DISTRIBUTION OF MICROPLASTICS IN FRESHWATER MUSSELS ACROSS A WATERSHED SCALE

A Thesis
by
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Abstract

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Microplastic research is still a fairly new field of study and we do not yet fully understand the scope or effects of microplastic pollution. Microplastics are ubiquitous in the environment, and have the potential to harm aquatic systems, however the field suffers from a lack of unified, accessible protocols and an ecosystems approach to evaluating microplastic contamination. During Autumn of 2020, I collected water, freshwater mussels, and sediment samples from 5 sites along the Yadkin-Pee Dee River Basin. These samples were digested, filtered, and analyzed for microplastic particles. I found significant differences in water ($F = 18.25$, $df = 4,10$ $P = <0.001$), mussel tissue in both particle abundance ($F = 10.42$, $df = 4,45$ $P = <0.001$) and concentration ($F = 7.44$, $df = 4,45$, $P = <0.001$ and sediment samples ($F = 31.64$, $df = 4,5$ $P <0.001$) among sites. Post hoc comparisons revealed that it was primarily low concentrations of microplastics at the high elevation Yadkin River site and high concentrations at the low elevation Pee Dee River site that were responsible for these differences. Correlation matrix analysis of water and sediment concentrations using drainage area and elevation as factors found that water concentrations were positively correlated ($P=<0.001$) with drainage area while sediment concentrations were negatively correlated with elevation ($P=<0.001$). Microplastic concentrations in mussels showed negative

correlations with mussel weight but were positively correlated with drainage area. The results of both analyses imply that factors affecting microplastic contamination are likely to be complex and that microplastic contamination does not always move readily between water, biota, and sediment. This highlights the need to approach microplastic research from an ecosystems perspective and the need for a protocol which can be applied to large-scale studies in a variety of locations. The protocol I developed has the potential to be a relatively low cost, accessible method for analyzing freshwater microplastics. Adaptation of this or similar protocols would allow for more research to be done in understudied regions, as well as provide results which are more readily compared between studies. I also showed the importance of a large-scale approach to understanding the transport, distribution, and severity of microplastic pollution.

Acknowledgements

I would first like to thank Michael M. Gangloff for accepting me into his lab for my graduate program. His patience and understanding in working through a pandemic and the extended amount of time needed to complete my research and degree motivated me to not give up on my project despite many frustrations. With the Covid-19 pandemic occurring in the middle of my degree, I don't know if I would have been willing to continue my research with another advisor who was not as approachable and sympathetic as him. His expertise and assistance also allowed me to complete my fieldwork efficiently in the brief window of time I was allowed and enabled me to evaluate the complex questions which arise from microplastics work. I would also like to thank Robert Creed whose input I have highly valued throughout my graduate degree. His freshwater ecology course gave me the solid foundational knowledge upon which I built my project and this thesis. He also always pushed me to think more deeply about my project, questions, and possible explanations, which helped me grow into the scientist I am now. I would like to thank Shea Tuberty for his feedback on my project, as well as his assistance in connecting me with various people and organizations that I was able to learn much from about the field of ecotoxicology and microplastic research. Last but certainly not least, I would like to thank my lab mates: Amber Olsen, Freddy Ortega, Rob Adams, Elijah Thompson, Sarah Hill, Paige Fulk, and Sam Fritz. Their contributions to brainstorming, fieldwork, venting frustrations, and friendship are among what I consider some of the most valuable things I acquired during my degree and all helped make this project happen in some way. I would like to thank Christophe Satterfield in particular, as without his help I would have spent countless additional hours working through plastic samples and would have likely taken even longer to finish this study.

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Foreword

The research detailed in this thesis will be submitted to the peer-reviewed journal *Freshwater Science*. This thesis has been prepared according to the style guidelines of that journal.

Introduction

The field of microplastic research is developing rapidly, with many studies being published just within the last decade (Akdogan and Guven 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, despite the fact that riverine systems are direct methods of transport to marine systems (Blettler et al. 2017, Wijnen et al. 2019). As a result of the relative lack of freshwater investigations, little is known about the distribution of microplastics throughout riverine networks and in North American freshwater biota. Microplastics found in freshwater ultimately end up in the ocean, as well as in sources of drinking water (Eerkes-Medrano et al. 2015). Determining the effects of these pollutants is difficult due to the lack of long-term studies. However, many compounds found in plastic or used in manufacturing, such as Bisphenol A (BPA), have been linked to health effects in both human and animal trials (Mandel et al. 2019). More recently, microplastics have been shown to adsorb per- and polyfluoroalkyl (PFAS) substances especially well in the presence of organic matter (Scott et al. 2021). These substances have been a recent area of focus by the EPA for their ability to cause various detrimental human health and environmental effects. Microplastic particles are readily consumed by many aquatic organisms and can also accumulate in tissues including gills, liver, and brain (Ding et al. 2018, Su et al. 2018). Despite the lack of freshwater studies, studies into the presence of microplastics in estuary and intertidal regions have shown that microplastic found throughout both the water column and sediment, providing numerous opportunities for uptake by organisms (Leads and Weinstein 2019).

Microplastics are plastic particles <5 mm in diameter (Wu et al. 2019). They come from a variety of sources, most often from wastewater or the breakdown of larger plastic litter (Karbalaei et al. 2018). Particles that are manufactured at diameters <5 mm are considered primary microplastics and they are most commonly found as microbeads that were used in many cosmetics until their banning by the Microbead-Free Waters Act of 2015 (Wu et al 2019). However, microbeads are still used in many products, including those used for scientific research, as the act only applies to cosmetic applications. Primary and other microplastics are transferred to waterways via wastewater discharges, as many treatment plants are not equipped to filter particles <5 mm from wastewater streams (Karbalaei et al. 2018). 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 that are commonly found in wastewater and waterways (Karbalaei et al. 2018).

Several studies have shown significant impacts of microplastic ingestion on other freshwater taxa, including other species of filter-feeders. Silva et al. (2019) found that ingestion of microplastics by *Chironomus riparius*, a deposit-feeding freshwater midge, led to reduced larval size and delayed emergence of adults. In addition, microplastics were found in dispersing adults suggesting that microplastic particles not only persist across multiple life stages but that they also can be transported by biological agents dispersing from aquatic to terrestrial environments. Another study found that microplastics consumed by *Daphnia magna*, a filter-feeding zooplankton, inhibited feeding rates and effectiveness (Colomer et al. 2019). This study also found that at higher rates of flow, microplastic exposure rates were also higher.

Filter-feeders play important foundational roles within many freshwater ecosystems and occupy basal trophic levels in many larger streams and rivers (Thorp and Delong 1994). These organisms are an important food source for many larger organisms and their microplastic burdens could result in bioaccumulation within species at higher trophic levels. Microplastic particles fall within the same size class as particles consumed by larger filter-feeders and so could present a mechanism for larger amounts of microplastic consumption.

Microplastic concentrations in Zebra mussels (family Dreissenidae) and other filter-feeding freshwater organisms vary widely among taxa. Although zebra mussels do not appear to be adversely affected by microplastic, negative effects have been observed in midges and cladocerans. However, no prior research has examined microplastic concentrations in freshwater pearly mussels (Order Unionoida) and it is possible that accumulation rates and effects vary widely across bivalve taxa. Freshwater mussels are among the most threatened groups of invertebrates on Earth and may provide a useful model for microplastic impacts because they are large and abundant filter-feeders that may process up to 6-8 l d^{-1} water while simultaneously interacting with, and obtaining food from, benthic substrates (Neves et al. 1997, Haag 2012, Williams et al. 2014). Mussels excrete processed inorganic matter and micro-plastics in the form of pseudofeces (i.e., undigestible organic matter mixed with sediment) that is deposited into adjacent sediments (Haag 2012).

Despite the abundance of studies focused on the impacts of microplastics to some freshwater groups, studies documenting the distribution of microplastics in freshwater across a watershed scale have not been conducted. Moreover, the majority of freshwater microplastic studies were conducted in Europe or Asia and few studies have examined microplastic abundance in Southeastern United States drainages where levels of freshwater

bivalve diversity and endangerment are both considerable (Neves et al. 1997). Because hydrologic and biotic attributes of river systems differ greatly across geographic scales, models of microplastic dispersal and abundance need to incorporate local physiochemical and climatological attributes to better understand how these novel contaminants may affect waterways and biota in the biodiverse freshwater ecosystems of southeastern North America.

I hypothesized that the concentration of microplastics should reflect changes in human population density in watersheds and I predicted that concentrations in water, sediment and mussel tissues will increase with decreasing elevation in the Pee Dee River Watershed. Moreover, position in the watershed and adjoining land use will also likely influence microplastic concentrations. By sampling along an elevational/drainage area gradient as well as collecting samples up and downstream of large impoundments I examined the role of longitudinal position and land use on microplastic concentrations in the environment and in filter-feeding bivalves.

Methods and Materials

To test these hypotheses, five sites were sampled across the Yadkin-Pee Dee River Basin. The Yadkin-Pee Dee River originates in the Blue Ridge Mountains near Blowing Rock, 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).

At each site, 10 *Elliptio spp.* of various sizes were collected by hand using mask and snorkel or tactile searches. Specimens of *Elliptio complanata*, *E. angustata* and *E. icterina* (*E. waccamawensis* form) were collected. Three 3.8-liter water samples and two 0.95-liter

sediment samples were collected from each site. Prior to processing, two 15 mL subsamples were taken from each sediment sample. Thus, total sample sizes for mussels, water, and sediment samples are 50, 15 and 10, respectively (Table 1).

Following collection, all mussels were tightly wrapped in aluminum foil to prevent release of water and pseudofeces, as well as to prevent microplastic contamination from outside sources. Wrapped mussels were labeled, placed into Ziploc bags, and stored in a cooler for transport to the lab where they were frozen at -20° C until processing. Sediment samples were collected in glass jars that were only opened during sediment collection. Because sediment deposition zones are spatially variable in rivers, samples were taken from within a 100-m radius of where mussels were found at each site. Sediment samples were frozen prior to sample processing. Water samples were collected from the water column in close proximity to the site of sediment sample collections. Water was collected from the water column 10-20 cm above the substrate, and jars were not opened until immediately before collection began. Water samples were placed in coolers and kept out of sunlight until processing and then placed in a freezer at -20° C.

Microplastic abundance in all samples was quantified using protocols modified from Thiele et al. (2019). Mussels were placed in a 10% KOH solution at 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. The mussels and KOH were combined in glass jars and covered with aluminum foil to prevent airborne contamination. Digestion was allowed to proceed for approximately 24 h on a shaker table that kept the solution in motion to assist in digestion. After 24 h, the digested tissue and solution was first filtered through a 50 µm sieve before being filtered using 90-mm borosilicate glass fiber filters in a vacuum filtration system. Filters were then placed in

covered glass petri dishes and allowed to dry under refrigeration. Sediment subsamples were placed in 50 ml of KOH solution and allowed to digest using same method as mussels. Water samples did not undergo digestion but were filtered using same sieve and vacuum filtration process as mussels and sediment. After drying, filters were placed under a dissecting scope and particles of suspected plastics counted. Each sample was examined for 30 minutes. Plastic particles were identified by prodding with a hot needle to determine if the particle melts, bends or otherwise reacts violently in a diagnostic manner indicative of plastics (Dehaut et al. 2018).

All handling of samples and filters, as well as tissue digestions, was done under controlled laboratory conditions to limit contamination as much as reasonably possible. All equipment was thoroughly rinsed with DI water between each sample. Gloves and cotton clothing were worn at all times and all samples were exposed to air only when under an active lab hood, or during the final analysis. When counting samples, care was taken to omit any particle that may have been recent contamination that occurred during the counting process. 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 were not considered in final counts. Because the distilled water used to create the KOH solution likely contains microplastics, several blanks without tissue (negative controls) were run to account for background contamination levels.

After the number of particles for each sample was quantified, analysis was run using 1-Way ANOVA (Tukey-Kramer post-hoc tests) using RStudio software to compare differences in microplastic abundance and concentration among sites (Posit Software 2023). A correlation matrix was used to examine the relationship between site elevation and

watershed area and the abundance of microplastics, as well as the relationship of mussel size and species on abundance using Jamovi software (The Jamovi Project 2022).

Results

Microplastics were detected in all mussel, water, and sediment samples analyzed (Table 2). Negative controls showed an average of 1.1 particles per replicate or 0.022 particles per ml of DI water (Table 3). This confirms that contamination originating within the KOH solution or from the lab during sample analysis was minimal and unlikely to affect results.

Microplastic abundance and concentrations in water samples were significantly different among sites (One-Way ANOVA df = 4, 10, F = 18.25, p=<0.001, Table 4). However, microplastics in Pee Dee River water samples were significantly higher than in samples from the other four sites. Water samples from the Yadkin River at Tom Dooley Road, the furthest upstream site examined, had the lowest level of microplastics detected but the concentrations were highly variable and not significantly different from the other sites (Figure 1). Correlation analysis showed a significant positive correlation between drainage area and concentration (Table 5). Elevation was not significantly correlated with concentration or drainage area.

Examination of microplastics in sediment samples revealed significant differences in both particle abundance and concentration among sites (1-Way ANOVA, df = 4, 5, F = 31.64, p =<0.001, Table 4). Microplastic abundance and concentration were lowest in the Yadkin River and highest in the Lumber River. Both the Yadkin and Lumber river sites were significantly different from the other 3 sites. However, the Lumber River site lacked a confidence interval due to the small sample size and similar sample means. Given how close

the Lumber River mean is to the other 3 low elevation sites, it would likely not be significantly different with additional samples (Figure 1). Correlation analysis revealed a significant positive association between elevation and sediment microplastic concentrations (Table 5). No significant correlation was observed between microplastic concentration and drainage area.

Microplastic concentrations in mussel tissues also significantly differed among sites for both abundance (One-Way ANOVA df = 4,45, F = 10.42, p=<.001) and concentration (One-Way ANOVA df = 4,45, F = 7.44, p=<.001, Table 4). Microplastic abundance and concentration were lowest in *Elliptio complanata* from the Yadkin and Pee Dee river sites and highest in mussels from the Waccamaw River. Microplastic abundance in Yadkin River mussels was significantly lower when compared to all other sites whereas concentrations were significantly lower in mussels from the Yadkin and Pee Dee rivers. Correlation analysis revealed significant negative correlations between microplastic abundance and mussel weight. Microplastic concentration was negatively correlated with weight, but positively correlated with drainage area, and elevation. Mussel tissue weight was significantly correlated with drainage area and elevation. Elevation and drainage area were not correlated (Table 6).

Correlation analyses were also performed with mussels separated into two groups: *E. complanata* and *E. angustata*. *E. complanata* are larger-bodied and more rhomboid shells whereas *E. angustata* have lanceolate shells that are generally smaller and lighter in comparison to *E. complanata*. Correlations revealed that in *E. complanata*, microplastic abundance was significantly negatively correlated with tissue weight and elevation, while concentration was significantly correlated with weight, drainage area, and elevation. In

contrast, *E. angustata* only showed a significant negative correlation between tissue weight and microplastic concentration (Table 7).

Discussion

My study shows that microplastic research should be done at an ecosystem level, looking at many variables to fully understand the patterns of transport and contamination within a system. The variation in significant factors among sample types shows that there are likely a range of complex interactions between the factors that determine microplastic concentration, and that the influence of these factors is not consistent across an entire system. The need for a unified, accessible approach to investigate microplastic abundance is also highlighted by my results. The development and adoption of standardized protocols are needed to better understand the extent and effects of microplastic pollution. My protocol provides an excellent starting point for large-scale research, and my results show that it is effective at determining patterns of microplastic pollution.

These data show that microplastics are widespread and abundant in aquatic habitats across the Pee Dee River Basin. I found a relatively high abundance of particles in water and sediment samples from across the drainage. Although microplastic concentrations vary considerably within water, sediment and mussel tissue samples, these particles appear to be ubiquitous in both the environment and the bivalve fauna of the Pee Dee system. Although there are many factors that may contribute to the heterogeneity of microplastics within a system including land use, discharge, time of year and the presence of impoundments, the abundance of particles in this system appears to increase at downstream sites with lower elevations and larger watersheds. These results suggest that particle abundance is driven by mostly geographical or hydrogeological rather than biological factors as the concentration of

plastics within mussels did not seem to be related to concentrations within water or sediment samples.

My initial predictions that plastic concentrations in all sample types would increase in a downstream direction were based largely on predictions of the River Continuum Concept (RCC) (Vannote et al. 1980). The RCC predicts that communities should be increasingly dependent on organic materials transported from upstream ecosystems and I hypothesized that concentrations of suspended microplastic particles should also increase downstream. However, microplastic concentrations were highest in the Pee Dee River and relatively low at all other sites. This does not support my initial hypothesis. There are several factors that may explain this discrepancy. First, microplastics do not degrade as readily as organic matter and may be deposited more rapidly or otherwise not move through aquatic systems in the same way as naturally occurring FPOM particles. Microplastic concentrations also likely vary greatly over time (Stanton et al. 2020). This temporal variation is not completely explained by seasonal flow rates and may be related to temporal differences in anthropogenic use of waterways (Stanton et al. 2020). My samples were collected over the course of several days and so I am unable to discern how season or water temperature may influence concentrations. More robust microplastic transport and accumulation models should be conducted over an extended period of time to temporal variability in concentrations. Additionally, Roebuck et al. (2020) noted that the RCC does not account for increasingly urbanized land use along all stream orders. Land use may alter input of allochthonous materials, including microplastics, and may change expectations based on the RCC.

The strong relationship between drainage area and the concentration of microplastic in water samples suggests that variability may be strongly influenced by runoff from urban

areas. Microplastics in water from the Pee Dee River were likely higher than those at other sites because the Pee Dee is the largest stream sampled and it had the largest drainage area as well (Table 1). The lack of a significant elevation effect indicates that upstream land use plays a more important role than geographical position. A study looking at a large river catchment in South America found similar results in 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).

I expected sediment concentrations to increase with drainage area and elevation because substrate deposition rates increase as streams became larger and slower moving (Nyman et al. 2020). I observed that only the relatively high elevation site on the Yadkin River had sediment microplastic concentrations that were statistically lower than all other sites. Sediment microplastic concentrations were significantly associated with elevation but were not related to drainage area. It is thus likely that the elevation may be serving as a proxy for local stream habitat characteristics. High elevation streams tend to have higher flows, coarser substrates, and lower rates of sediment deposition relative to lower-elevation streams in the Pee Dee 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 localized differences in streambed topography could be influencing this pattern and artificially depressing concentrations of microplastics in sampled reaches.

The concentration of microplastics within mussel tissues showed perhaps the most interesting result. I expected that mussels would serve as an intermediary between water and sediment and their role as filter-feeders led me to hypothesize that the amount of plastic

within the mussels should be correlated with the abundance of plastic in water and sediment samples. This was not the case however, as the Yadkin and Pee Dee sites had similar concentrations of microplastics per gram of mussel tissue despite having statistically different water and sediment concentrations. However, the number of particles per mussel was higher in the Pee Dee compared to the Yadkin River. There was a significant difference in the size of mussels collected from the Pee Dee and Yadkin sites relative to those in other streams. 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). This is likely because larger-bodied mussels were sampled at both the Yadkin and Pee Dee sites. Although there was a slight difference in the mean size of the Pee Dee and Yadkin mussels, it was not significant due to high variation in mussel sizes collected at both sites. This likely contributed to the lower microplastic concentrations (particles per gram of tissue) reported from these sites, despite the fact that microplastic concentrations (particles per organism) were significantly different at these sites.

It is interesting that Pee Dee River mussels still had similar microplastic abundance levels compared to the other low elevation sites, despite the concentration of microplastics in water samples being much higher. This may be due to the ability of mussels to selectively exclude particles and deposit them as pseudofeces. In large streams with higher particulate loads, mussels may become more selective in their feeding habits which may mediate their consumption of plastic particles. This could explain why I saw no differences in microplastic concentration among lower elevation sites, despite differences in water concentration. This

would also mean that although high-elevation streams appear to be cleaner, mussels and other filter-feeders in these reaches may be more vulnerable to microplastic pollution as they are less selective in their consumption when particulate matter is scarce (Frau et al. 2016, Mistry and Ackerman 2018).

There is also an ontogenic shift in feeding strategy between juvenile and adult *E. complanata* (Forbes-Green and Cyr 2023). Younger mussels tend to use pedal-feeding, using their foot to pull in organic material from the sediment in which they remain buried for several years early in their lifecycle. There is a large amount of variability in regard to the size and age at which *E. complanata* emerge from the sediment, but they do exhibit a gradual shift in feeding strategy as they age from pedal to filter-feeding (Forbes-Green and Cyr 2023). This is seen in many other species of mussel as well, although some continue to utilize both methods of feeding throughout their life (Yeager et al. 1994). This shift in feeding strategy could explain the relationships observed between weight and microplastic concentration. It is possible that younger, small 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 through their method of filter-feeding. Given the variation in growth rate based on environment and the variability in size and age at emergence, it is difficult to determine which size class of mussels that I collected would be pedal-feeding versus filter-feeding. However, with the prior study showing that *E. complanata* likely exhibits a gradual shift in feeding strategy, it follows logically that the gradual shift in microplastic concentration correlated with weight implies a similar gradual shift rather than a discrete transition. This is particularly concerning as it is well documented in the literature

that, in general, juvenile organisms of many taxa tend to be the most susceptible to toxicity and pollution.

There is also a seasonal shift that has been observed in the verticality of *E. complanata* (Cyr 2009). It is unlikely that this would have affected my results given that all samples were collected during the same season. However, seasonality can differ across geographic location, and a shift in depth within the sediment could also correspond with a shift in feeding strategy as well. This highlights the need for long-term studies of microplastics in unionids as well as other biota which may exhibit seasonal behaviors that could influence microplastic exposure.

One possible source of heterogeneity involves the use of multiple mussel taxa in this study. Although only one species was examined at each site, several species were included in this analysis. Mussels at the Yadkin and Pee Dee River sites were *Elliptio complanata.* In contrast, *Elliptio angustata* was sampled in the Lumber and Waccamaw River sites and the animals collected from Lake Waccamaw were *Elliptio icterina* (waccamawensis shell morph) which is likely a synonym of *E. complanata* (Fagundo 2016). It is likely that filtering rates differ among species and mussel sizes, and this could have affected the abundance or concentration of microplastics within the mussel samples (Hoellian et al. 2017, Spooner and Vaughn 2008). Despite this, 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 Pee Dee and Yadkin sites. With the strong correlation between species and weight, as well as between weight and both abundance and concentration, it is likely that any effect of species is likely to be an effect of body size on filtering rates or selective feeding ability.

The data obtained in my study shows the need for ecosystem-scale approach to microplastic monitoring and research. 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. My data shows that the relationship between environment and organismal contamination is likely to be the result of a multitude of complex factors rather than a simple correlation between microplastic abundance and bio-accumulation. Looking at a single aspect of a system is not an effective method for determining the potential movement, input, or uptake of microplastics by organisms. 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 as a whole suffers from a lack of unified or widely accepted protocols to investigate plastic concentrations in various samples. My protocol was modified from methods described by Thiele et al. (2019) and I believe it will provide other researchers with a rapid and cost-effective protocol that is still robust enough to investigate many different questions regarding microplastics. The large number of different methods that have been developed to detect microplastics in aquatic systems can 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 the scope of any citizen science-based monitoring efforts. Protocols such as the one I developed, which used easily accessible materials and equipment, can help facilitate the long-term, large-scale studies needed to properly assess the impacts and dynamics of microplastic pollution.

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Tables and Figures

Site	Elevation (m)	Drainage Area (km^2)	Mussels
1. NC: Wilkes Co. Yadkin River at Tom Dooley Road Boat Ramp (36.085, -81.369)	327	468.9	*E. complanata* n=10
2. NC: Richmond Co. Pee Dee River shoals downstream of US Highway 74 crossing (34.932, -79.862)	37	17819.1	*E. complanata* n=10
3. NC: Robeson Co. Lumber River at NC Highway 72 boat ramp (34.592, -78.984)	33	1867.4	*E. angustata* n=10
4. NC: Columbus Co. Lake Waccamaw along southwestern shore (34.261, -78.524)	13	238.3	*E. complanata* n=10
5. NC: Columbus Co. Waccamaw River at NC Highway 904 boat ramp (34.015, -78.632)	6	2527.8	*E. angustata* n=10

Table 1. List of study site locations, their elevation, drainage area, and sample sizes. Sediment sample sizes are 2 with 4 subsamples for all sites, and water sample sizes are 3 for all sites.

Water	Yadkin River	Pee Dee River	Lumber River	Lake Waccamaw	Waccamaw River
Mean Abundance	3.33	17.67	6	8	6.33
Mean Concentration	0.88	4.67	1.59	2.11	1.67
Sediment					
Mean Abundance	2.25	5.75	6.5	5.5	5.75
Mean Concentration	0.15	0.38	0.43	0.36	0.38
Mussels					
Mean Abundance	3.6	5.9	6.9	6.8	7.6
Mean Concentration	0.28	0.34	1.37	1.50	1.00
Mean Wet Mass (g)	18.76	21.52	6.50	5.21	8.17

Table 2. Table of descriptive statistics of water, sediment, and mussel samples collected from 5 sites along the Yadkin-Pee Dee River Basin in 2020. All samples include mean abundance (particles per sample) and concentration in particles per L (water), cm^3 (sediment), and g (mussels) for each site. Mean wet mass for mussel samples is also included for each site.

Negative	Particles	Particles/ mL
1	0	0
2	3	0.06
3	3	0.06
4	2	0.04
5	1	0.02
6	1	0.02
7	0	0
8	0	0
9	1	0.02
10	0	0
Mean	1.1	0.022

Table 3. List of negative control replicates and their abundance and concentration of particles in #/mL. Means for abundance and concentration are listed at the bottom.

Sediment	**Df**	**Sum Sq.**	**Mean Sq.**	**F Value**	**P Value**
Site	4	0.098	0.024	31.64	<.001
Residuals	5	0.0038	0.00079		
Water					
Site	4	25.47	6.37	18.25	<.001
Residuals	10	3.49	0.35		
Mussel (Abundance)					
Site	4	96.52	24.13	10.42	<.001
Residuals	45	104.20	2.32		
Mussel (Concentration)					
Site	4	12.91	3.23	7.44	<.001
Residuals	45	19.54	0.43		

Table 4. Results of one-way ANOVA analysis of sediment, water, and mussel samples collected from 5 sites along the Yadkin-Pee Dee River Basin in 2020. Mussel sample results are reported using both particles per organism(abundance) and particles per gram(concentration). Sediment and Water samples are reported using concentration (#/cm^3 and #/L respectively).

	Concentration	Drainage Area	Elevation
Concentration	- -	r = 0.889* p <0.001	r = -0.421 p = 0.118
Drainage Area	r = 0.266 p = 0.458	- -	r = -0.248 p = 0.372
Elevation	r = -0.940* p <0.001	r = -0.248 p = 0.489	- -

Table 5. Correlation matrix of water (above diagonal) and sediment (below diagonal) samples collected from 5 sites along the Yadkin-Pee Dee River Basin in 2020 showing correlations between 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.

	Abundance	Concentration	Weight	Drainage Area
Weight	r = 0.510* p <0.001	r = 0.612* p <0.001	- -	
Drainage Area	r = 0.010 p = 0.946	r = 0.334* p = 0.018	r = 0.482* p <0.001	- -
Elevation	r = 0.657* p <0.001	r = 0.403* p <0.001	r = 0.394* p = 0.005	r = -0.248 p = 0.082

Table 6. Correlation matrix of mussel samples collected from 5 sites along the Yadkin-Pee Dee River Basin in 2020 showing correlations between abundance (particles of microplastic per organism), concentration (particles per gram of tissue), weight in grams, Species, Drainage Area (km2), and Elevation (m). Significant correlations ($p<0.05$) are marked with an asterisk.

	Abundance	Concentration	Weight	Drainage Area	Elevation
Abundance	- -	r = 0.209 p = 0.376	r = -0.134 p = 0.574	r = 0.278 p = 0.236	r = -0.278 p = 0.236
Concentration	r = 0.628* p <0.001	- -	r = -0.825* p <0.001	r = -0.216 p = 0.360	r = 0.216 p = 0.360
Weight	r = -0.444* p = 0.014	r = -0.680* p <0.001	- -	r = 0.414 p = 0.069	r = -0.414 p = 0.069
Drainage Area	r = 0.152 p = 0.424	r = -0.375* p = 0.041	r = 0.418* p = 0.022	- -	r = -1.000* p <0.001
Elevation	r = -0.635* p <0.001	r = -0.471* p = 0.009	r = 0.274 p = 0.142	r = -0.429* p = 0.018	- -

Table 7. Correlation matrix of mussel samples collected from 5 sites along the Yadkin-Pee Dee River Basin in 2020, separated by species, showing correlations between abundance (particles of microplastic per organism), concentration (particles per gram of tissue), tissue weight (g), drainage area (km^2), and elevation (m). *E. angustata* correlations are listed above the diagonal, and *E. complanata* below. Significant correlations (p=.05) are marked with an asterisk.

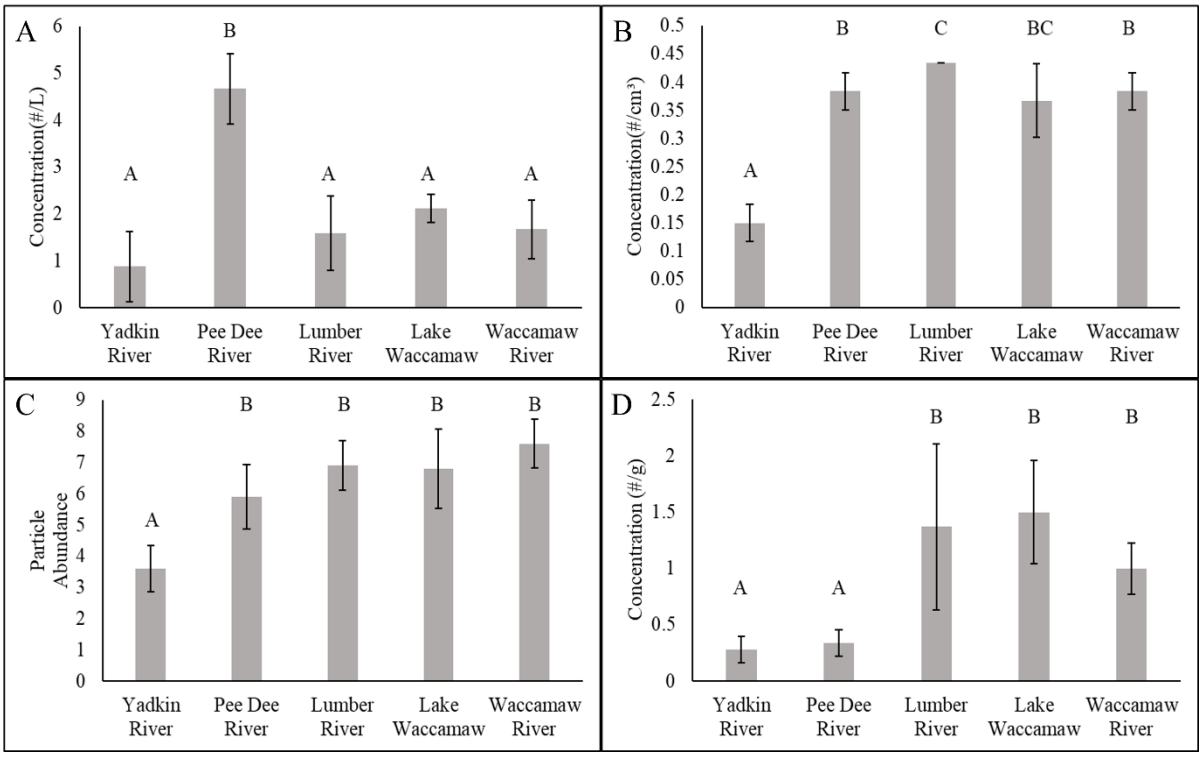

Figure 1. Results of post-hoc analysis of water particle concentration (A), sediment particle concentration (B), mussel particle abundance (C), and mussel particle concentration (D) from samples collected from 5 sites along the Yadkin-Pee Dee River Basin in 2020. Groupings are labeled above each bar.

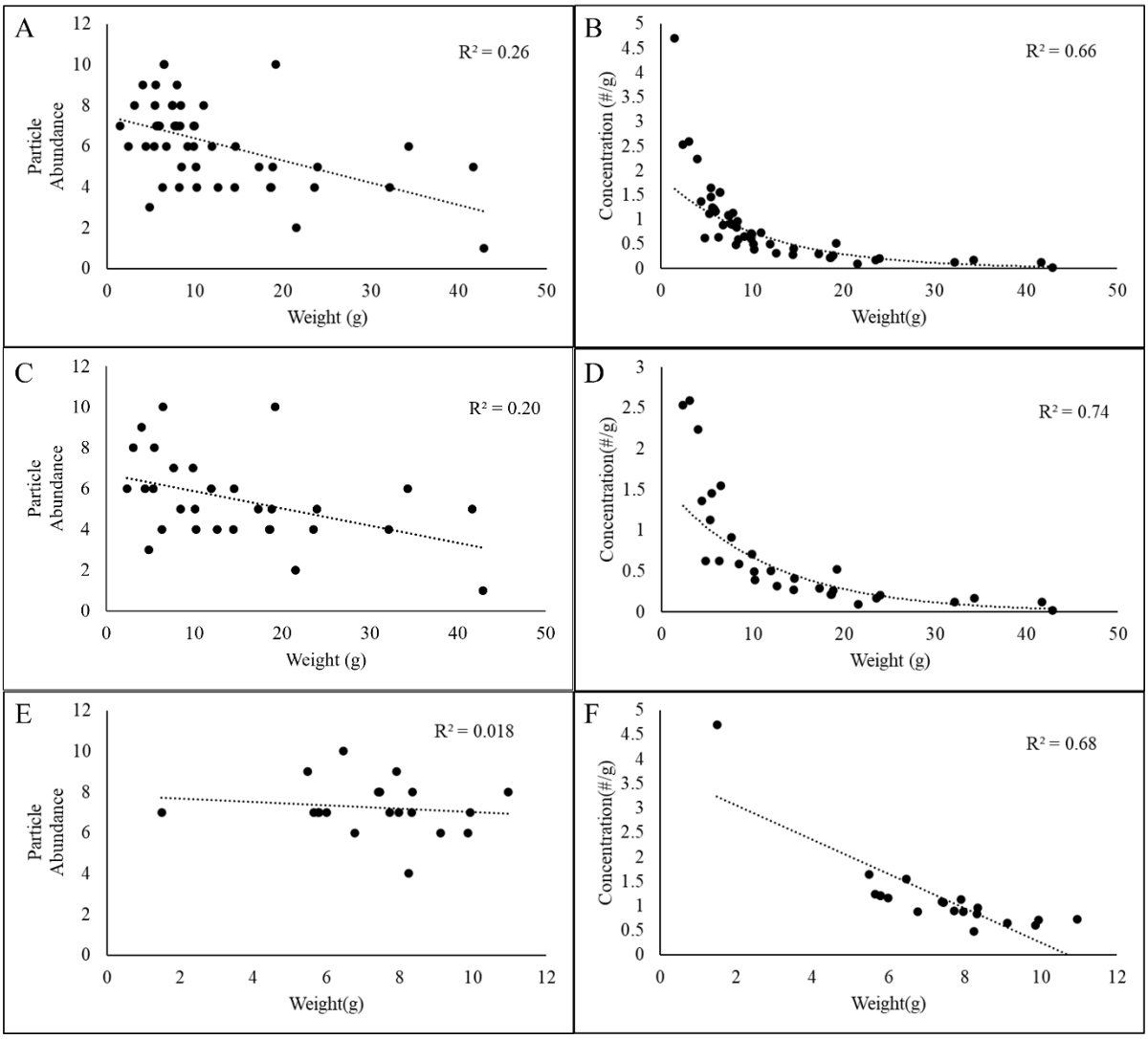

Figure 2. Scatterplots showing the relationships between freshwater mussel wet mass and particle abundance (A) and concentration (B) in all mussels, *E. complanata* (C & D) and *E. angustata* (E & F) collected from 5 sites in the Yadkin-Pee Dee River Basin in 2020.

Vita

James Brandon Williams was born in Durham, North Carolina to Kitsy Williams and James M. Williams Jr in October of 1995. In 2014 he began studying at Appalachian State University in pursuit of a Bachelor of Science degree in Ecology, Evolution, and Environmental Biology. He was awarded his degree in May of 2019. Prior to that, he began working with the Aquatic Conservation Research Lab in the Spring of 2019, assisting with fieldwork and then joining the lab as a graduate student in Fall of 2019. He pursued a Master of Science degree in Ecology and Environmental Biology and was awarded the degree in May of 2023.