931/FMBC.V22I2.2019.43-60
- Haag, W. R., and J. D. Williams. 2014. Biodiversity on the brink: an assessment of conservation strategies for North American freshwater mussels. *Hydrobiologia* 735:45–60. doi: 10.1007/S10750-013-1524-7
- Hinzmann, M. F. 2016. Studies on immunity of freshwater bivalves (Unionidae family): Characterization, antibacterial activity and applications as biomarkers. Doctoral dissertation, University of Porto, Portugal. doi: 10.13140/RG.2.2.12669.87520
- Hinzmann, M., M. Lopes-Lima, F. Cerca, A. Correia, J. Machado, and M. Vilanova. 2017. Identification of distinct haemocyte populations from the freshwater bivalves swan mussel (*Anodonta cygnea*) and duck mussel (*Anodonta anatina*) using wheat-germ agglutinin. *Canadian Journal of Zoology* 95:937–947. doi: 10.1139/cjz-2017-0006
- Kataoka, C., and S. Kashiwada. 2021. Ecological risks due to immunotoxicological effects on aquatic organisms. *International Journal of Molecular Sciences* 22:8305. doi: 10.3390/IJMS22158305
- King, W. L., N. Siboni, T. Kahlke, M. Dove, W. O'Connor, K. R. Mahbub, C. Jenkins, J. R. Seymour, and M. Labbate. 2020. Regional and oyster microenvironmental scale heterogeneity in the Pacific oyster bacterial community. *FEMS Microbiology Ecology* 96:fiaa054. doi: 10.1093/FEMSEC/FIAA054
- Lapointe, J. F., G. B. Dunphy, and C. A. Mandato. 2012. Hemocyte–hemocyte adhesion and nodulation reactions of the greater wax moth, *Galleria mellonella* are influenced by cholera toxin and its B-subunit. *Results in Immunology* 2:54–65. doi: 10.1016/j.rnim.2012.02.002
- Levine, J. F., M. Law, and T. Ben-Horin. 2022. Bivalves. Pages 203–234 in G. A. Lewbart, editor. *Invertebrate Medicine*, 3rd ed. John Wiley & Sons, Hoboken, New Jersey. doi: 10.1002/9781119569831.CH10
- Liu, S., S.-C. Zheng, Y.-L. Li, J. Li, and H.-P. Liu. 2020. Hemocyte-mediated phagocytosis in crustaceans. *Frontiers in Immunology* 11:268. doi: 10.3389/fimmu.2020.00268
- Lubawy, J., and M. Słocińska. 2020. Characterization of *Gromphadorhina coquereliana* hemolymph under cold stress. *Scientific Reports* 10:12076. doi: 10.1038/s41598-020-68941-z
- Matozzo, V., C. Ercolini, L. Serracca, R. Battistini, I. Rossini, G. Granato, E. Quagliari, A. Perolo, L. Finos, G. Arcangeli, D. Bertotto, G. Radaelli, B. Chollet, I. Arzul, and F. Quaglio. 2018. Assessing the health status of farmed mussels (*Mytilus galloprovincialis*) through histological, microbiological and biomarker analyses. *Journal of Invertebrate Pathology* 153:165–179. doi: 10.1016/J.JIP.2018.02.018
- Meier, C. J., S. Ahmed, J. S. Barr, T. Y. Estévez-Lao, and J. F. Hillyer. 2024. Extracellular matrix proteins Pericardin and Lonely heart mediate peritrophic hemocyte aggregation in the mosquito *Anopheles gambiae*. *Developmental & Comparative Immunology* 159:105219. doi: 10.1016/j.dci.2024.105219

- Mueller, M. E., C. E. LePrevost, and W. G. Cope. 2025. Finding the pulse of healthy native freshwater mussels (order Unionida): insights from propagation biologists. *Journal of Aquatic Animal Health* 37:47–53. doi: 10.1093/jahafs/vsae001
- Nguyen, T. V., and A. C. Alfaro. 2019. Applications of flow cytometry in molluscan immunology: Current status and trends. *Fish & Shellfish Immunology* 94:239–248. doi: 10.1016/J.FSI.2019.09.008
- Putney, J. W., and T. Tomita. 2012. Phospholipase C signaling and calcium influx. *Advances in Biological Regulation* 52:152–164. doi: 10.1016/j.advenzreg.2011.09.005
- Ramos Martínez, J. I., M. González-Riopedre, and R. Barcia. 2012. Role of protein kinases C (PKC) in the relationship between the neuroendocrine and immune systems in marine mussels: the model of *Mytilus galloprovincialis* Lamarck (1819). *Italian Journal of Zoology* 79:162–168. doi: 10.1080/11250003.2011.600729
- Richling, I., and C. Krause. 2022. Minimally invasive DNA sampling by hemolymph extraction minimizes risk to freshwater mussels: The case of endangered *Unio crassus* Philipsson, 1788 (Bivalvia: Unionidae). *Malacologia* 64:309–313. doi: 10.4002/040.064.0212
- Salimi, L., S. Jamili, A. Motalebi, P. Eghtesadi-Araghi, M. Rabbani, and M. Rostami-Beshman. 2009. Morphological characterization and size of hemocytes in *Anodonta cygnea*. *Journal of Invertebrate Pathology* 101: 81–85. doi: 10.1016/J.JIP.2009.03.003
- Shozawa, A., and C. Suto. 1990. Hemocytes of *Pomacea canaliculata*: I. reversible aggregation induced by Ca^{2+} . *Developmental & Comparative Immunology* 14:175–184. doi: 10.1016/0145-305x(90)90089-w
- Song, L. 2016. Immune defence mechanisms of bivalves. *Fish & Shellfish Immunology* 53:80–81. doi: 10.1016/J.FSI.2016.03.109
- Song, L., L. Wang, L. Qiu, and H. Zhang. 2010. Bivalve immunity. *Advances in Experimental Medicine and Biology* 708:44–65.
- Steinagel, A. C., M. J. Burkhard, K. F. Kuehn, G. T. Watters, P. J. Rajala-Schultz, K. H. Valentine, and B. A. Wolfe. 2018. Hematological and biochemical assessment of two species of freshwater mussels, *Quadrula quadrula* and *Amblema plicata*, following translocation. *Journal of Aquatic Animal Health* 30:119–129. doi: 10.1002/AAH.10016
- Tang, S.H., H.C. Lin, J.-B. Chang, Y.-S. Chan, H.-F. Tang, F.-Y. Chang, T.-S. Chiueh, and B.H. Yang. 2022. Preservation of red blood cell antigenicity in a new storage solution in vitro. *Annals of Medicine* 55:168–174. doi: 10.1080/07853890.2022.2157476
- Van der Oost, R., J. Beyer, and N. P. E. Vermeulen. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: A review. *Environmental Toxicology and Pharmacology* 13:57–149. doi: 10.1016/S1382-6689(02)00126-6
- van Niekerk, G., S. M. Hattingh, and A. M. Engelbrecht. 2016. Invertebrates: Why no adaptive immune system? *Scandinavian Journal of Immunology* 83:160–161. doi: 10.1111/SJI.12400
- Vaughn, C. C. 2018. Ecosystem services provided by freshwater mussels. *Hydrobiologia* 810:15–27. doi: 10.1007/S10750-017-3139-X
- Waller, D. L., and W. G. Cope. 2019. The status of mussel health assessment and a path forward. *Freshwater Mollusk Biology and Conservation* 22: 26–42. doi: 10.31931/FMBC.V22I2.2019.26-42
- Wendling, C. C., and K. Mathias Wegner. 2015. Adaptation to enemy shifts: Rapid resistance evolution to local *Vibrio* spp. in invasive Pacific oysters. *Proceedings of the Royal Society B: Biological Sciences* 282:20142244. doi: 10.1098/RSPB.2014.2244
- Yao, C.-L., and G. N. Somero. 2012. The impact of acute temperature stress on hemocytes of invasive and native mussels (*Mytilus galloprovincialis* and *Mytilus californianus*): DNA damage, membrane integrity, apoptosis and signaling pathways. *Journal of Experimental Biology* 215:4267–4277. doi: 10.1242/jeb.073577
- Zannella, C., F. Mosca, F. Mariani, G. Franci, V. Folliero, M. Galdiero, P. G. Tiscar, and M. Galdiero. 2017. Microbial diseases of bivalve mollusks: Infections, immunology and antimicrobial defense. *Marine Drugs* 15:182. doi: 10.3390/MD15060182

REGULAR ARTICLE

DISTRIBUTION OF MICROPLASTICS IN FRESHWATER MUSSELS ACROSS A WATERSHED

James Brandon Williams and Michael M. Gangloff*

Department of Biology, 572 Rivers Street, Appalachian State University, Boone, NC 28608-2027

ABSTRACT

Microplastic research is an emerging field of study, and we do not yet fully understand the scope or effects of microplastic pollution. Microplastics are ubiquitous in the environment, but the field suffers from a lack of unified, accessible protocols and an ecosystems approach to evaluating microplastic contamination. During autumn 2020, we collected water, mussel, and sediment samples from five sites along the Yadkin–Pee Dee River Basin, North Carolina. These samples were digested, filtered, and analyzed for microplastic particles. We found significant differences in microplastic particles in water, mussel tissue, and sediment samples among sites. Low microplastic concentrations at the upstream site and high concentrations at the downstream site contributed to these differences. Water sample microplastic concentrations were positively correlated with drainage area, and sediment concentrations were negatively correlated with elevation. Mussel microplastic concentrations were negatively correlated with weight but were positively correlated with drainage area. These results reveal that microplastics do not always move readily between water, biota, and sediment. Our study highlights the need for a standardized and accessible protocol that can be used to process and analyze samples of water, sediment, and biota. The protocol we developed was effective and relatively low-cost; it may facilitate microplastic research in understudied regions and provide results that are more readily comparable across studies.

KEY WORDS: Unionidae, *Elliptio*, North Carolina, Yadkin–Pee Dee River Basin, watershed

INTRODUCTION

In the past two decades, an increasing number of studies have focused on monitoring microplastics within the soft tissues of aquatic organisms and have attempted to synthesize the effects of microplastics on biota (Akdogan and Guven 2019; Bom and Sa 2021; Moyo 2022). Microplastics are plastic particles < 5 mm in diameter, and they arrive in freshwater from a variety of sources (Karbalaie et al. 2018; Wu et al. 2019). Prior research has revealed that microplastics are ubiquitous in both terrestrial and marine environments. However, the primary focus of most research at this point has been marine ecosystems, even though riverine systems are direct conduits of microplastic transport to marine systems (Blettler et al. 2017; van Wijnen et al. 2019). Consequently, little is known about the distribution of microplastics throughout riverine networks and in North American freshwater biota. A better understanding of this distribution may assist in both cleaning up and mitigating microplastic contamination in riverine systems. It also might aid future

research to predict which biota may be most heavily impacted, where point source pollution may end up, and what the potential is for impacts on human health.

Microplastics found in freshwater have the potential to affect a wide range of biota. Freshwater can transport microplastics over long distances, with most ultimately ending up in the ocean (Eerkes-Medrano et al. 2015). Contaminated groundwater also can become a source of microplastics to freshwater systems. Determining the sources and effects of these pollutants can be difficult due to the lack of long-term studies and widespread monitoring. However, many compounds found in plastic, such as bisphenol A (BPA), have been linked to health effects in human and animal trials (Mandel et al. 2019). More recently, microplastics have been shown to adsorb per- and polyfluoroalkyl (PFAS) substances in the presence of organic matter (Scott et al. 2021). Due to the detrimental effects of microplastics, BPA, and PFAS on human health and the environment, the EPA recently has focused on these substances.

Primary and secondary microplastics are transferred to waterways via wastewater discharges, as many treatment

*Corresponding Author: gangloffmm@appstate.edu

plants are not equipped to filter particles < 5 mm from wastewater streams (Karbalaei et al. 2018). Microbeads are the most common form of primary microplastic despite their ban by the Microbead-Free Waters Act of 2015. Secondary microplastics are derived from the breakdown of larger plastic objects within waterbodies or as runoff from terrestrial systems. Fibers shed from synthetic clothing during washing are an example of secondary microplastics commonly found in wastewater and waterways (Karbalaei et al. 2018).

Microplastic particles are readily consumed by many aquatic organisms and can accumulate in the gills, liver, and brain (Ding et al. 2018; Su et al. 2018). Microplastic particles are found throughout nearly all of Earth's aquatic ecosystems, but they are seemingly most abundant in urban environments as well as estuaries associated with rivers draining densely populated watersheds (Leads and Weinstein 2019). The few studies that have looked at microplastic distributions across a watershed have speculated that atmospheric deposition, urbanization, and wastewater water effluent may be the largest contributors of microplastics to river networks (Elnahas et al. 2024; Miller et al. 2024; Williams et al. 2024). However, targeted studies examining wastewater and land use impacts are not always clear. Microplastic levels in Northern Hogsucker (*Hypentelium nigricans*) guts were correlated with agricultural land use as well as the concentration of *Escherichia coli* in water samples (Tuzzio et al. 2025).

Microplastic ingestion impacts a wide range of filter feeders. The ingestion of microplastics by *Chironomus riparius*, a deposit-feeding freshwater midge, led to reduced larval size, delayed emergence, and adult-transported microplastic particles moving from freshwater to terrestrial environments and biota (Silva et al. 2019). Similarly, microplastic consumption by *Daphnia magna* inhibited feeding rates and particle capture effectiveness (Colomer et al. 2019). Studies of freshwater bivalves have proven more equivocal, with some studies finding that microplastics are associated with changes to burrowing behavior or feeding effectiveness; acute impacts were seldom detected (Weber et al. 2021; Kudla et al. 2024). This may be due to the ability of bivalves to selectively remove or depurate microplastics from their tissues as they grow or to the need for more sophisticated metabolomic assays to detect microplastic effects on cellular and subcellular processes (Wu et al. 2022; Chen et al. 2026).

Although several recent studies document the effects of microplastic on unionids, the distribution of microplastics on the landscape has only recently begun to be appreciated. Few studies have examined microplastic abundance in southeastern US drainages where freshwater mollusk diversity and endangerment is considerable (Neves et al. 1997). Because hydrologic and biotic attributes of river systems differ across geographic scales, models of microplastic dispersal and abundance should incorporate local physiochemical and climatological attributes to better understand how these novel contaminants affect waterways and biota in the biodiverse freshwater ecosystems of this region (sensu Williams et al. 2024).

Filter feeders play foundational roles within many freshwater ecosystems and occupy basal trophic levels in many larger streams and rivers (Thorp and DeLong 1994). The microplastic burdens of these filter feeders may contribute to bioaccumulation at higher trophic levels. Moreover, bivalves were among the most likely taxonomic groups to be impacted by microplastics, and microplastics had the greatest impacts on filter feeders, omnivores, and predators (Moyo 2022). Microplastics have been observed in a range of filter-feeding organisms including zebra mussels (family Dreissenidae) and basket clams (Corbiculidae) (Weber et al. 2021).

Until recently, few studies had examined microplastic concentrations in freshwater pearly mussels (Order Unionoida). However, recent work by Weber et al. (2021) in Germany, Alemeshal et al. (2022) in Hungary, Della Torre et al. (2023) in Italy, and Gedik and Atasaral (2022) in Türkiye, as well as Wardlaw and Prosser (2020) and Doucet et al. (2021) in Canada have all found microplastics present in unionids. Given the broad range of habitats unionids occupy and their ecological sensitivities, accumulation rates and their effects might vary widely across freshwater pearly mussel taxa. Unionids may provide a useful model for microplastic impacts because they are large and abundant filter feeders that can process up to $6\text{--}8\text{ L d}^{-1}$ water while simultaneously interacting with, and obtaining food from, benthic substrates (Neves et al. 1997; Haag 2012; Williams et al. 2014). Freshwater mussels excrete microplastics in the form of pseudofeces (i.e., undigestible organic matter mixed with sediment) that is deposited into adjacent sediments (Ringwood 2021).

Because the abundances of fine sediments and organic matter generally increase in a downstream direction (sensu Vannote et al. 1980), we expected microplastic concentrations to be higher at lower elevation sites dominated by fine substrates. We hypothesized that the concentration of microplastics should be associated with higher human population density in watersheds, and we predicted that microplastic concentrations in water, sediment, and mussel tissues should increase downstream (with decreasing elevation and drainage area) in the Yadkin–Pee Dee River Basin. Those higher concentrations likely result from both increased wastewater discharge from areas of higher population density and from the flow contributed by tributaries funneling plastics into the main channel. We also hypothesized that larger mussels would be associated with higher microplastic concentrations due to increased filtration and water intake. Moreover, position in the watershed and adjoining land use also would likely influence microplastic concentrations. By sampling along an elevational/drainage area gradient, we examined the role of longitudinal position and land use on microplastic concentrations in the environment and in filter-feeding bivalves.

METHODS

To test our hypotheses, we sampled five sites across the Yadkin–Pee Dee River Basin. The Yadkin–Pee Dee River originates in the Blue Ridge Mountains near Blowing Rock,

Table 1. List of study site locations, their elevation, drainage area, and sample sizes. For all sites, sediment sample sizes are two with four subsamples and water sample sizes are three. Note that *Elliptio* collected at Site 4 (*Elliptio waccamawensis* shell morph) are presumed to be *Elliptio complanata*.

Site	Elevation	Drainage	Mussels ($n = 10$)
1. NC: Wilkes Co. Yadkin River at Tom Dooley Road Boat Ramp (36.085, -81.369)	327 m	469 km ²	<i>E. complanata</i>
2. NC: Richmond Co. Pee Dee River shoals downstream of US Highway 74 crossing (34.932, -79.862)	37 m	17,819 km ²	<i>E. complanata</i>
3. NC: Robeson Co. Lumber River at NC Highway 72 boat ramp (34.592, -78.984)	33 m	1,867 km ²	<i>E. angustata</i>
4. NC: Columbus Co. Lake Waccamaw along southwestern shore (34.261, -78.524)	13 m	238 km ²	<i>E. complanata</i>
5. NC: Columbus Co. Waccamaw River at NC Highway 904 boat ramp (34.015, -78.632)	6 m	2,528 km ²	<i>E. angustata</i>

North Carolina, and flows east and then southwest to its confluence with the Atlantic Ocean near the North Carolina–South Carolina border. Major tributaries include the Little Pee Dee, Lumber, Uwharrie, and Waccamaw rivers (Table 1 and Fig. 1). Study sites ranged from a relatively high-gradient, upland fifth-order stream (the Yadkin River) to a large, sandy, and very shallow coastal plain lake (Lake Waccamaw). All sites were in rural areas without large human populations nearby. However, the Yadkin–Pee Dee River system includes several larger municipalities including Wilkesboro and Winston-Salem.

At each site, we opportunistically collected 10 adult *Elliptio* spp. with shell lengths > 30 mm using mask and snorkel or via tactile searches. We collected specimens of *Elliptio complanata*, *Elliptio angustata*, and *Elliptio waccamawensis*. We also collected three 3.8-L water samples and two 0.95-L

sediment samples from each site. Prior to processing, we took two 15-mL subsamples from each sediment sample. Thus, total sample sizes for mussels, water, and sediment samples were 50, 15, and 20, respectively (Table 1).

Following collection, we tightly wrapped all mussels in aluminum foil to prevent the release of water and pseudofeces and to prevent microplastic contamination from outside sources. We placed the wrapped mussels into Ziploc bags, labeled them, and stored them in a cooler for transport to the lab where they were frozen at -20°C until processing. We collected sediment samples in glass jars that were opened only during sediment collection. Because sediment deposition zones are spatially variable in rivers, we collected samples from within a 100-m radius of where mussels were found at each site. We collected sediments of all size classes and froze them until processing. We then sifted the sediment samples

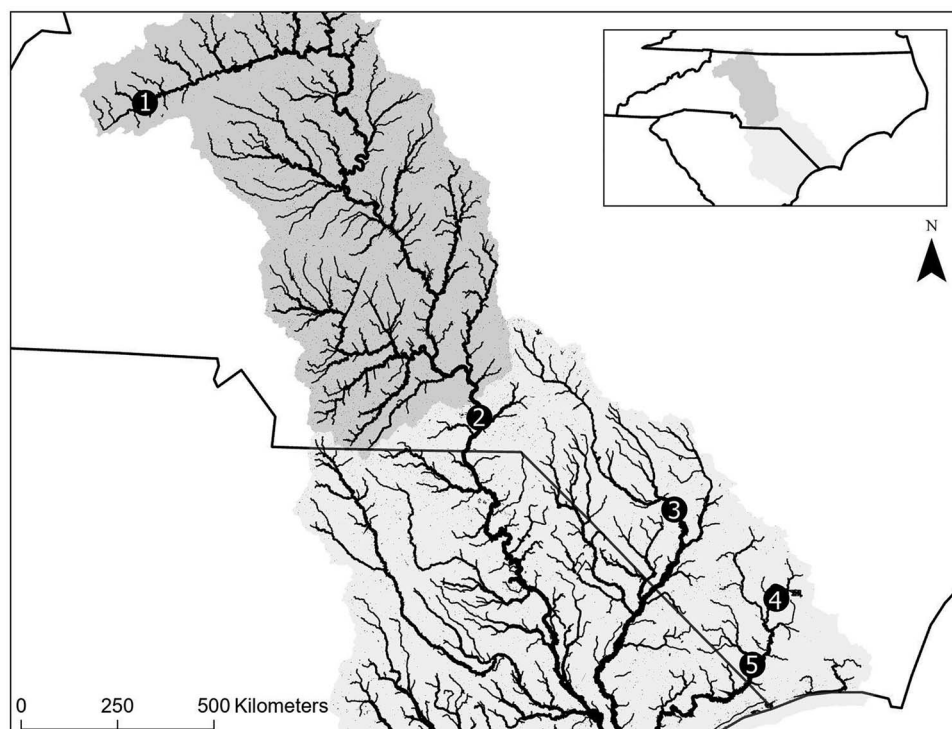


Figure 1. Map depicting the location of study sites in the Pee Dee River Drainage of North Carolina. Inset map shows area of detail in North and South Carolina. Numbers correspond to sites described in Table 1.

down to particles of < 5 mm to coincide with the defined size range of microplastics. Subsamples of ~ 10 mL were taken from the sifted sediment due to the volume capacity of the filtration apparatus. We collected water samples from the water column (10–20 cm above the substrate) near the site of sediment sample collections. We did not open the jars until immediately before collection began. We placed water samples in coolers, out of sunlight, and then in a freezer at -20°C until processing to prevent degradation of plastics.

We quantified microplastic abundance using protocols modified from Thiele et al. (2019). We placed mussels in a 10% KOH solution at a ratio of 2–3 mL KOH to 1 g of tissue. For example, a mussel weighing 13.5 g would have been digested in 30 mL of KOH. We combined the mussels and KOH in glass jars and covered them with aluminum foil to prevent airborne contamination. Digestion proceeded for ~ 24 h on a shaker table that kept the solution in motion to assist in digestion. After 24 h, we filtered the digested tissue, first through a 50- μm sieve which was then rinsed using DI water onto 90-mm diameter borosilicate glass fiber filters with a particle retention of 1.6 μm in a vacuum filtration system. We then placed the filters in covered glass Petri dishes and dried them under refrigeration. We placed sediment subsamples in 50 mL of KOH solution and digested them using the same method as mussels. We did not digest water samples but filtered them using the same sieve and vacuum filtration process as mussels and sediment. After drying, we placed the filters under a dissecting scope and counted particles of suspected plastics for 30 min. We identified plastic particles by prodding the particle with a hot needle to determine if it melted, bent, or otherwise reacted violently in a diagnostic manner indicative of plastics (Dehaut et al. 2018).

All handling of samples and filters, as well as tissue digestions, was conducted under controlled laboratory conditions to limit contamination as much as reasonably possible. We thoroughly rinsed all equipment with DI water between each sample. Gloves and cotton clothing were always worn, and all samples were exposed to air only when under an active lab hood or during analysis. When counting samples, we omitted any particle that may have been recently contaminated from the counting process. We did not include in the final counts any fibers that were not adhered to the filter, were outside of the ring where the flask sat on the filter, or matched the color of observers' clothing. Because the distilled water used to create the KOH solution likely contains microplastics, blanks without tissue (negative controls) were run to account for background contamination levels ($n = 10$).

We conducted preanalysis data processing and all statistical analyses using Jamovi software (Jamovi Project 2026). We tested normality with Shapiro–Wilk tests and examined the homogeneity of variance assumption with Levine tests. Microplastic abundance and concentration data from sediment and water samples satisfied the assumptions of standard one-way (Fisher's) ANOVA, as did the microplastic abundance in mussel data. However, mussel weight was not normally distributed and

did not exhibit equal variances among groups. A Kruskal–Wallace one-way ANOVA and Dwass–Steel–Critchlow–Fligner pairwise comparisons were used to assess differences in mussel weight among sites. Additionally, mussel microplastic concentration (particles per unit weight) violated the equal variance assumption, so we used a Welch's one-way ANOVA and Games–Howell post-hoc tests to examine the differences among sites.

We examined correlations between site elevation, watershed area, and the abundance of microplastics ($n = 10$) in mussels using Pearson correlations; for correlations between mussel size and microplastic abundance in all mussels as well as within individual species (*E. complanata*, $n = 30$; *E. angustata*, $n = 20$), we used Spearman correlations (Jamovi Project 2026). *Elliptio complanata* is a larger-bodied animal, and its shells are typically more rhomboid in profile, whereas *E. angustata* and other lances have more lanceolate shells that are generally more elongate and thinner in comparison (Johnson 1970; Williams et al. 2014). Finally, we used lines from the best fitting equations (highest R^2 among linear, logarithmic, parabolic, and negative exponential regressions) to model and visualize the relationships between mussel body size, microplastic abundance, and concentrations.

RESULTS

We detected microplastics in all water, sediment, and mussel samples analyzed (Table 2). Negative controls had an average of 1.1 particles per sample or a concentration of 0.022 particles per mL of DI water, confirming that contamination originating within the KOH solution or from the lab during sample analysis was minimal and unlikely to significantly affect results.

Microplastic abundance and concentrations in water samples differed significantly among sites ($F_{4,10} = 18.25$, $P \leq 0.001$; Table 3), with microplastics in Pee Dee River water samples ~ 2 – $5\times$ more abundant than in samples from the other four sites. Water samples from the Yadkin River, the furthest upstream site examined, had the lowest level of microplastics detected but the concentrations were highly variable and did not differ significantly from the other sites (Fig. 2). There was a significant positive correlation between drainage area and concentration of microplastics in the water column, but elevation was not significantly correlated with concentration or drainage area (Table 4).

Microplastic abundance and concentration in sediment samples also differed significantly among sites ($F_{4,5} = 31.64$, $P \leq 0.001$; Table 3). Microplastic abundance and concentration were lowest in the Yadkin River and highest in the Lumber River, differing significantly from the other three sites. However, we could not compute confidence intervals for the Lumber River site due to the small sample size and low variability. Given how close the Lumber River mean is to the other three low-elevation sites, it is likely not significantly different from them (Fig. 2). Sediment microplastic concentrations were negatively correlated with elevation but not with drainage area (Table 4).

Table 2. Mean $\pm$ standard error of the abundance (particles per sample, n) and concentration of microplastic particles recovered from water, sediment, and mussel samples collected from five sites along the Yadkin–Pee Dee River Basin in 2020. Mean wet mass for mussel samples is also included for each site. At each site, we examined three water, four sediment, and 10 mussel samples.

	Yadkin River	Pee Dee River	Lumber River	Lake Waccamaw	Waccamaw River
Water					
Abundance (n)	3.3 ± 1.45	17.7 ± 1.45	6.0 ± 1.53	8.0 ± 0.58	6.33 ± 1.2
Concentration (n/L)	0.88 ± 0.38	4.7 ± 0.38	1.6 ± 0.4	2.1 ± 0.15	1.67 ± 0.32
Sediment					
Abundance (n)	2.25 ± 0.48	5.8 ± 0.48	6.5 ± 0.29	5.5 ± 0.5	5.75 ± 0.63
Concentration (n/cm^3)	0.15 ± 0.03	0.38 ± 0.03	0.43 ± 0.02	0.37 ± 0.03	0.38 ± 0.04
Mussels					
Abundance (n)	3.6 ± 0.37	5.9 ± 0.53	6.9 ± 0.41	6.8 ± 0.65	7.6 ± 0.40
Concentration (n/g wet mass)	0.28 ± 0.06	0.34 ± 0.06	1.37 ± 0.38	1.5 ± 0.23	1.0 ± 0.12
Mussel wet mass (g)	18.8 ± 3.5	21.5 ± 3.1	6.5 ± 0.63	5.2 ± 0.59	8.2 ± 0.59

Microplastics in mussel tissues differed significantly among sites in abundance ($F_{4,45} = 10.42$, $P \leq 0.001$) and concentration ($F_{4,45} = 7.44$, $P \leq 0.001$; Table 3). Microplastic abundance and concentration were lowest in *E. complanata* from the Yadkin and Pee Dee River sites and highest in mussels from the Waccamaw River. Microplastic abundance in *Elliptio* species in the Yadkin River was significantly lower when compared to all other sites, whereas concentrations were significantly lower in *Elliptio* species from the Yadkin and Pee Dee rivers (Fig. 2). Microplastic abundance and concentration were negatively correlated with mussel weight (Table 5 and Fig. 3). Microplastic concentration was positively correlated with drainage area and elevation (Table 5).

Elliptio complanata microplastic abundance was significantly negatively correlated with weight and elevation, and microplastic concentration was significantly negatively correlated with weight, drainage area, and elevation (Tables 4–6). In contrast, *E. angustata* microplastic concentration was

negatively correlated only with tissue weight (Table 6 and Fig. 3).

DISCUSSION

Site-level differences in water, sediment, and mussel microplastic concentrations suggest that multiple interacting factors that vary across a watershed likely contribute to microplastic concentration. Land use and the abundance of point-source inputs, such as reservoirs and wastewater outfalls, likely have a strong influence on the distribution and abundance of microplastics. Additionally, flow conditions (e.g., depth and velocity) as well as the trophic position and body size of biota are also likely important factors. As such, future studies designed to model the abundance of microplastics in a watershed should examine multiple taxa and trophic levels to fully understand its associated patterns of transport and contamination. The development and adoption of standardized protocols are also needed to better understand the

Table 3. Results of analyses comparing the concentration of microplastics in water, sediment, and mussel samples collected from five sites in the Yadkin–Pee Dee River Basin in 2020. A Fischer's one-way ANOVA was used to compare differences in water and sediment samples as well as the number of particles detected in each mussel. However, because the concentration of plastic particles per mussel was not normally distributed, we used a Kruskal Wallance non-parametric ANOVA to examine differences among sites, and so a χ^2 is reported in place of an F here. Mussel sample results are reported using both particles per organism (abundance) and particles per gram (concentration). Sediment and water samples are reported using concentration ($no./cm^3$ and $no./L$ respectively).

Parameter	Factors	df	Sum sq.	Mean sq.	F	P Value
Particles/ cm^3 sediment	Site	4	0.098	0.024	31.6	$P < 0.001$
	Residuals	5	0.0038	0.00079		
Particles/L water	Site	4	25.5	6.37	18.3	$P < 0.001$
	Residuals	10	3.49	0.35		
Particle abundance	Site	4	96.5	24.1	10.4	$P < 0.001$
	Residuals	45	104.2	2.32		
Particle concentration	Site	4	12.9	3.23	$\chi^2 = 33.3$	$P < 0.001$
	Residuals	45	19.5	0.43		

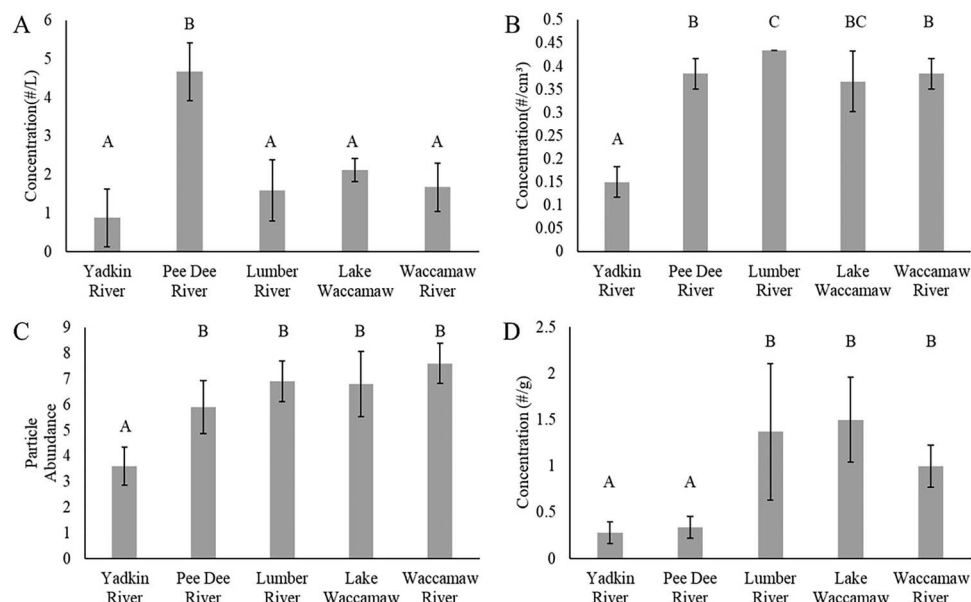


Figure 2. Results of post-hoc analysis of water particle concentration (A), sediment particle concentration (B), mussel particle abundance (C), and mussel particle concentration (D) from samples collected from five sites along the Yadkin–Pee Dee River Basin in 2020. Groupings are labeled above each bar. At each site, we collected three water, four sediment, and 10 mussel samples.

extent and effects of microplastic pollution in freshwater biota (Bom and Sá 2021). Our study relied upon a simple, cost-effective method for quantifying microplastic abundance in freshwater systems and organisms that could be used by a wide range of stakeholders and provide insights into the flow of these contaminants through watersheds.

Microplastics are widespread and abundant in water, sediment, and mussel-tissue samples across the Yadkin–Pee Dee River Basin and appear to increase in a downstream direction. Although microplastic concentrations vary considerably across sites, these particles appear to be ubiquitous in both the environment and its bivalve fauna. Although many factors may contribute to the heterogeneity of microplastics within a system including land use, discharge, time of year, and the

presence of impoundments, microplastic abundance in this system is higher at downstream sites with lower elevations and larger drainage areas. Because patterns of microplastic concentrations within mussels were not similar to the patterns of microplastic concentrations within water or sediment samples, our results suggest that particle abundance is driven by mostly geographical or hydrogeological factors rather than biological ones.

Our prediction that microplastic concentrations in all sample types would increase downstream were based on the River Continuum Concept (RCC; sensu Vannote et al. 1980), which projects that downstream communities should be increasingly dependent on organic materials transported from upstream ecosystems. However, microplastic concentrations in water samples were highest in the Pee Dee River and relatively low

Table 4. Correlation matrix of water ($n = 3$ per site, data above diagonal) and sediment ($n = 4$ per site, data below diagonal) samples collected from five sites along the Yadkin–Pee Dee River Basin in 2020 showing Pearson coefficients for correlations between microplastic concentration (particles per liter of water or cubic centimeter of sediment), drainage area (km^2), and elevation (m). Significant correlations ($P < 0.05$) are marked with an asterisk. Note that abundance (particles per sample) was not reported in this table as samples were all of standardized volume; therefore, correlations between microplastic abundance and concentration with other variables are identical.

	Concentration	Drainage Area	Elevation
Concentration	—	$r = 0.89^*$ $P < 0.001$	$r = -0.42$ $P = 0.12$
Drainage area	$r = 0.27$ $P = 0.46$	—	$r = -0.25$ $P = 0.37$
Elevation	$r = -0.94^*$ $P < 0.001$	$r = -0.248$ $P = 0.49$	—

Table 5. Correlation matrix of mussel samples ($n = 50$) collected from five sites along the Yadkin–Pee Dee River Basin in 2020 showing Spearman coefficients (ρ) and P values for correlations between abundance (particles of microplastic per organism), concentration (particles per gram of tissue), weight in grams, drainage area (km^2), and elevation (m). Pearson coefficients are reported for correlations between drainage area and elevation. Significant correlations ($P < 0.05$) are marked with an asterisk.

	Abundance	Concentration	Weight	Drainage Area
Weight	$\rho = 0.51^*$ $P < 0.001$	$\rho = 0.92^*$ $P < 0.001$	—	
Drainage Area	$\rho = 0.13$ $P = 0.95$	$\rho = 0.30^*$ $P = 0.035$	$\rho = 0.48^*$ $P < 0.001$	—
Elevation	$\rho = -0.65^*$ $P < 0.001$	$\rho = 0.69^*$ $P < 0.001$	$\rho = 0.60^*$ $P < 0.001$	$r = -0.25$ $P = 0.082$

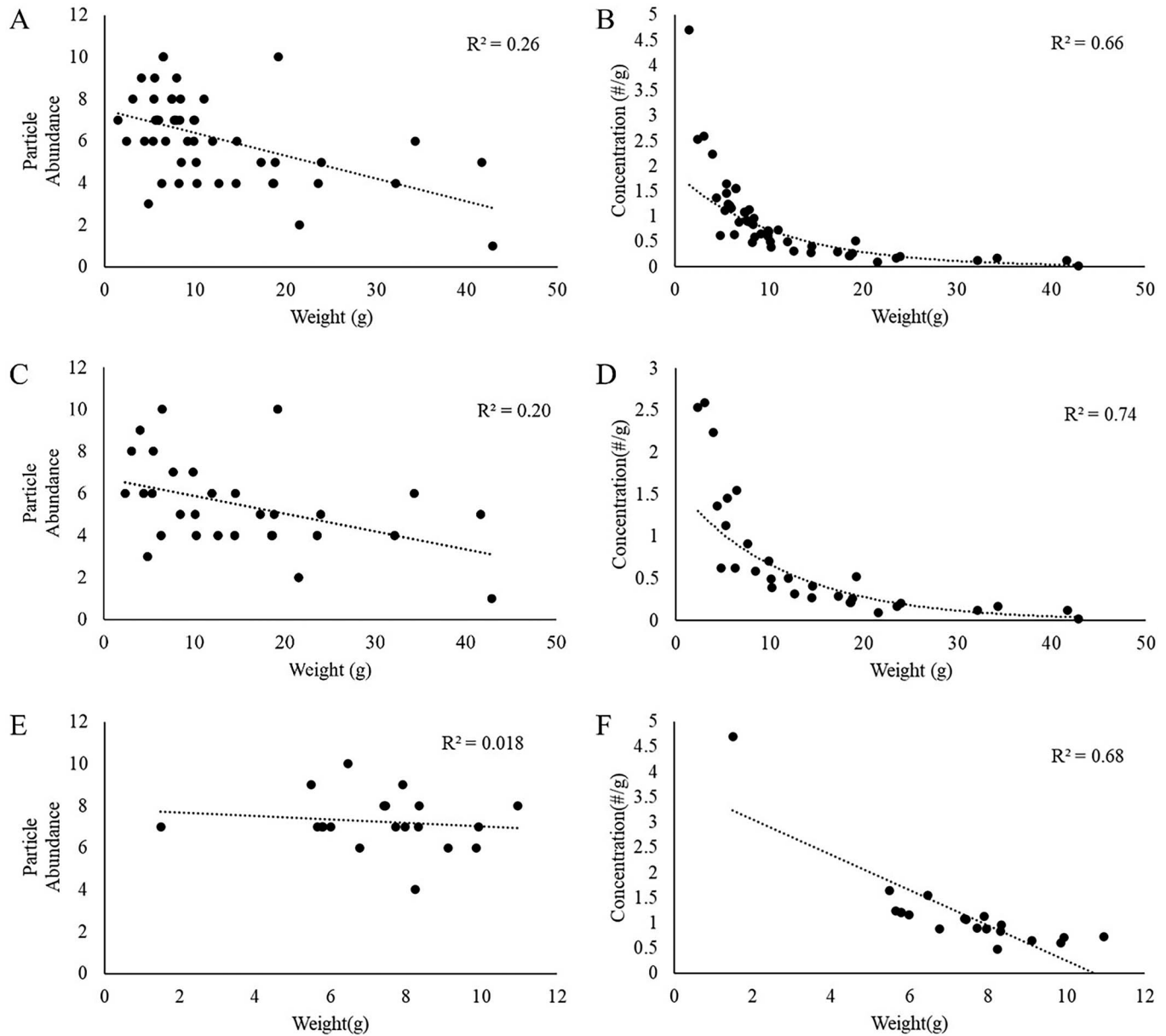


Figure 3. Scatterplots showing the relationships between freshwater mussel wet mass and particle abundance (A) and concentration (B) in all mussels, *Elliptio complanata* (C and D), and *Elliptio angustata* (E and F) collected from five sites in the Yadkin–Pee Dee River Basin in 2020. R^2 values represent the strength of the visualized relationships for best fit lines.

at all other sites. This does not support our initial hypothesis. Several factors may explain this discrepancy. First, microplastics do not degrade as readily as organic matter and may be deposited more quickly or otherwise not move through aquatic systems in the same way as naturally occurring fine-particulate organic matter.

Additionally, we collected our data in a short time frame, and the data represent a snapshot of microplastic concentrations at that time. Microplastic concentrations are likely to vary over time as discharge fluctuates (Stanton et al. 2020; Della Torre et al. 2023). It is unclear how variation in microplastic abundance is related to other environmental factors, including substrate composition, seasonal variability in flow

rates, or anthropogenic use of waterways (Stanton et al. 2020). Our samples were collected over the course of several days, and we are thus unable to discern how season or water temperature may influence concentrations. More robust microplastic transport and accumulation models should use data from sites that were sampled seasonally or following flood events to better understand temporal variability in microplastic concentrations.

Land use may alter input of allochthonous materials, including microplastics, and may change expectations based on the RCC. Additionally, the RCC does not account for increasingly urbanized land use along all stream orders (Roebuck et al. 2020). The strong relationship between drainage area and microplastic concentration in water samples suggests that urban runoff

Table 6. Correlation matrix of mussel samples ($n = 50$) collected from five sites along the Yadkin–Pee Dee River Basin in 2020, separated by species, showing Spearman coefficients (ρ) for relationships between abundance (particles of microplastic per organism), concentration (particles per gram of tissue), tissue weight (g), drainage area (km^2), and elevation (m). *Elliptio angustata* ($n = 18$) correlations are listed above the diagonal, and *Elliptio complanata* ($n = 28$) below. Pearson coefficients (r) are reported for correlations between drainage area and elevation. Significant correlations ($P < 0.05$) are marked with an asterisk.

	Abundance	Concentration	Weight	Drainage Area	Elevation
Abundance	—	$\rho = 0.55$ $P = 0.012$	$\rho = -0.19$ $P = 0.431$	$\rho = 0.24$ $P = 0.313$	$\rho = -0.24$ $P = 0.313$
Concentration	$\rho = 0.70^*$ $P < 0.001$	—	$\rho = -0.89^*$ $P < 0.001$	$\rho = -0.17$ $P = 0.465$	$\rho = 0.17$ $P = 0.465$
Weight	$\rho = -0.45^*$ $P = 0.014$	$\rho = -0.94^*$ $P < 0.001$	—	$\rho = 0.38$ $P = 0.007$	$\rho = -0.38$ $P = 0.097$
Drainage area	$\rho = 0.16$ $P = 0.403$	$\rho = -0.64^*$ $P < 0.001$	$\rho = 0.75^*$ $P < 0.001$	—	$r = -1.000^*$ $P < 0.001$
Elevation	$\rho = -0.64^*$ $P < 0.001$	$\rho = -0.72^*$ $P < 0.001$	$\rho = 0.62$ $P < 0.001$	$r = -0.43^*$ $P = 0.018$	—

increases microplastic abundance. Microplastics in water from the Pee Dee River were likely higher than those at other sites because the Pee Dee was the largest stream sampled, and it had the largest drainage area. The lack of a significant elevation effect indicates that upstream land use plays a more important role than geographical position. A study in a large, South American river catchment found that highland and midland sites did not differ in microplastic concentrations, but lowland sites that flowed through more populous areas had significantly higher microplastic concentrations (Correa-Araneda et al. 2022).

We expected sediment concentrations to increase with drainage area and elevation because substrate deposition rates increase as streams become larger and slower moving (Nyman et al. 2020). Based on low sediment microplastic concentrations at our highest elevation site and a positive correlation between sediment microplastic concentration and elevation, elevation may be serving as a proxy for local stream characteristics. High-elevation streams tend to have higher flows, coarser substrates, and lower rates of sediment deposition relative to lower-elevation streams in the Yadkin–Pee Dee River Basin. In these systems, larger microplastic particles would likely remain suspended until transported downstream to larger, slower reaches. There is also a possibility that local variation in mesohabitat (i.e., riffles, runs, and pools) abundance related to differences in streambed topography could be influencing this pattern and artificially depressing concentrations of microplastics in sampled reaches.

Mussels' mediation between water and sediment through their filter feeding could mediate microplastic abundance in water and sediment samples. This did not appear to be the case: the Yadkin and Pee Dee sites had similar microplastics per gram of mussel tissue despite having statistically different water and sediment concentrations. However, although the Pee Dee River had the highest concentrations of microplastics in water samples and relatively high numbers of particles per mussel, the larger sizes of mussels collected from this river meant that particle densities were relatively low. A similar

pattern was observed in Yadkin River mussels (although particle densities in water samples from this site were low). Larger-bodied mussels at the Yadkin and Pee Dee sites may have contributed to the lower microplastic concentrations (particles per gram of tissue) reported from these sites, despite significant differences in microplastic abundance (particles per organism) observed between these sites.

Across two, different-sized species, the smaller-sized species had higher concentrations of microplastic particles; however, it is difficult to discern if microplastic concentration variation was the result of size, feeding habit, or some other species-specific characteristic (Almeshal et al. 2022). Several authors have demonstrated that a still poorly understood “growth-dilution” effect may occur with larger-bodied and presumably older bivalves and fishes exhibiting lower somatic concentrations of plastics as they grow larger (Wu et al. 2022; Collins et al. 2025). Mussels might selectively exclude particles and deposit them as pseudofeces; however, as yet, this has not been documented. In large streams with higher particulate loads, mussels may become more selective in their feeding habits, which could reduce their consumption of plastic particles. Selective feeding in the presence of abundant particulate loads could explain why we saw no differences in microplastic concentration among lower elevation sites despite differences in water concentration. This could also mean that, although high-elevation streams appear to have less microplastics, mussels and other filter feeders in these reaches may be more vulnerable to such pollution if they are less selective in their consumption when particulate matter is scarce (Frau et al. 2016; Mistry and Ackerman 2018).

Uptake of plastic particles from sediment and associated organic matter early in life could explain declining plastic concentrations as mussels age and grow. Freshwater mussels undergo ontogenetic shifts in diet and feeding strategies (Forbes-Green and Cyr 2023). For several years early in their life-cycle, young mussels tend to use pedal feeding, using their foot to pull in organic material from the sediment in which they are buried. The size and age at which *E. complanata*

emerge from the sediment varies, but mussels generally exhibit a gradual shift in feeding strategy as they age from pedal to filter feeding (Forbes-Green and Cyr 2023). Many other mussels undergo similar dietary shifts, although some taxa do continue to use both methods of feeding throughout their life (Yeager et al. 1994). Although our mussels were adults and likely relied primarily on filter feeding (all were > 30 mm total length), shifts in feeding strategy could explain the negative relationship observed between weight and microplastic concentration. Possibly, younger mussels are more likely to uptake plastic particles due to their reliance on sediment feeding. Larger, adult mussels may be able to more selectively exclude microplastics via filter feeding. Thus, we should expect a gradient of microplastic concentrations and a general decrease as mussels grow larger. If this is the case, it is particularly concerning because juvenile mussels (and the juveniles of many other aquatic taxa) tend to be the most susceptible to environmental toxins (Raimondo et al. 2016).

Mussels also exhibit seasonal variation in vertical and horizontal movement and burial patterns (Cyr 2009). It is unlikely that this would have affected our results given that all samples were collected during the same season. However, seasonality can differ across geographic location, and a shift in mussel depth within the sediment could also correspond with a shift in feeding strategy as well. Long-term studies of microplastics in unionids could detect seasonal behaviors that influence microplastic exposure.

One possible source of heterogeneity in mussel microplastic concentrations involves the use of multiple taxa in this study. Although only one species was examined at each site, two species were included in this analysis. Species were site-dependent, with only one species collected at each site. Filtering rates vary among species and individual mussels and could affect the abundance or concentration of microplastics within mussels (Spooner and Vaughn 2008; Hoellein et al. 2017). Accumulation rates of microplastic fibers can differ significantly among even species of the same genus (Almeshal et al. 2022). In our study, the abundance of microplastics was relatively consistent across mussels in multiple taxa, whereas concentrations were lowest in the larger-bodied species, *E. complanata*, from the Yadkin and Pee Dee rivers.

An ecosystem-scale approach to microplastic monitoring and research can reveal landscape and biota-derived differences in microplastic abundance. In addition to the lack of long-term studies, many microplastic studies look at the concentrations in water or sediment and assume that these concentrations are indicative of their potential effects on biota. Our data show that the relationship between an environment and contamination levels are likely to be the result of multiple complex factors rather than a correlation between microplastic abundance and bio-accumulation. A single aspect of a system does not fully describe the potential movement, input, or uptake of microplastics by organisms (Gessner and Tlili 2016). Microplastic exposure and assimilation rates vary among species, highlighting the need to sample a broader

range of taxa and trophic levels to better understand the scope of microplastic impacts within watersheds (Almeshal et al. 2022). Future research should be focused on long-term analysis of systems with regard to target organisms, sediment, and water.

Long-term studies are, of course, often difficult to implement due to funding and other limiting factors. In addition, microplastic research suffers from a lack of unified or widely accepted protocols to investigate plastic concentrations in various samples. Our protocol was modified from methods described by Thiele et al. (2019), and we believe it will provide other researchers with a rapid and cost-effective procedure robust enough to investigate many different questions regarding microplastics. Differing methods for detecting microplastics in aquatic systems lead to difficulties in comparing data from different sources or studies. Many approaches, including those used in experimental studies, often greatly overestimate the amount of microplastics in a system. For example, some studies report microplastic concentrations in the gut contents of a 10 g fish as particles per kilogram, a gross over-extrapolation (Masoudi et al. 2022). Moreover, some experimental studies documenting effects of microplastics expose test organisms to concentrations that are several magnitudes higher than what is environmentally relevant (Colomer et al. 2019; Wu et al. 2019). Other methods, such as μ -FTIR analysis, are prohibitively expensive or require expensive or difficult-to-work-with chemicals that place these methods out of reach for smaller research labs and citizen science-based monitoring efforts. Protocols using easily accessible materials and equipment, such as the one we developed, can facilitate the long-term, large-scale studies needed to properly assess the impacts and dynamics of microplastic pollution.

LITERATURE CITED

- Akdogan, Z., and B. Guven. 2019. Microplastics in the environment: A critical review of current understanding and identification of future research needs. *Environmental Pollution* 254:113011.
- Almeshal, W., A. Takacs, L. Aradi, S. Sandil, P. Dobosy, and G. Zaray. 2022. Comparison of freshwater mussels *Unio tumidus* and *Unio crassus* as biomonitors of microplastic contamination of Tisza River (Hungary). *Environments* 9:122.
- Blettler, M., M. Ulla, A. Rabuffetti, and N. Garello. 2017. Plastic pollution in freshwater ecosystems: macro-, meso-, and microplastic debris in a flood-plain lake. *Environmental Monitoring and Assessment* 189:1–13.
- Bom, F. C., and F. Sa. 2021. Concentrations of microplastics in bivalves of the environment: A systematic review. *Environmental Monitoring and Assessment* 193:846.
- Chen, Z., S. Fan, W. Guo, M. Hu, J. S. Lee, and Y. Wang. 2026. Unveiling the hidden impacts: A comprehensive review of microplastic effects on marine bivalves. *Aquatic Toxicology* 291:107567.
- Collins, S. F., L. Soliz, G. Tucker, and A. Norton. 2025. Evidence for the 'growth-dilution' of microplastics and microfibers in urban stream fish populations. *Science of the Total Environment* 998:180249–180258.
- Colomer, J., M. F. Müller, A. Barcelona, and T. Serra. 2019. Mediated food and hydrodynamics on the ingestion of microplastics by *Daphnia magna*. *Environmental Pollution* 251:434–441.
- Correa-Araneda, F., J. Pérez, A. M. Tonin, C. Esse, L. Boyero, M. E. Díaz, R. Figueroa, R. Santander-Massa, A. Cornejo, O. Link, E. Jorquera, and M. A. Urbina. 2022. Microplastic concentration, distribution, and dynamics along one of the largest Mediterranean-climate rivers: A whole watershed approach. *Environmental Research* 209:112808.

- Cyr, H. 2009. Substrate and fetch affect the emergence of freshwater mussels from lake sediments. *Journal of the North American Benthological Society* 28:319–330.
- Dehaut, A., L. Hermabessiere, and G. Duflos. 2018. Current frontiers and recommendations for the study of microplastics in seafood. *Trends in Analytical Chemistry* 116:346–359.
- Della Torre, C. N. Riccardi, S. Magni, V. Modesto, M. Fossati, and A. Binelli. 2023. First comparative assessment of contamination by plastics and non-synthetic particles in three bivalve species from an Italian subalpine lake. *Environmental Pollution* 330:121752.
- Ding, J., S. Zhang, R. M. Razanajatovo, H. Zou, and W. Zhu. 2018. Accumulation, tissue distribution, and biochemical effects of polystyrene microplastics in the freshwater fish red tilapia (*Oreochromis niloticus*). *Environmental Pollution* 238:1–9.
- Doucet, C. V., A. J. Labaj, and J. Kurek. 2021. Microfiber content in freshwater mussels from rural tributaries of the Saint John River, Canada. *Water, Air and Soil Pollution* 232:32.
- Eerkes-Medrano, D., R. C. Thompson, and D. C. Aldridge. 2015. Microplastics in freshwater systems: a review of the emerging threats, identification of knowledge gaps and prioritization of research needs. *Water Research* 75:63–82.
- Elnahas, A., A. Gray, J. Lee, N. AlAmiri, N. Pokrel, S. Allen, and H. Foroutan. 2024. Atmospheric deposition of microplastics in South Central Appalachia in the United States. *ACE EST Air* 2:64–72.
- Forbes-Green, M. J., and H. Cyr. 2023. The effects of physical disturbance and sediment refuge on the growth of young native freshwater mussels *Elliptio complanata* (Eastern Elliptio). *Hydrobiologia* 850:1123–1140.
- Frau, D., F. Molina, and G. Mayora. 2016. Feeding selectivity of the invasive mussel *Limnoperna fortunei* (Dunker, 1857) on a natural phytoplankton assemblage: What really matters? *Limnology* 17:47–57.
- Gedik, K., and S. Atasarl. 2022. The microplastic pattern in Turkish lakes: Sediment and bivalve samples from Cildir Lake, Almus Dam Lake and Kartalkaya Dam Lake. *Turkish Journal of Zoology* 46:396–408.
- Gessner, M. O., and A. Tilili. 2016. Fostering integration of freshwater ecology with ecotoxicology. *Freshwater Biology* 61:1991–2001.
- Haag, W. R. 2012. *North American Freshwater Mussels: Natural History, Ecology, and Conservation*. Cambridge University Press, Cambridge, England 505 p.
- Hoellein, T. J., C. B. Zarnoch, D. A. Bruesewitz, and J. DeMartini. 2017. Contributions of freshwater mussels (Unionidae) to nutrient cycling in an urban river: Filtration, recycling, storage, and removal. *Biogeochemistry* 135:307–324.
- Jamovi Project. 2026. *jamovi* (Version 2.7) [Computer Software]. <https://www.jamovi.org>. Accessed 15 August 2026.
- Johnson, R. I. 1970. The systematics and zoogeography of the Unionidae (Mollusca: Bivalvia) of the Southern Atlantic Slope Region. *Bulletin of the Museum of Comparative Zoology* 140(6):263–420.
- Karbalaei, S., P. Hanachi, T. R. Walker, and M. Cole. 2018. Occurrence, sources, human health impacts and mitigation of microplastic pollution. *Environmental Science and Pollution Research* 25:36046.
- Kudla, Y. M., M. M. Ijzerman, C. J. Bennett, P. L. Gillis, K. A. Kidd, and R. S. Prosser. 2024. Quantifying effects and ingestion of several pristine microplastics in two early life stages of freshwater mussels. *Environmental Toxicology and Chemistry* 43:2557–2568.
- Leads, R. R., and J. E. Weinstein. 2019. Occurrence of tire wear particles and other microplastics within the tributaries of the Charleston Harbor Estuary, South Carolina, USA. *Marine Pollution Bulletin* 145:569–582.
- Mandel, N. D., B. Gamboa-Loira, M. E. Cebrián, A. Mérida-Ortega, and L. López-Carrillo. 2019. Challenges to regulate products containing bisphenol A: Implications for policy. *Salud Pública de México* 61:692–697.
- Masoudi, E., A. Hedayati, T. Bagheri, A. Salati, R. Safari, M. Gholizadeh, and M. Zakeri. 2022. Different land uses influenced on characteristics and distribution of microplastics in Qarasu Basin rivers, Gorgan Bay, Caspian Sea. *Environmental Science and Pollution Research* 29:64031–64039.
- Miller, J., N. Barrett, J. Love, A. Gray, R. Youker, C. Hall, N. Meiri, M. Gaesser, G. Randall, R. Jarrett, and J. Spafford. 2024. Temporal and spatial variations in microplastic concentrations in small headwater basins in the Southern Blue Ridge Mountains, North Carolina, USA. *Environments* 11:240–264.
- Mistry, R., and J. D. Ackerman. 2018. Flow, flux, and feeding in freshwater mussels. *Water Resources Research* 54:7619–7630.
- Moyo, S. 2022. An enigma: A meta-analysis reveals the effect of ubiquitous microplastics on different taxa in aquatic systems. *Frontiers in Environmental Science* 10:999349.
- Neves, R.J., A. E. Bogan, J. D. Williams, S. A. Ahlstedt, and P. D. Hartfield. 1997. Status of aquatic mollusks in the southeastern United States: A downward spiral of diversity. Pages 43–64 in G. W. Benz and D. E. Collins, editors, *Aquatic fauna in peril: The Southeastern perspective*. Southeast Aquatic Research Institute Special Publication 1, Lenz Design & Communications, Decatur, GA.
- Nyman, P., W. A. C. Box, J. C. Stout, G. J. Sheridan, S. D. Keesstra, P. N. J. Lane, and C. Langhans. 2020. Debris-flow-dominated sediment transport through a channel network after wildfire. *Earth Surface Processes and Landforms* 45:1155–1167.
- Raimondo, S., C.R. Lilavois, L. Lee, T. Augspurger, N. Wang, C.G. Ingersoll, C. Bauer, E. Hammer, and M.G. Barron. 2016. Assessing variability in chemical acute toxicity of unionid mussels: Influence of intra- and interlaboratory testing, life stage, and species. *Environmental Toxicology and Chemistry* 35:750–758.
- Ringwood, A. H. 2021. Bivalves as biological sieves: Bioreactivity pathways of microplastics and nanoplastics. *Biological Bulletin* 241:185–195.
- Roebuck, J. A., Jr., M. Seidel, T. Dittmar, and R. Jaffe. 2020. Controls of land use and the river continuum concept on dissolved organic matter composition in an anthropogenically disturbed subtropical watershed. *Environmental Science and Technology* 54:195–206.
- Scott, J. W., K. G. Gunderson, L. A. Green, R. R. Rediske, and A. D. Steinman. 2021. Perfluoroalkylated substances (PFAS) associated with microplastics in a lake environment. *Toxics* 9:106.
- Silva, C. J., A. L. P. Silva, C. Gravato, and J.L. Pestana. 2019. Ingestion of small-sized and irregularly shaped polyethylene microplastics affect *Chironomus riparius* life-history traits. *Science of the Total Environment* 672:862–868.
- Spooner, D. E., and C. C. Vaughn. 2008. A trait-based approach to species' roles in stream ecosystems: Climate change, community structure, and material cycling. *Oecologia* 158:307–317.
- Stanton, T., M. Johnson, P. Nathanail, W. MacNaughtan, and R. L. Gomes. 2020. Freshwater microplastic concentrations vary through both space and time. *Environmental Pollution* 263:114481.
- Su, L., H. Cai, P. Kolandhasamy, C. Wu, C. M. Rochman, and H. Shi. 2018. Using the Asian clam as an indicator of microplastic pollution in freshwater ecosystems. *Environmental Pollution* 234:347–355.
- Thiele, C. J., M. D. Hudson, and A. E. Russell. 2019. Evaluation of existing methods to extract microplastics from bivalve tissue: Adapted KOH digestion protocol improves filtration at single-digit pore size. *Marine Pollution Bulletin* 142:384.
- Thorp, J. H., and M. D. Delong. 1994. The riverine productivity model: A heuristic view of carbon sources and organic processing in large river ecosystems. *Oikos* 70:305–308.
- Tuzzio, I. M., B. A. Murry, and C. C. Arantes. 2025. Widespread microplastic pollution in Central Appalachian streams: Implications for freshwater ecosystem sustainability. *Sustainability* 17:2926–2943.
- van Wijnen, J., A. M. J. Ragas, and C. Kroeze. 2019. Modelling global river export of microplastics to the marine environment: Sources and future trends. *Science of the Total Environment* 673:392–401.
- Vannote, R. L., G. W. Minshall, K. W. Cummins, J. R. Sedell, and C. E. Cushing. 1980. The river continuum concept. *Canadian Journal of Fisheries and Aquatic Sciences* 37:130–137.

- Wardlaw, C., and R. S. Prosser. 2020. Investigation of microplastics in freshwater mussels (*Lasmigona costata*) from the Grand River watershed in Ontario, Canada. *Water Air and Soil Pollution* 231:405.
- Weber, A., N. Jeckel, C. Weil, S. Umbach, N. Brennholt, G. Reifferscheid, and M. Wagner. 2021. Ingestion and toxicity of polystyrene microplastics in freshwater bivalves. *Environmental Toxicology and Chemistry* 40: 2247–2260.
- Williams, J. D., N. A. Johnson, G. L. Warren, and R. S. Butler. 2014. *Freshwater Mussels of Florida*. University of Alabama Press, Tuscaloosa 498 p.
- Williams, W. A., A. Arunprasad, and S. Aravamudhan. 2024. Application of a modified set of GoogLeNet and ResNet-18 convolutional neural networks towards the identification of environmentally derived-MPLs in the Yadkin-Pee Dee River Basin. *Environmental Systems Research* 13:53–70.
- Wu, P., J. Huang, Y. Zheng, Y. Yang, Y. Zhang, F. He, and B. Gao. 2019. Environmental occurrences, fate, and impacts of microplastics. *Ecotoxicology and Environmental Safety* 184:109612.
- Wu, Y., J. Yang, Z. Li, H. He, Y. Wang, H. Wu, L. Xie, D. Chen, and L. Wang. 2022. How does bivalve size influence microplastic accumulation? *Environmental Research* 214:113847–113846.
- Yeager, M. M., D. S. Cherry, and R. J. Neves. 1994. Feeding and burrowing behaviors of juvenile rainbow mussels, *Villosa iris* (Bivalvia: Unionidae). *Journal of the North American Benthological Society* 13:217–222.



Freshwater Mollusk Biology and Conservation

© 2026

ISSN 2472-2944

Editorial Board

EDITOR IN CHIEF

Caryn Vaughn, University of Oklahoma

MANAGING EDITOR

Ani Escobar, Limestone Valley RC&D

ASSOCIATE EDITORS

Serena Ciparis, U.S. Fish & Wildlife Service

Traci DuBose, U.S. Geological Survey

Susan Geda, FL Fish & Wildlife Conservation Commission

Jonathan Lopez, University of Mississippi

Michele Melchior, Earth Sciences New Zealand

Kathryn Perez, University of Texas Rio Grande Valley

Ryan Prosser, University of Guelph

Ieva Roznere, Watters Aquatic Conservation Center

Jeremy Tiemann, EnviroScience, Inc

Alexandra Zieritz, University of Nottingham