Surveillance of Microplastic Pollution in Central Virginia Freshwater Ecosystems

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Abstract

Aquatic organisms often mistake microplastic particulates (MP) as food and inadvertently ingest the particulates, which can biomagnify through the food chain. While MP ingestion is well-researched in the marine environment, little is known about microplastics in freshwater ecosystems. This project explores MP occurrence in an ecologically significant freshwater invertebrate: crayfish. Crayfish from two Central Virginia streams are collected to identify MP in the digestive tracts and gill filaments, and characterize the MP using analytical chemistry techniques. It was determined that MP were present in the digestive tracts and gill filaments of crayfish collected from both streams, and that MP frequency in the urban stream was greater than that of the rural stream due to its location near the dominant thoroughfare.

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1. Introduction

First synthesized in 1907, plastics have facilitated the majority of human advances. Cheap to produce and requiring little energy expenditure, more than 20 major types of plastics constitute numerous household and commercial products (North & Halden, 2013). From 1950 to 2014, the amount of plastics produced globally exploded from 1.5 million tons to 299 million tons (Nelms et al., 2015). Of this plastic waste, approximately eight million tons of plastic are estimated to enter the world's oceans annually. The majority of this marine debris is comprised of plastic items originating from landfills and human activities such as fishing. However, the effluent of municipal and industrial wastewater treatment plants has also been recognized as a significant contributor of microplastics into the aquatic environment, and particularly the freshwater environment, by producing microplastic contaminants such as synthetic clothing microfibers and microbeads from cosmetic items (Ziajahromi, Kumar, Neale, & Leusch, 2018). Despite the numerous benefits provided by plastics, the disposable material has devastating implications for the health of aquatic organisms. Microplastic pollution of water sources and its impacts on aquatic organisms is an emerging topic in the scientific community. Aquatic organisms often mistake microplastic particles as food sources, therefore inadvertently ingesting the particulates, which can biomagnify through the food chain (Figure 1); (Garneau, 2016). With an estimated 1.3 billion tons of plastic waste produced annually, the implications for aquatic life are drastic (North & Halden, 2013).

Figure 1. REMOVED FOR COPYRIGHT. Biomagnification of microplastics in freshwater ecosystems (Medrano, Thompson, & Aldridge, 2015, p. 76).

Across the globe, plastic pollutants are considered to be the most pervasive form of anthropogenic debris in both the oceans and freshwater ecosystems (Andrade et al., 2019). As plastics are persistent contaminants, it is inevitable that marine species will encounter plastic items and be impacted through ingestion and entanglement. Although these effects can be easily observed when organisms become entangled in larger plastic items, there are less obvious repercussions from minuscule pieces of plastics known as microplastics. Pieces of larger plastics are converted into microplastics through wave movements, ultraviolet light, and physical abrasion (Nelms et al., 2015). These small particulates become readily bioavailable and directly impact aquatic life through respiration, ingestion, gastric obstruction, physiological effects, chemical transfer, and trophic transfer, potentially causing liver toxicity, endocrine disruption, decreased fecundity, and lower survival rates (Lusher et al., 2016). These microplastics are then transported through the food chain and the interacting food webs, causing these contaminants to biomagnify throughout higher trophic levels (Nelms et al., 2015).

While entanglement in plastic items is readily observed, ingestion of microplastics is less obvious despite having dramatic physiological impacts. Although plastic ingestion has been documented in a variety of marine species, marine turtles are especially at risk due to their extremely mobile behaviors and have become emblematic for raising public awareness of the plastic pollution crisis. The two avenues through which turtles may ingest microplastics are through direct or indirect ingestion, of which the former has been documented in all marine turtle species (Nelms et al., 2015). When plastic pollution is interspersed with targeted food items, marine turtles inadvertently ingest plastic particulates. For example, studies have discovered that

juvenile green turtles have consumed plastics as they intentionally target macroalgae for consumption (Di Beneditto & Awabi, 2014). Furthermore, accidental ingestion of plastics can occur when the turtles mistake the plastic items as prey. Turtles are predominately visual foragers, and therefore they are susceptible to mistaking items like balloons and shopping bags as prey to actively consume (Nelms et al., 2015). For instance, studies have identified plastic bottle lids in loggerhead turtles, as these lids float at the water's surface and resemble the neustonic prey of the turtles (Hoarau, Ainley, Jean, & Ciccione, 2014). In addition to direct ingestion, indirect ingestion can occur when invertebrate prey like mollusks or crustaceans ingest microplastics (Nelms et al., 2015). As species like marine turtles ingest these organisms, the plastic particulates bioaccumulate through the trophic levels and can produce significant physiological effects. However, it is challenging to identify and attribute sublethal physiological effects to indirect ingestion rather than other water quality problems.

Despite the deleterious implications of trophic transfer of pollutants and the consequent increased awareness about plastics in the environment, the demand for plastics has continued to rise over the past sixty years due to their durability and low cost (Kitamoto et al., 2011). Nylon and other synthetic fibers are now being used in place of natural fibers, further increasing the surplus of smaller plastic contaminants in the aquatic environment (Gregory, 2009). Evidence has demonstrated that all continents are affected by plastic pollution, and consequently the crisis of the pollution of water sources has dramatically intensified. In fact, one study estimates that over 250,000 tons of plastics are floating in the world's oceans alone (Eriksen et al., 2014). As larger marine organisms are being negatively impacted by plastic pollution, interest in plastics in the marine environment has been piqued (Parker, 2018).

Although larger plastics have negative implications for aquatic organisms through ingestion and entanglement, it is the degradation of larger plastics into smaller pieces known as microplastics that poses the greatest threat to aquatic life. Microplastics are classified according to the physical dimensions, with particles under five millimeters (mm) considered to be microplastics (Figure 2); (Oberbeckmann, Loder, & Labrenz, 2015). These particles are known to be ingested by a diverse array of marine organisms, ranging from whales, dolphins, and sea turtles, to zooplankton, mussels, and sea cucumbers (Brown, Dissanayake, Galloway, Lowe, & Thompson, 2008); (Graham & Thompson, 2009). The two most common chemical constituents of plastics, polyethylene which comprises plastic bags and polypropylene which comprises plastic bottles, have been found in the digestive tracts of marine organisms (Lusher, McHugh, & Thompson, 2013). Therefore, the investigations of this study focused on the presence of particles of polyethylene and polypropylene in freshwater organisms, primarily in crayfish digestive tracts and gill filaments.

Figure 2. REMOVED FOR COPYRIGHT. Isolated microplastic fragments less than 5 mm (Oberbeckmann et al., 2015, p. 554).

As a prolific environmental contaminant throughout both marine and freshwater ecosystems, microplastics have deleterious implications for the health of these ecosystems and their inhabitants, as well as human health (Lusher, Welden, Sobral, & Cole, 2017). Plastics can absorb many toxic chemicals, an example of which is pesticides (Webb, Arnott, Crawford, & Ivanova, 2012). These toxic chemicals can then be consumed by a variety of other organisms and consequently biomagnify through the food chain with detrimental implications for ecosystems (Eriksen et al., 2013). The inadvertent ingestion of microplastic particulates by marine organisms and consequent movement through the marine biota is thoroughly researched. However, research

regarding the impacts of microplastic contamination in freshwater ecosystems is scarce. Despite this understanding and the increasing evidence of microplastic pollution in freshwater ecosystems, there is little research concerning the uptake of microplastic particulates by invertebrates in freshwater ecosystems (Scherer, Brennholt, Reifferscheid, & Wagner, 2017). Therefore, this research project explores the occurrence of local microplastic pollution in an abundant and ecologically important invertebrate species: crayfish. As research regarding microplastic contamination in freshwater invertebrates is scarce, this project contributes to the knowledge of freshwater microplastic contamination and its ecological significance, particularly for crayfish organisms (Lusher et al., 2017).

2. Material and methods

2.1. Sample collection

The crayfish organisms collected in this investigation were of the species *Cambarus bartonii,* the common crayfish species that is native to eastern North America (Figure 3). Crayfish specimens were obtained from two stream locations in Central Virginia: (1) Rock Castle Creek, an urban stream located near a major commercial center, and (2) Opossum Creek, a rural stream located in a wooded mountain region with minimal pollution. The urban stream location was filled with plastic pollution in the form of bags, bottles, and plastic furniture after recent flooding events, and thus the stream was selected to represent a freshwater ecosystem impacted by plastic pollution (Figure 4). The crayfish were collected from each stream using a kick seine technique and were then euthanized either by transferring the crayfish into an Eppendorf tube containing isopropyl alcohol or by immediately sacrificing the crayfish on ice. As the physical stress caused by field sampling can induce gut evacuation in the crayfish organisms, which can decrease the number of microplastics observed prior to examination, the

time interval between field sampling and analysis of the animal was minimized (Lusher et al., 2017). Additionally, special care was taken to minimize handling stress of the crayfish in the lab in order to decrease microplastic regurgitation (Lusher et al., 2017). In addition to collection of crayfish, representative water samples and sediment samples were collected using Whirl-Pak™ sampling bags in order to examine the substrate for the presence of microplastics. It was recognized that these sterile polyethylene bags, as well as the Eppendorf tubes, could be contributing sources of microplastic particulates to the crayfish, water, and sediment samples. However, a manufacturer of the Eppendorf tubes reports that the company does not use slip agents, plasticizers, or biocides – substances demonstrated to leach from plastic items into a sample – during the manufacturing process in order to minimize contamination by leaching into samples (Pipette.com, 2020).

Figure 3. Image of the crayfish organism (*Cambarus bartonii*) collected from each study site.

Figure 4. Recent flooding events in 2018 showing evidence of plastic pollution.

2.2. Sample measurements and dissection

Prior to dissection, each crayfish was measured from rostrum to tail and the length was recorded in millimeters (mm). Additionally, the gender and blotted wet mass in grams (g) of each specimen was recorded. For the Opossum Creek crayfish organisms, the average blotted wet mass was 3.39 g and the average total length was 46.24 mm. In contrast, for the Rock Castle Creek crayfish organisms, the average blotted wet mass was 7.33 g and the average total length was 43.30 mm. Before conducting analytical chemistry analysis of the microplastic debris, the crayfish were dissected and examined under the dissecting microscope in order to investigate microplastic debris located in the digestive tract as well as the gill filaments. First, the appendages and caraparace were removed and discarded. The gill filaments and digestive system were extracted, which included the pyloric and cardiac stomachs, intestine, and hepatopancreas. After investigation of the tissues of interest under the dissecting microscope, the tissues were then transferred to separate glass microscope slides to further search for potential contaminants under a Scannning Electron Microscope (SEM). Upon completion of visual analysis, the digestive system and gill filaments were then transferred into glass jars rinsed with 70% ethanol. Each digestive tract and gill tissue sample was then placed in its own ethanol-rinsed glass jar. Next, each glass specimen jar was filled with enough 70% ethanol to fully immerse the sample by one centimeter.

2.3.Sample preparation for FTIR-ATR analysis

After dissection of the crayfish and visual investigation using the two categories of microscopes, analytical chemistry tools were utilized to determine the identity of any foreign particulates and determine their frequency within the gill filaments and digestive tracts of the crayfish specimens. Each digestive tract and gill tissue immersed in ethanol in its individual jar was ultrasonicated for five minutes using an ultrasonic cleaning bath filled with water. The technique of ultrasonication was utilized to gently separate any microplastic contaminants from the biological tissue. The ethanol from each specimen was decanted from each sonicated digestive tract, as well as the gill filaments, into an ethanol-rinsed glass container. Each specimen was then membrane filtered through a 25-mm stainless steel Millipore microsyringe filter holder with a new 25-mm and 0.2 μm pore size Anodisc™ membrane disc filter for each specimen in order to eliminate contamination between the samples. The purpose of this membrane was to capture any potential microplastic particulates that could then be examined using microscopy and analytical chemistry analysis. Finally, the filters were then dried within the glass jars prior to further analysis.

2.4. Chemical analysis with FTIR-ATR

Following these sample preparation and isolation techniques, the analytical chemistry tool Fourier-Transform Infrared Spectroscopy (FTIR) with the Attenuated Total Reflection accessory (ATR) was used to identify the chemical "fingerprints," or functional groups, of the contaminants. The general consensus among the scientific community upholds that FTIR-ATR spectroscopy is an accurate and effective technique to determine the chemical identities of plastic polymers (Lusher et al., 2017). FTIR-ATR spectroscopy is a useful method for determining the

chemical composition of microplastic debris as it is able to analyze both solid and liquid particles and does not require extensive sample preparation (Jung et al., 2018). Expert judgement and reference spectra were used in order to identify the chemical constituents according to the specific absorption bands of the functional groups, with polypropylene and polyethylene as the most commonly identified polymers (Figure 5); (Ory et al., 2018). With the implementation of this analytical chemistry technique, microplastics could be identified in the crayfish organisms. Any isolated microplastics were then classified into categories such as granules and pellets, rigid fragments, or films (Figure 6); (Cozar et al., 2017).

Figure 5. REMOVED FOR COPYRIGHT. IR spectra produced by the microplastic constituents polypropylene and polyethylene (Ory et al., 2018, Supplementary Material p. 6).

Figure 6. REMOVED FOR COPYRIGHT. Different categories used to characterize microplastic particulates (Cozar et al., 2017, Figure 4).

3. Results

3.1. Preliminary investigation: FTIR-ATR analysis

In order to determine the best dissection and tissue preservation techniques, two crayfish specimens collected from Opossum Creek were initially dissected. Their body lengths were recorded, and the genders of both specimens were determined to be female. Furthermore, a male crayfish from the Carolina Biological Supply Company was examined. Upon dissection of this specimen, the digestive tract was extracted, and two hard particles were discovered at the junction of the stomach, intestine and hepatopancreas (Figure 7). These particles were speculated to be foreign contaminants, and thus chemical investigation was performed to determine the identity of the particulates. The unknown particle was examined using FTIR-ATR spectroscopy and compared with the spectra produced by a plastic pipette that contains polyethylene. The

foreign particle and the plastic pipette displayed similar stretches from 2750-3000 cm^{-1} and 1250-1500 cm^{-1}, which was indicative of shared functional groups (Figure 8). Additional resolution of the spectra displayed distinctly comparable stretches from 2900-2920 cm^{-1} and 2840-2860 cm^{-1}, which further indicated a similar chemical identity (Figure 9). A final resolution of the spectra displayed comparable stretches from 1400-1500 cm^{-1} and 700-750 cm^{-1} that also demonstrated a similar chemical composition between the plastic pipette constituents and the foreign particle (Figure 10). Moreover, the broad band at approximately 1400 cm^{-1} as well as the strong and sharp band at 873 cm^{-1} was indicative of the presence of calcium carbonate in its common calcite crystal form (Figure 10).

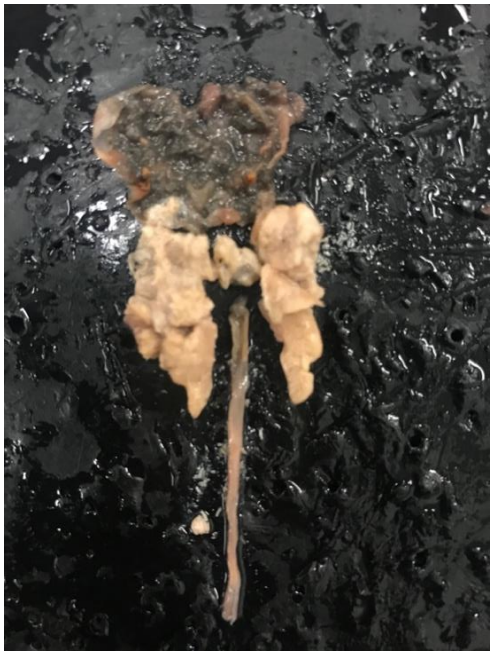

Figure 7. Two hard particles discovered at the junction of the stomach, intestine and hepatopancreas.

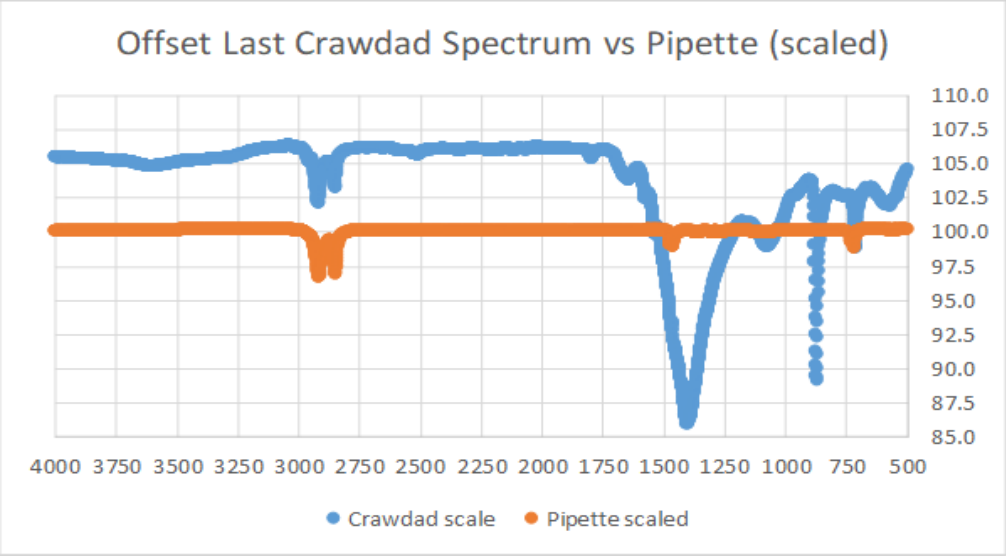

Figure 8. Initial spectra produced by a plastic pipette and the foreign particle in the crayfish digestive system.

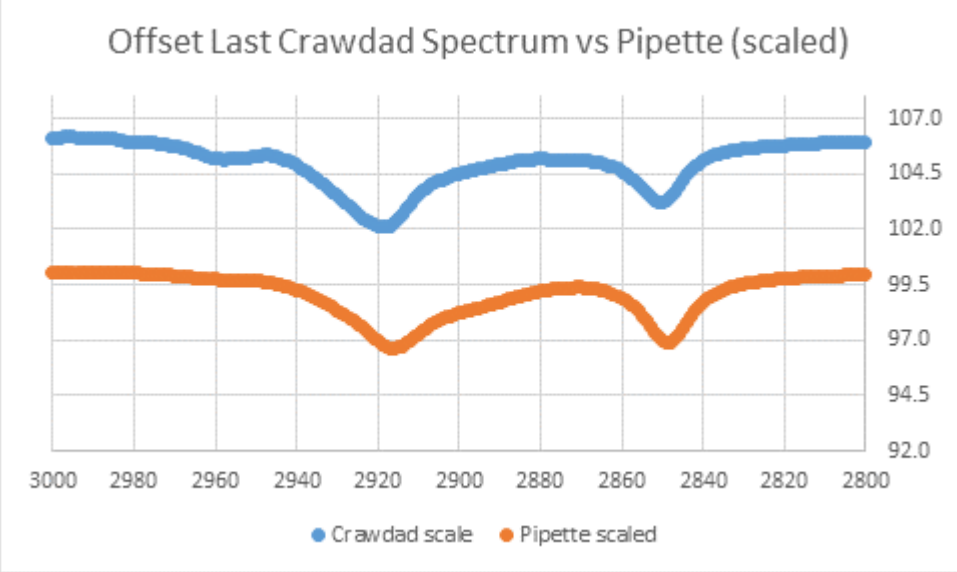

Figure 9. Initial scaled spectra produced by a plastic pipette and the foreign particle in the crayfish digestive system.

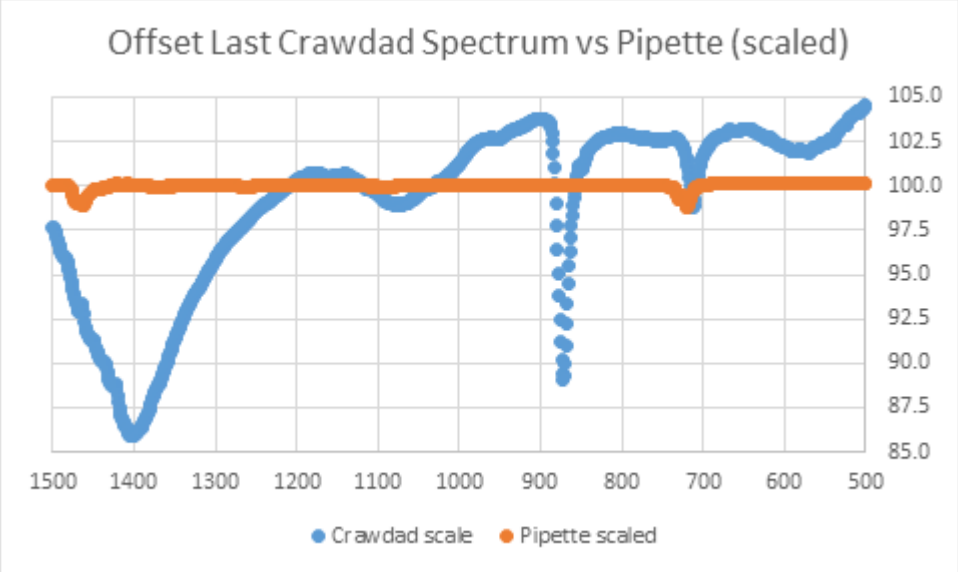

Figure 10. Additional initial scaled spectra produced by a plastic pipette and the foreign particle in the crayfish digestive system.

3.2. Preliminary investigation: Sediment analysis

Moreover, the preliminary investigation included collection and subsequent analysis of sediment samples from the Rock Castle Creek study site. Three Whirl-Paks™ of sediment samples were collected at this study site using ethanol-rinsed, 1000-micron filters. In order to collect more representative samples of the study site, the three sediment samples were collected at different locations across the length of the creek. The GPS coordinates of the three collection sites in Lynchburg, Virginia, were as follows: 37°21’17”N and 79°11’1”W at 810 ft elevation, 37°21’16”N and 79°11”1”W at 770 ft elevation, and 37°21’17”N and 79°10’59”W at 760 ft elevation. After collection of the sediment samples in the field, the samples were transferred to ethanol-rinsed glass dishes. The sediment samples were investigated for any potential microplastic particles under the dissecting microscope. Any particulates that appeared to be of synthetic origin were retrieved and collected in Eppendorf tubes for analysis. However, none of

the suspected items were determined to be of plastic identity. Furthermore, a variety of plastic items was collected from the heavily polluted Rock Castle Creek study site in order to establish a plastic library as a reference for sample analyses. These items included a green fishing line, Styrofoam cup, soda bottle, large tarp that was submerged in the water, plastic Walmart shopping bag, and a black trash bag. FTIR-ATR analysis was performed on each item and their respective spectra were saved in order to create the plastic library for future reference.

3.3. Initial Rock Castle Creek dissection findings

Based on the findings of this preliminary investigation, as well as practice of the dissection protocol, collection of crayfish specimen from the field was conducted. First, five crayfish specimens were collected from the urbanized Rock Castle Creek study site. Upon extraction of the digestive tract and gill filaments, the tissues were examined under both the dissecting and scanning electron microscopes. Performance of these visual analysis techniques on four of the specimens did not reveal any foreign particulates in the tissues of interest. However, performance of the visual analysis techniques on one of the five specimens revealed a foreign fiber embedded in the digestive tract (Figure 11). Moreover, the hepatopancreas of the crayfish specimen was granulated and hard. The foreign fiber embedded in the digestive tract was of an opaque color and was clearly entwined in the stomach tissue. This fiber was analyzed using FTIR-ATR and its spectrum was compared with that of a commercial translucent polypropylene lid. The spectra exhibited identical resonances in the 2800-3000 cm^{-1} and 1400-1500 cm^{-1} regions (Figure 12). Therefore, it was concluded that the identity of this foreign fiber was polypropylene. It was speculated that the resonances in the fingerprint region were due to another component in the fiber such as a mineral or trace organic material from the crayfish.

Thus, this substantiated result supported the hypothesis that crayfish organisms are ingesting microplastic particulates in their aquatic environments.

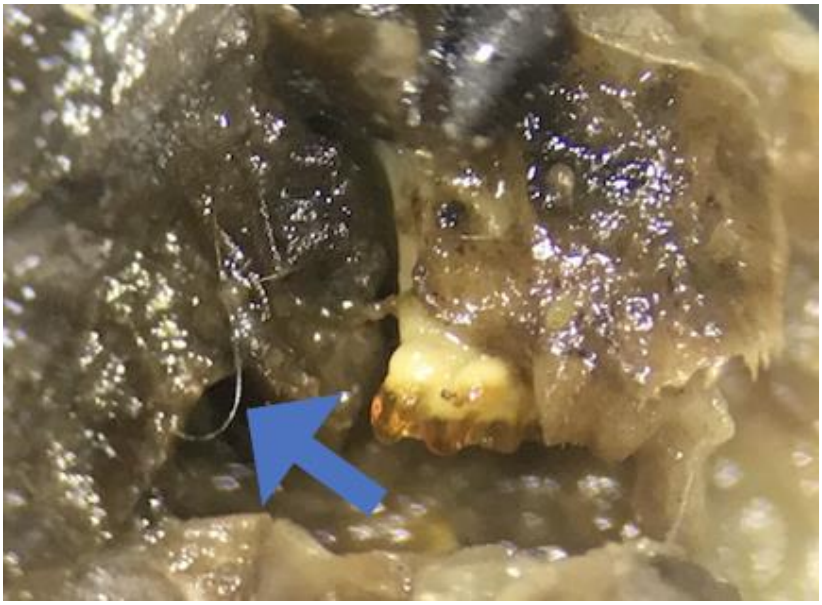

Figure 11. Plastic fiber entwined in the gastric stomach of a Rock Castle crayfish.

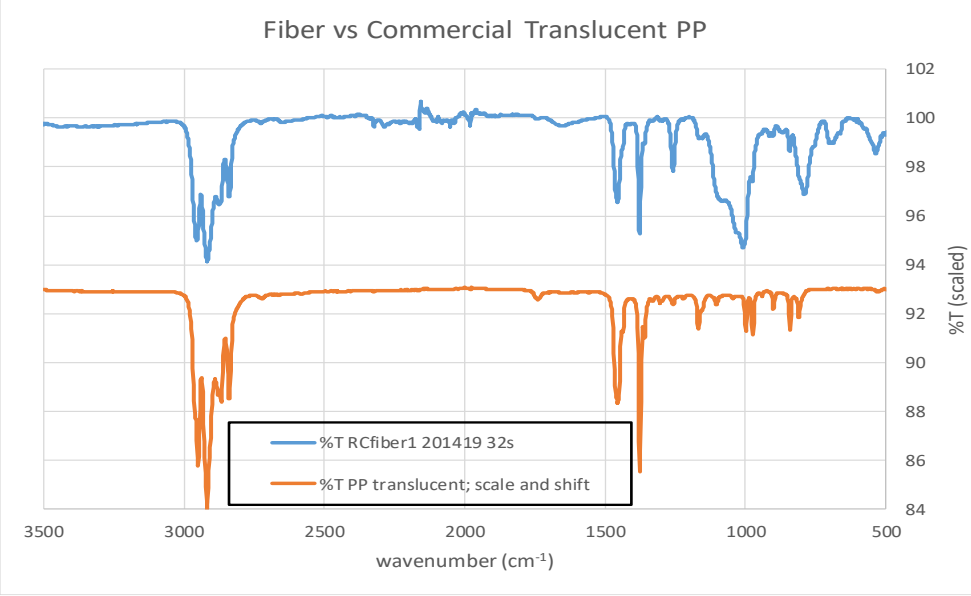

Figure 12. FTIR spectra of the gastric stomach fiber compared to a polypropylene lid.

3.4. Tank study experimental design

After the discovery of the polypropylene fiber in the crayfish specimen collected from Rock Castle Creek, it was evident that crayfish organisms can ingest microplastics that they encounter in their environment. However, it was not known whether this particulate was inadvertently ingested through normal feeding behavior, or if the particulate was mistaken as a food item and thus intentionally ingested. Therefore, a tank study was performed to investigate the feeding behaviors of crayfish organisms in relation to microplastics. In order to minimize the potential compounding factor of microplastic contaminants being already present in the digestive tracts, the crayfish specimens used in the tank study were collected from the rural study site, Opossum Creek. This was due to the understanding that as an aquatic ecosystem not situated near a dominant thoroughfare like Rock Castle Creek, Opossum Creek should have little to no plastic pollution. Thus, the crayfish should then possess minimal, if any, microplastic particulates in their digestive tracts. Therefore, eight crayfish were collected from Opossum Creek and their gender, length, and blotted wet mass were recorded. Of these crayfish specimens, four were male and four were female. The crayfish were then transferred into eight individual glass aquaria that were randomized to a plastic treatment and labeled from 1 to 8 (Figure 13). The crayfish were alloted two weeks to acclimate to their new aquatic environments prior to experimental treatment. After the acclimation period, two different plastic treatments with one control treatment were performed across the eight different glass aquaria. First, tanks 1, 6, and 8 served as the control tanks and received no plastic treatment. Three, two by two inch squares of a synthetic green fabric were submerged in tanks 4, 5, and 7. Lastly, three, two by two inch squares of polypropylene shopping bags were submerged in tanks 2 and 3.

Figure 13. Set-up of the eight class aquaria utilized for the experimental tank study.

3.5. Gill filament analysis

Furthermore, as crayfish continually filter water over their gill filaments, it was speculated that microplastic particulates could become trapped within the gill tissue. In fact, it has been demonstrated that aquatic organisms may be threatened more by ingestion of fibers than other types of microplastics (Collard et al., 2018). Therefore, the investigations of this study included examination of the gill tissue for potential microplastic fiber contaminants. During analysis of a crayfish specimen collected from the rural Opossum Creek study site, a blue fiber was identified in the gill filaments under the scanning electron microscope at a magnification power of 40X (Figure 14). This blue fiber was then transferred to an Eppendorf tube for analysis using FTIR-ATR. However, due to the microscopic size of this fiber and the difficulty of

transferring the fiber to the FTIR-ATR instrument, the identity of the fiber was unable to be determined. However, this fiber was characterized by fibril edges that were consistent with characteristics of synthetic fibers like those found in fabric or plastic tarps that are commonly identified pollutants in freshwater ecosystems. Therefore, it was speculated that the identity of this fiber was of synthetic origin.

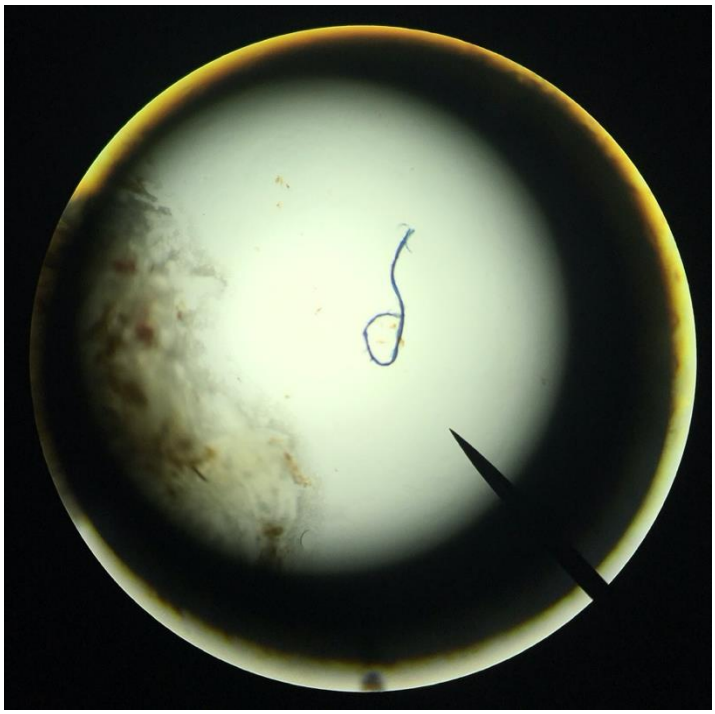

Figure 14. Foreign fiber identified in the gill filaments of an Opossum Creek crayfish.

Based on the discovery of the potentially synthetic blue fiber in the gill filaments of the Opossum Creek crayfish specimen, ten additional crayfish were collected from the Rock Castle Creek study site and their gill filaments investigated for potential microplastic pollutants. Of the crayfish collected, numerous foreign fibers were identified in the gill filaments of one of the crayfish specimens. Using the dissecting microscope, three fibers were identified in the gill filaments. The gill filaments were then transferred to an ethanol-rinsed glass microscope slide and examined under the scanning electron microscope. Using this microscope, two blue fibers

and one red fiber were visualized. (Figure 15). This was the first documentation of a red fiber in the investigations conducted in this study (Figure 16). Moreover, utilizing the same techniques, a blue plastic bag-like translucent fiber was identified in the gill filaments of another crayfish specimen from this collection. Unlike the other fibers that were observed in this study, which have exhibited fibril characteristics like fabric, this foreign particulate resembled the translucent texture of a plastic bag (Figure 17).

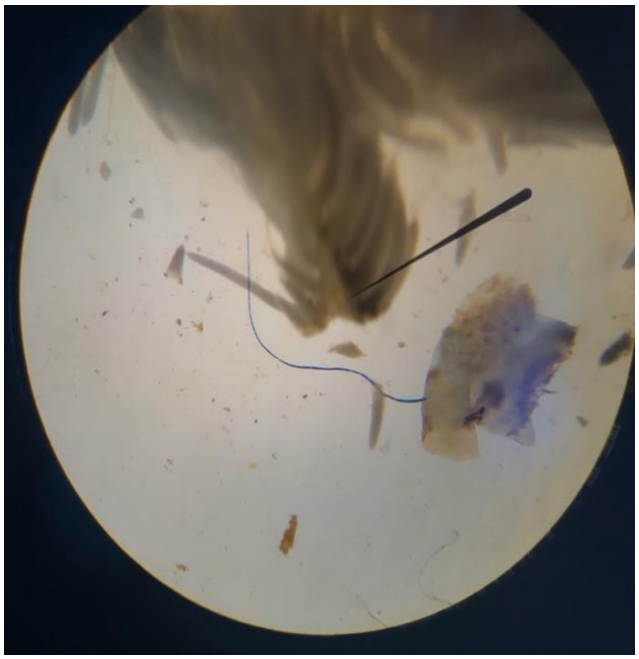

Figure 15. Foreign fiber identified in the gill filaments of a Rock Castle Creek crayfish.

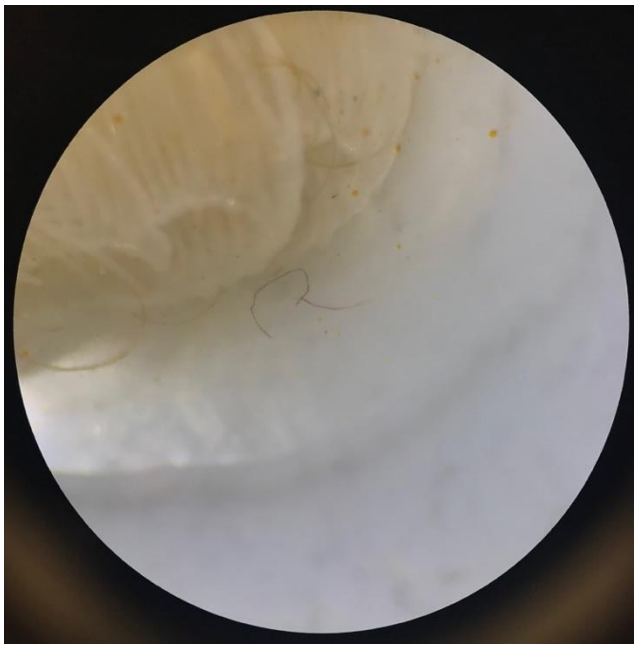

Figure 16. Red and blue foreign fibers identified in the gill filaments of a Rock Castle Creek crayfish.

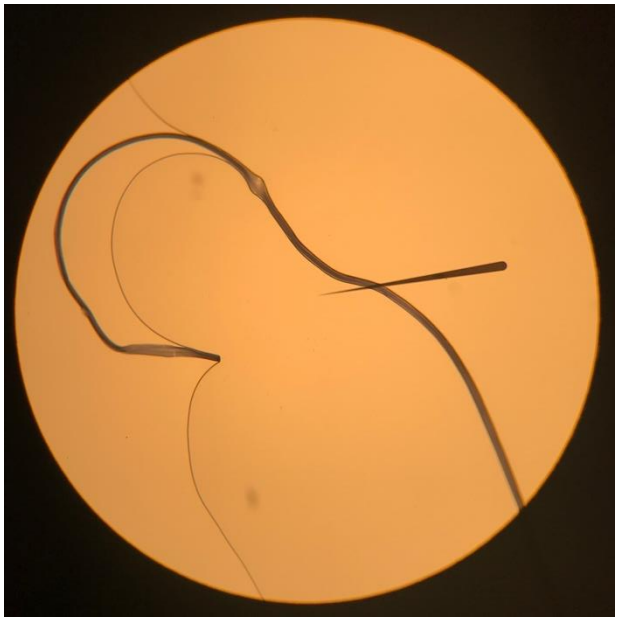

Figure 17. Blue foreign fibers identified in the gill filaments of a Rock Castle Creek crayfish.

3.6. Tissue filtration analysis

Furthermore, filtration of the digestive tissues and gill filaments of ten Rock Castle Creek crayfish organisms and ten Opossum Creek crayfish organisms was performed to isolate any potential microplastic particulates. Following filtration, a number of suspect particles that became entrapped on the membrane filter were discovered in the Rock Castle Creek individuals that were evaluated. First, in the digestive tissue of one Rock Castle Creek individual, a dark fiber was identified (Figure 18). Secondly, a bright blue particle was isolated from the gill filaments of another Rock Castle Creek individual (Figure 19). An opaque film was then isolated from the gill filaments of an additional Rock Castle Creek crayfish, resembling that of a plastic bag (Figure 20). However, across the digestive tissues and gill filaments of the ten Opossum Creek crayfish organisms that were filtered, no microplastic particulates were identified.

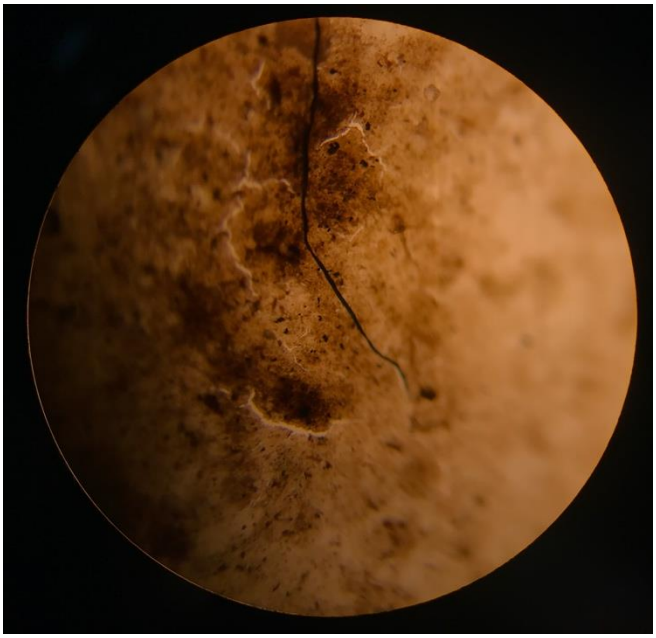

Figure 18. Dark fiber identified in the digestive tissue of a Rock Castle Creek crayfish.

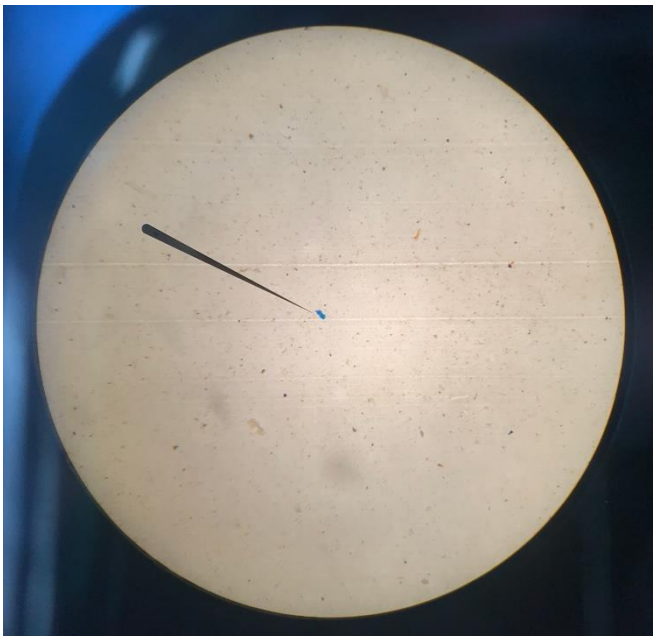

Figure 19. Blue particle identified in the gill filaments of a Rock Castle Creek crayfish.

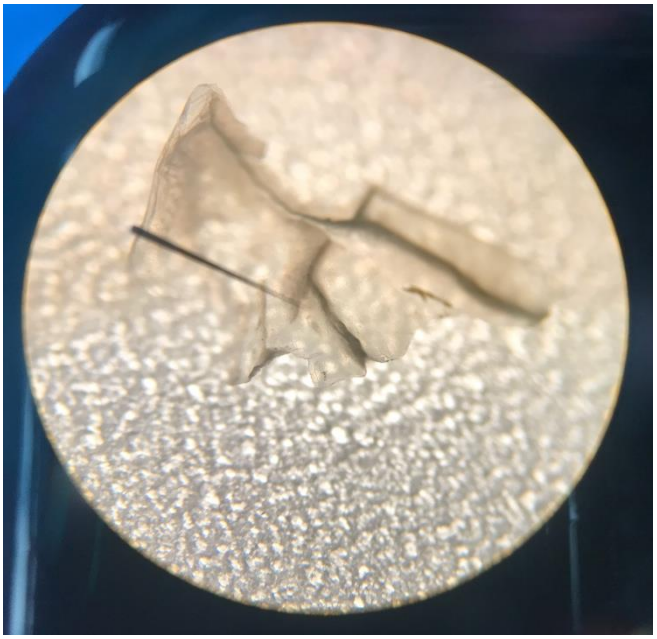

Figure 20. Opaque film identified in the gill filaments of a Rock Castle Creek crayfish.

4. Discussion

4.1. Preliminary investigation

Following the preliminary investigation, with the data gathered and compared with the available literature, the spectrum produced by the foreign particle resembled polyethylene filled with calcium carbonate, which is a common filler of polyethylene used in the popular white lawn furniture as a substitute for the expensive polymer fillers (Ozen, Simsek, & Eren, 2013). The FTIR spectra produced suggested the presence of calcium carbonate in its calcite crystal form. Calcite, which is a common crystal form of calcium carbonate, is the most common mineral filler used to generate the desired whitened and filled quality of polyethylene furniture pieces. Therefore, these substantiated results from the preliminary investigation provided compelling evidence for microplastic contaminants in the digestive tracts of crayfish. Moreover, due to the FTIR spectra produced, it was determined that the identity of the two particles identified in the Carolina Biological Supply Company specimen were not components of the gastroliths. The gastroliths are synthesized as a pair in the stomach wall of crayfish during their pre-molt life stage (Luquet et al., 2016). The purpose of these structures is to store calcium ions that can be readily accessed after molting in order to calcify the exoskeleton. In particular, the calcium is deposited as amorphous calcium carbonate (ACC) within the gastrolith pair (Luquet et al., 2016). During phases of molting, crustaceans like crayfish have the ability to synthesize and resorb calcium-containing minerals. As crayfish possess an inelastic exoskeleton, they are required to molt in order for growth to occur. Like most crustaceans, crayfish achieve hardening of the exoskeleton through calcification by precipitation of calcium carbonate. Although calcium ions are generally accessible in the aquatic biotopes, crustaceans like the crayfish most often utilize calcium ions stores through the development of gastroliths in the stomach wall of the gastric

stomach (Luquet et al., 2016). However, the amorphous calcium carbonate of the gastroliths would not produce the sharp peak at 873 cm^{-1} that was observed in the FTIR spectrum. In light of this information, it was concluded that the identity of the two particles discovered in the Carolina Biological Supply Company specimen were not components of the gastroliths. Therefore, it was speculated that the identity of the foreign particles was a man-made polyethylene particle filled with calcium carbonate that the crayfish organism likely consumed.

4.2. Tank study

As other crayfish studies were simultaneously being conducted in the laboratory by other research teams at the time of this investigation, many crayfish were housed in glass aquaria for extended periods of time. Upon weekly feeding of these crayfish specimens, it was observed that some of the crayfish from the other studies were shredding the sponge filters that were present in the aquaria. Therefore, the tank study was performed to investigate this observed shredding behavior and explore its potential association with microplastic ingestion. As omnivores and scavengers, crayfish often shred any material that they encounter in their environment. For example, crayfish play a critical role in the shredding of leaf litter and decaying material, therefore making vital nutrients available to other trophic levels of their aquatic ecosystem. Therefore, the observation of crayfish shredding the sponge filters in their glass aquaria was consistent with the shredding behavior that is observed in their natural habitats. Consequently, it was hypothesized that crayfish may be exhibiting this same shredding behavior towards plastic contaminants that they encounter in their freshwater ecosystems like the Rock Castle Creek and Opossum Creek study sites.

After a period of five months, the eight crayfish from the tank study were dissected and their digestive tracts and gill filaments examined for microplastics. However, using the visual

analyis techniques, no microplastic particulates were identified in the tissues of interest that were extracted. It was determined that either the crayfish did not target the submerged plastics as potential food sources, or that if the crayfish did ingest any plastic fibers they were able to excrete them. However, it was concluded that the noninteraction was more likely to have occurred because the plastic items submerged did not most accurately represent the physical nature of plastic pollutants that crayfish might encounter in the field. However, performance of the tank study provided important information about the feeding behaviors of crayfish in association with plastic pollutants.

4.3. Gill filament and digestive tissue analysis

Upon dissection of the digestive tissue and gill filaments of the ten Rock Castle Creek organisms and ten Opossum Creek organisms, numerous suspect foreign particulates were visualized under the scanning electron microscope. Following membrane filtration of both the digestive tissue and gill filaments of the crayfish organisms, the membrane filters were again visualized under the scanning electron microscope to characterize any isolated particles. These isolated particles were speculated to be of foreign origin, such as from microplastic pollution. However, due to the microscopic size of the fibers compared to that of the FTIR-ATR device probe, the foreign particulates were unable to be transferred to the FTIR-ATR device for accurate chemical identification. Therefore, the chemical identities of the suspect contaminants were unable to be determined by FTIR-ATR analysis. Nonetheless, it was speculated that these isolated fibers were of foreign origin, specifically of microplastic pollutant identity, such as polyethylene. Although microplastic particulates in aquatic environments exhibit various densities, it is typically expected that polymers such as polyethylene, which has a lower density than water, will float at the surface compared to denser plastics that will sink to the bottom

sediment. However, recent studies have revealed that the majority of the polymer categories of microplastics, including polyethylene, will ultimately sink to the bottom sediment of aquatic ecosystems where benthic invertebrates are likely to encounter and consequently ingest the particulates either through direction ingestion or gill filament entrapment (Ziajahromi et al., 2018). For instance, one study discovered that numerous low-density microplastics, including polyethylene, settled in the deep-sea sediment (Ziajahromi et al., 2018). It is suggested that this phenomenon results from ecological processes like association with organic material and microorganisms that can alter microplastic properties, causing increased density and eventual settling to the bottom sediment (Ziajahromi et al., 2018). However, due to the methodological limitations of this study, the chemical identities of these foreign particulates could not be determined and these final observations were speculative.

Furthermore, the identification of foreign particulates in the gill filaments of the crayfish organisms was consistent with the understanding that the filtering action of crayfish organisms can result in the entrapment of microplastic pollutants. For aquatic organisms engaged in respiration, the gill filaments are the first tissue exposed to anthropogenic particulates (Karami, Golieskardi, Bin Ho, Larat, & Salamatinia, 2017). Therefore, respiration action through the gill tissue greatly increases the potential for microplastic particulates to become entrapped in the gill filaments (Karami et al., 2017). For example, one study found that upon exposure to high-density polyethylene fragments, these particles became entrapped in the gill filaments of the blue mussel (*Mytilus edulis*) (von Moos, Burkhardt-Holm, & Köhler, 2012). An additional study discovered that waterborne exposure of zebra fish (*Danio rerio*) to polystyrene microspheres resulted in accumulation of the particles in the gill tissues (Lu et al., 2016). Moreover, the majority of current studies concentrate on the presence of plastic particulates in organisms that occupy

higher tropic levels, such as fish, rather than in freshwater invertebrates (Windsor, Tilley, Tyler, & Ormerod, 2019). However, freshwater invertebrates, and freshwater benthic invertebrates like the crayfish in particular, have an increased risk of exposure due to the sinking of microplastics into the sediment substrate (Redondo-Hasselerharm, Falahudin, Peeters, & Koelmans, 2018). As this investigation discovered foreign particulates in both the digestive tissue and gill filaments of the crayfish, these findings were consistent with the current literature regarding the ingestion and gill entrapment of anthropogenic particulates by freshwater invertebrates.

5. Conclusions

As a prolific environmental contaminant throughout both marine and freshwater ecosystems, microplastics have deleterious implications for the health of these ecosystems and their inhabitants, as well as human health. The inadvertent ingestion of microplastic particulates by marine organisms and consequent movement through the marine biota is well researched. However, research regarding the impacts of microplastic contamination in freshwater ecosystems is scarce. With increasing evidence of microplastic pollution in freshwater ecosystems, there is little research concerning the uptake of microplastic particulates by invertebrates in freshwater ecosystems (Triebskorn, 2019). Therefore, this project contributes to the knowledge of freshwater microplastic contamination and its ecological significance, particularly for crayfish organisms.

This research project functions as a surveillance of microplastic pollution in the Central Virginia region and is consequently driven by field collection of samples. In order to establish a baseline of comparison between microplastic occurrence in urban and rural freshwater ecosystems, crayfish were collected from two stream sites, the digestive tracts and gill filaments of the crayfish were removed, and the tissues were examined under dissecting and scanning

electron microscopes in order to identify microplastic contaminants. Any suspect particles were then examined using analytical chemistry tools to determine their chemical identities. The dominant analytical chemistry technique employed in this project was Fourier-Transform Infrared Spectroscopy (FTIR) with the Attenuated Total Reflection Accessory (ATR), which is regarded as a proven technique for identifying larger microplastic contaminants. Using these techniques, microplastic contaminants were isolated and their identities confirmed in the crayfish organisms. Therefore, this approach will be further continued and expanded for the examination of future samples.

With recent observations of plastic pollution in the Rock Castle Creek study site and speculations about uptake by crayfish organisms, the preliminary results of this study support the investigation of microplastics in the digestive tracts and gill filaments of crayfish organisms. Visual observation of the Rock Castle Creek study site indicated the frequency of plastic pollution, while dissection of the digestive tracts and gill filaments of collected specimens indicated the uptake of these contaminants. Using an internal plastic library, the chemical identities of the fibers and suspect particles retrieved from the digestive tracts were determined using FTIR-ATR analysis. Moreover, recent examination of gill filaments in additional organisms revealed colorful foreign fibers embedded in the gill tissues. The substantiated results from this investigation provide compelling evidence for microplastic contaminants in the digestive tracts, as well as the gill filaments, of crayfish. Upon future investigations, it is expected that: (1) the presence and frequency of MP will be confirmed in the digestive tracts and gill filaments of crayfish collected from both study sites; (2) the plastic library will assist in identifying the contaminants extracted from the crayfish samples; and (3) the occurrence of MP in the urban stream will be greater than that of the rural stream due its location near commercial

businesses and the dominant thoroughfare. With the most recent findings of foreign fibers in the gill filaments of Rock Castle crayfish, it is expected that more microplastic particulates will be isolated from the crayfish specimens in addition to those identified in the digestive tracts.

With the increasing body of literature revealing the threats of plastic pollution to global aquatic ecosystems, continued study of microplastic contamination of both marine and freshwater ecosystems is paramount to addressing this environmental crisis. Both laboratory and field evaluations have demonstrated that the ingestion and movement of microplastic particulates through trophic levels can negatively impact aquatic organisms such as fish, birds, zooplankton, and invertebrates (Windsor et al., 2019). However, current research has largely focused on marine organisms rather than on freshwater organisms, despite their closer association to terrestrial microplastic pollution sources. In fact, there are only a few recent studies that have investigated and confirmed the ingestion of microplastic contaminants by freshwater invertebrates such as Tubificid worms, *Gammarus pulex* and *Hyalella azteca* (Windsor et al., 2019). Although controlled laboratory exposure of freshwater invertebrates like *G. pulex* and *H. Azteca* to microplastic contaminants have not revealed overt toxicity at environmentally relevant concentrations, these studies have focused on broad-scale effects such as growth and reproduction, as well as mortality, rather than the chronic effects across less severe biological endpoints that pose risks to the health of freshwater invertebrates (Redondo-Hasselerharm et al., 2018). Therefore, it is of critical importance that studies begin to concentrate efforts on comprehensively understanding the ingestion of microplastics by freshwater invertebrates, as these organisms are primary consumers that play significant roles in the entry of microplastic particulates into freshwater food chains (Windsor et al., 2019).

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