

Downstream Dilemma: Navigating Microplastic's Impacts on Freshwater Symbiosis in the Anthropocene

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ACADEMIC ABSTRACT

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Annually, it is estimated that 82 million tons of global plastic waste is either mismanaged or littered, bypassing waste management practices. This mismanagement causes the permeation of plastic debris into the environment, which then undergoes natural degradation processes. These degradation processes result in the proliferation of miniscule plastic particles known as microplastics. Due to the inherent proximity to sources of anthropogenic waste, concerns of microplastic pollution and its impact on freshwater ecosystems have recently increased. Until recently, microplastic research has primarily been focused on the toxicological effects on an individual organism rather than the interactions that occur between taxa. Only focusing on the toxicological impact experienced by an individual organism turns a blind eye to the communities that maintain ecosystem health and stability. To that end, our experiment is the first study to assess the impact of microplastics on a freshwater symbiosis. Until recently, symbioses in the scope of toxicokinetic studies have primarily been dominated by that of terrestrial and marine relationships. This knowledge gap poses a concern as previous literature suggests freshwater systems are more contaminated than that of any other aquatic habitat. To address this knowledge gap, we conducted mesocosm-based exposure-response assays, exposing a crayfish-branchiobdellidan symbiosis to microplastics while varying symbiont densities. We used the crayfish-branchiobdellidan model system in our study due to its amenability to laboratory monitoring and manipulation. The crayfish *Cambarus appalachiensis*, common in the New River Basin of Virginia, USA, served as host to obligate ectosymbiotic annelids in the order Branchiobdellida. Previous research using the crayfish-branchiobdellidan symbiosis demonstrated that the interaction is a cleaning symbiosis, where hosts benefit from reduced gill fouling while symbionts benefit from increased resource availability. We observed the physical and behavioral changes of the crayfish-branchiobdellidan symbiosis over a 172-day chronic exposure assay. Our results show that crayfish hosts with higher symbiont densities experienced decreased physical growth when exposed to microplastics compared to the control. This alteration in host growth was the result of increased antagonistic symbiont behavior in the form of gill tissue consumption. Our results suggest microplastics caused a reduction in epibiont abundance, thus decreasing symbiotic resource availability. This reduction in resources resulted in a shift in context dependency, resulting in increased parasitic symbiont behavior. This study demonstrates as freshwater ecosystems continue to be inundated with plastic pollution, the presence of microplastics have the capability to shift symbiotic context from a mutualism to a parasitism.

GENERAL AUDIENCE ABSTRACT

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Every year, due to improper waste management, approximately 82 million tons of global plastic waste ends up in the environment. Once in the environment, this plastic waste naturally breaks down into tiny particles known as microplastics. Recently, there has been growing concern about how these microplastics affect freshwater habitats. One particular worry is the impact that this contaminant can have on the relationships between different organisms living in freshwater environments. Currently, multiple scientific experiments have studied the effects of microplastics on marine and land-based organismal relationships; however, none have studied the effect of microplastics on freshwater organismal relationships. To explore this, we used the relationship of crayfish and a type of worm called Branchiobdellida, due to their naturally occurring symbiotic relationship. In fact, these Branchiobdellida worms live on the outside of crayfish and keep the gills of the crayfish clean by consuming any matter that has grown or lodged itself on the gill filaments. As such, the worms rely on the crayfish as a home and means of collecting food. Depending on food availability, the relationship can be mutually beneficial for both the crayfish and worm. Due to the worm's reliance of the crayfish and the mutually beneficial relationship they facilitate, the crayfish-branchiobdellidan relationship is known as a symbiosis. To best understand how microplastics affect the crayfish-branchiobdellidan symbiosis, we conducted a 180-day experiment where we exposed crayfish with varying numbers of worms to a controlled dosage of microplastics within artificial environments called mesocosms. Each microplastic dosage contained different types of microplastics that mimicked the type and amount commonly found in nature. We found that crayfish with higher worm densities grew less when exposed to microplastics compared to those not exposed to microplastics. We also noticed that when microplastics were present, the worms exhibited more aggressive behavior, like consuming the gills of the crayfish. Our findings suggest that microplastics reduce the resource availability to the worms, which then triggers a change in the relationship the symbionts experience with the crayfish host. Instead of being helpful, the worms start behaving more like parasites when exposed to microplastic. Ultimately, this study shows as plastic pollution continue to worsen in freshwater systems, microplastics have the capability to change the way different organisms interact, turning a beneficial symbiosis into a harmful one.

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My academic career at Virginia Tech has spanned a total of eight years, of which, I've had the blessing of working and befriending some of the most knowledgeable, understanding, and plain incredible individuals. These eight years at Virginia Tech not only facilitated my growth as a scientist and academic but acts as a personal metamorphosis.

Honestly, whenever I get the chance to sit and ponder my “academic” journey here in Blacksburg, I don't quite recognize the Cameron Braswell that started it. As a previously homeschooled child, my inclinations and perceptions of the world were molded purely from my upbringing. However, upon arriving at Virginia Tech, my “understanding” of the world was severely challenged to say the least. The dynamic ecosystem that is Virginia Tech, provided me with countless instances of personal growth interlaced with intellectual and professional development. In my opinion, the college experience should facilitate just that. An atmosphere where social development thrives, intellectual ideals and opinions are challenged, and experiences build self-assurance. So that, at the end of your “academic” journey you've become personally and intellectually unrecognizable to your previous self.

Now, although this journey is one of personal and individualistic growth, it is not done alone – quite the opposite. It is the people who lead you and the people that experience the journey alongside you, who help guide the trajectory of your overarching “academic” journey. In fact, that's what this portion of my paper is all about – to acknowledge those who mentored me along the way and to those who endured this tumultuous journey alongside me.

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With my foundation of scientific knowledge established and solidified, I transitioned into one of the most incredible working environments I could have every asked for – the Brown Lab. Bryan Brown doesn't just facilitate a well-functioning lab, but curates a unique community of students that stands the test of time. While in The Brown Lab, my close friend and colleague Cameron Lockett were blessed to work alongside a group of incredibly capable and impressive undergrads we know call friends – all of which hand-picked by Bryan.

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expertise. It is with a heavy heart, I conclude my time not only in the Brown Lab, but under the utterly unmatched mentorship of Bryan Brown.

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Chapter 1

Exploring the Ecological Nexus: Plastic Pollution, Plastic Degradation, Microplastics, and the Role of Symbiotic Relationships in Freshwater Systems

This chapter aims to provide a comprehensive overview of the research topics, focusing on plastic pollution, plastic degradation, and microplastics, as well as the role of organismal relationships in freshwater ecosystems. This chapter presents a detailed background of the sources, prevalence, and impacts of environmental plastic pollution, while also elucidating the processes and formation of microplastics through plastic degradation and the subsequent environmental challenges. Furthermore, this chapter examines the current understanding of the effects of microplastics on symbiotic relationships and assesses knowledge gaps in the influence of microplastics on freshwater organismal interactions. Finally, this chapter will define the specific research questions and hypotheses driving the experiment while also delineating the scope and limitations of the research presented.

Plastic pollution stands as an undeniable ecological and societal threat (Law et al. 2020, Macleod et al. 2021). On a global scale, an estimated 79% of total plastic production has been landfilled or mismanaged, thus infiltrating the environment (Geyer et al. 2017). As of 2016, the annual influx of plastic waste entering aquatic and terrestrial systems ranged from 9 to 25 million metric tons (MMT) (Borelle et al. 2020, Lau et al. 2020, and MacLeod et al. 2021). Such plastic debris has been found in a wide variety of locations spanning deep sea sediments, mountain tops, open oceans, and even arctic snow (Barnes et al. 2009, Van Cauwenberghe et al. 2013, Bergmann et al. 2019). The widespread presence of plastic pollution is directly attributed to the low cost and manufacturing capability of this petroleum biproduct (Ostle et al. 2019). In fact, the

production of plastic has only increased over the last 70 years, plateauing at nearly 359 MMT in 2018 (Plastic Europe. 2019, Statista. 2020, and Okoffo et al. 2021).

The extent of plastic pollution has forced costly cleanup operations across a multitude of countries (Dauvergne, P. 2018). The most affected areas are that of small island states, most notably those found within the Indian Ocean (Burt et al. 2020 and Mathis et al 2022). Due to the presence of degraded plastic waste, fragments, and subsequent leachates, these island states also pose as a benchmark for ecosystem resilience and health, when faced with intensive toxicological pressures from plastic pollution (Gove et al. 2019, Asakura et al. 2004, Bejgarn et al. 2015, Li et al. 2016, Gregory et al. 1991, Wright et al. 2013, Cartraud et al. 2019). During the International Union for the Conservation of Nature (IUCN), French Minister Berangere Abba, was famously quoted saying, “If the world fails to act there will be more plastic in the ocean than fish by 2050” (World Economic Forum 2016).

As the severity and presence of this contaminant continues, concerns oriented around water scarcity, agricultural impacts, and long-lasting ecological consequences have garnered considerable attention within research institutions, industrial agriculture, and water management entities (Koelmans et al. 2019). Plastic waste not only poses as a formidable environmental pollutant but has garnered considerable attention as a potential societal health-risk. These initial concerns are now met with alarming findings, where miniscule plastic particulates are being found within human blood (Leslie et al. 2022), breast milk (Ragusa et al. 2022), as well as lung (Amato-Lourenco et al. 2021), and placenta tissue (Ragusa et al. 2021). To that end, plastic pollution poses a significant threat to not only societies around the globe, but ecosystems worldwide (Shen et al. 2020).

Economic Drivers of Plastic Pollution

The presence of plastics as a societal and environmental contaminant can be explained by its multiple applications within the global economy, spanning agricultural, commercial, industrial, and health care uses. However, while employed across these numerous economic sectors, their extensive applications in agriculture and commerce are particularly notable for explaining the prevalence of environmental plastic waste (Shen et al. 2020, Wu et al. 2020). For example, the presence of terrestrial plastic can be partly linked to advancements in agricultural techniques that utilize plastic-based tools and products for weed suppression, temperature manipulation, irrigation, and soil aeration (Duis et al. 2016). Plastics commonly used in agricultural settings can be improperly disposed of, eventually fragmenting into plastic particulates which further contaminate agricultural soils (Nizzetto et al. 2016).

Commercial fishing commonly uses plastic-based nets, ropes, buoys, and even cages that are used to catch and retrieve sellable products (Wu et al. 2020). As this equipment is used, it experiences natural degradation processes resulting in the proliferation of plastic particulates into the surround aquatic environment (Corcoran et al 2019). In both cases, the use of plastic equipment and techniques within the commercial and agricultural sectors, allow for the introduction of plastics waste into vital terrestrial and aquatic habitats.

The Interconnectivity of Plastic Pollution Across Biomes

Although plastic contamination within terrestrial habitats is a serious ecological and societal concern (Nizzetto et al. 2016, Van den Berg et al. 2020, Leifheit et al. 2021), it's currently estimated 80% of aquatic plastic debris originates from land-based sources and is now prevalent in both oceanic and freshwater environments around the globe (Mendenhall et al 2018, Ostle et

al. 2019). Land-based plastics can find its way into aquatic systems through storm events that can wash loose plastic debris from soil, roads, and drainage systems into the closest body of water, in the form of runoff (Han et al. 2022, Beni et al. 2023). Despite current mitigation practices, aquatic ecosystems have accumulated millions of metric tons (MMT) of plastic pollution (Mihai et al. 2022). However, aquatic ecosystems as a term, encompass both freshwater and marine environments which experience plastic pollution in different forms and scales.

In 2010, an assessment incorporating 192 coastal countries estimated 4.8 to 12.7 MMT of plastic debris infiltrated the ocean (Jambeck et al. 2015). Only six years later, in 2016, this amount nearly doubled, as approximately 19 to 23 MMT of additional plastic waste entered the same oceanic habitats (Borrelle et al. 2020). Although these oceanic concentrations are alarming, it is argued due to the proximity of anthropogenic development, concentrations of environmental contaminants are far worse in freshwater systems than that of marine (Nava et al. 2023). In fact, a recent study suggests a conglomeration of rivers and streams around the world have contributed 80% of the plastic pollution that has entered the ocean (Meijer et al. 2021).

The heightened concentration of plastic pollution within freshwater systems is likely due to the proximity to human development and activities (Li et al. 2020, Nava et al. 2023). For instance, plastic debris can permeate into freshwater systems through storm water infrastructure, water treatment plants, as well as industrial and agricultural endeavors (van der Wal et al. 2015, Horton et al. 2017a, Horton et al. 2017b). Relative proximity to anthropogenic inputs, such as these, have been shown to magnify the concentration of plastic litter in freshwater environments (Li et al. 2020).

Although our understanding of plastic fate and flux within freshwater networks is in its infancy, we now know, freshwater ecosystems act as delivery systems for terrestrially derived

plastic debris to marine ecosystems (Meijer et al. 2021). Moreover, it is theorized that riverine catchments not only serve as the main source of aquatic plastic influx but may act as a natural environmental entanglement (Free et al. 2014, Jiang et al. 2019). To summarize, as run-off plastic debris enters freshwater systems, this movement is commonly hindered by the natural hydrology and vegetation trapping the plastic litter (Meijer et al. 2021). In these scenarios, the accumulation of debris will continue to build overtime, until the next storm event raises water levels resulting in a downstream surge of plastic, which will eventually reach the ocean (Lebreton et al. 2017, Meijer et al. 2021). Based on the riverine emissions observed in these studies, freshwater systems will continue to transport terrestrially based plastic pollution into our oceans for the foreseeable future. However, until mitigation and waste-management practices adequately evolve, freshwater ecosystems will continue to be inundated with anthropogenically-sourced plastic.

Despite the push for ecological conservation, humanity faces a societal and environmental challenge on its horizon. Not only is this pollutant incredibly costly, across a multitude of ecological and societal margins, but as more time passes the ability to effectively mitigate plastic pollution becomes exponentially harder (Lebreton et al. 2019).

Types of Plastic Degradation: Mechanical, Chemical, and Microbial

The decreasing effectiveness of plastic pollution mitigation is due to the multitude of degradation processes that can occur to macroplastics, such as bags, bottles, and nets (Lebreton et al. 2019, Mihai et al. 2022). Despite the inherent resilience of plastic products, over time, the structural integrity begins to falter. Once exposed to environmental weathering, breakdown and fragmentation can be divided into biotic and abiotic processes (Zhang et al. 2021). As plastic debris permeates into surrounding landscapes, this litter undergoes three main means of physical

modification: mechanical, chemical, and microbial (Corcoran et al 2021). Typically, abiotic degradation occurs before biotic degradation, usually in the form of chemical or physical alterations (Zhang et al. 2021). These initial stressors are commonly represented through exposure to water, light, heat, and mechanical stress. In fact, it is well-established that these degradation processes occur prior to biotic degradation due to the inherently low bioavailability of plastic (Andrady 2015).

Mechanical Degradation

When found in a dynamic landscape, where movement is inherent, plastic debris experiences mechanical modification such as abrasion and erosion when kinetic interactions occur (Zhang et al. 2021). This type of degradation process can occur by multitudes of differing substrates and surfaces. Across both terrestrial and aquatic environments, common organic interactions include that of woody debris, sand, and shells (Zhang et al. 2021). These forms of degradation commonly result in the formation of uniform, rounded fragments with consistent surface texture weathering (Zbyszewski and Corcoran 2011). For instance, visual analyses using a Scanning Electron Microscope (SEM), show consistent grooves and conchoidal fractures throughout the surface of the plastic particle (Zbyszewski and Corcoran 2011, Cooper and Corcoran 2010, Wang et al. 2017, Zhou et al. 2018, Liu et al. 2020). Due to these consistently spaced grooves, such degradation patterns support claims that weathered plastic debris may act as a vector for pollutants, non-indigenous species, and even promote harmful algal distribution (Barnes et al. 2009).

Chemical Degradation

In addition to mechanical degradation, plastic waste can undergo chemical transformations when exposed to sunlight, such as photo-oxidation (Kalogerakis et al. 2017). In fact, photo-degradation is one of the most important degradation processes that initializes the breakdown of plastic debris (Zhang et al. 2021). This type of degradation is commonly caused by high and medium energy ultraviolet (UV) irradiation due to accessory chemical reactions (Liu et al. 2019). During this form of degradation, chemical bonds found within the polymer-chains are cleaved, subsequently destroying the interconnectedness of the plastic chemical lattice.

However, such degradation processes react with different polymer types in unique ways. These reactions are purely dependent on the chemical structure of the plastic in question. For instance, polyethylene (PE) is known to be resistant against UV degradation due to the lack of susceptible chemical linkages, while polyvinyl chloride (PVC) rapidly degrades when exposed to the same UV irradiation. (Zhang et al 2021). These susceptible linkages within the chemical structure are known as chromophores, or UV absorbent aspects found within the polymer (Tolinski et al. 2015). Across multiple plastic types (PE, PP, PVC), specific chemical properties found within the polymer act as chromophores that catalyze the structural breakdown when exposed to UV irradiation. These chemical properties range from the carbonyl groups found in the backbone of PE, the tertiary carbons integrated into the structure of polypropylene (PP), and the unsaturated carbon double bonds found in PVC (Weber et al. 201, Fairbrother et al. 2019). However, a recent study found that physical defects, such as impurities, manufacturing defects, or early forms of deterioration can also act as chromophores (Fairbrother et al. 2019). Once exposed, the synthesis of free radicals, chain scissions, and cross-linkages are common chemical alterations that occur during the degradation process (He et al. 2019, Su et el. 2019). With

enough time and exposure, the structural integrity of the plastic will become compromised resulting in more rapid fragmentation.

Microbial Degradation

Apart from abiotic degradation, plastic also undergoes biotic deterioration. Biotic degradation can include breakdown processes such as, chewing, digesting, or enveloping (Cadée 2002, Dawson et al. 2018, Jang et al. 2018, Porter et al. 2019, Cau et al. 2020, Mateos-Cardenas et al. 2020, 2022). Specifically, microbial envelopment is conducted by microorganisms that live off the bioavailable carbon found within or plastic polymer (Crawford and Quinn 2017, Tiwari et al. 2019). Over time microbes develop into an expansive biofilm that catalyzes the degradation of plastic's structural integrity (Andrady et al. 2017). The unique envelopment and degradation process have created a new ecological niche, wherein plastic debris acts as a conducive substrate for microorganism colonization while also serving as a carbon source for microbial production (Yuan et al. 2020). In fact, microbial-based remediation strategies suggest using bacterial and fungal strains to encourage controlled plastic breakdown. Both bacterial and fungal colonies can even alter chemical properties of the plastics, like functional group structures and hydrophobicity (Yuan et al. 2020).

However, smaller microorganisms cannot digest macromolecule polymers and commonly need to use extracellular enzymes to aid in the breakdown process (Chen et al. 2019). This initial resiliency of plastic is commonly why abiotic degradation precedes that of biotic (Andrady 2015). Abiotic degradation is known to produce smaller, more fragmented plastic debris that has been shown to catalyze the initiation and pace of biotic or microbial deterioration. For example, if plastic debris is consumed by a larger taxon, the physical breakdown induced by chewing will prime the bioavailability and increase the surface area of the subsequent plastic fragments for the

microbial and chemical based digestive degradation (Cadée 2002, Dawson et al. 2018, Cau et al. 2020, Mateos-Cardenas et al. 2020).

When are Plastic Particulates Deemed Microplastics?

When experienced in unison, these forms of weathering can cause the breakdown of robust plastic products, such as water bottles, plastic bags, as well as fishing and farming equipment into smaller more ubiquitous plastic particles (Arthur et al. 2009, Corcoran et al. 2009). Once these fragments reach a size of less than 5 millimeters in diameter, they are classified as microplastics (Thompson et al. 2004, Arthur et al. 2009). These microscopic particles can then be further classified as either *primary* or *secondary*. If categorized as primary, these plastic entities have been intentionally manufactured into minuscule size ($\leq 5\text{mm}$). Conversely, if a microplastic particle is considered secondary, this is the outcome of degradation where a previously larger piece of debris has progressively deteriorated over time.

Microplastics Prevalence in Freshwater Systems

Due to their small scale and ubiquitous nature, the concern of freshwater microplastic pollution has warranted considerable alarm, and with good reason. Mentioned previously, the collection or entanglement effect that occurs within freshwater networks, suggests freshwater environments harbor far more plastic pollution than that of marine ecosystems (Nava et al. 2023). In addition, due to the combination of abrasive interactions, ultra-violet and microbial exposure, it is believed freshwater systems hold the highest concentrations of microplastics within the water column and sediment than any other form of aquatic systems (Hurley, Woodward, Rockwell et al. 2018, Mani et al. 2015). However, despite these findings, microplastic research on freshwater ecosystems is still in its infancy, especially when compared

to that of marine environments. (Gallagher et al. 2016, Sadri and Thompson, 2014, Vendel et al., 2017). In fact, a bibliometric analysis of previous microplastic research found that 87% of cumulative scientific literature between 1980 and 2015 was focused on marine ecosystems, while only 13% was focused on freshwater ecosystems (Blettler et al. 2018). This knowledge gap poses a major concern, as freshwater ecosystems, which are among the most heavily polluted aquatic environments, and have only recently begun to receive scientific attention, despite their critical ecological vulnerability. (Wagner et al. 2014, Eerkes-Medrano et al. 2015, Lambert and Wagner 2018).

The Importance of Freshwater Ecosystems

Although freshwater environments cover less than 1% of global surface area, these ecosystems play a fundamental role in not ecological biodiversity but pivotal societal services. (Nyingi et al. 2013, Green et al. 2015, Kumari et al. 2022). Some of these services include water supply, water quality control, flood regulation, and carbon sequestration. Furthermore, freshwater ecological services provide erosion prevention, habitat conservation, soil fertility, groundwater recharge and nutrient cycling, just to name a few (Nyingi et al. 2013, Reid et al. 2020). The complexity and heterogeneity of freshwater environments allow a disproportionate amount of earth's biodiversity to inhabit these ecosystems. In fact, approximately 10% of earth's biodiversity inhabits these systems while only representing 0.01% of the earth's surface area (Reid et al. 2020). Despite the current focus of plastic pollution within marine ecosystems, freshwater habitats, and their subsequent plastic pollution, are home to a disproportionate amount of global biodiversity. Within such freshwater environments hide unique community and population dynamics, that help intertwine the expansive array of organismal diversity. On a global scale, the influence of these population and community dynamics, bolstered by the vast

diversity of earth's ecosystems, shapes the heterogeneity and complexity of earth's biosphere (Hom et al. 2021).

Organismal Fate and Effects When Exposed to Microplastics

As microplastic concentrations continue to rise within both freshwater and marine ecosystems, organismal ingestion, whether intentional or inadvertent, has been found to concurrently rise (Weiss et al., 2006, Lusher et al. 2013, Cauwenberghe and Janssen 2014, Lusher, A. 2015, Desforges et al. 2015). Organisms covering multiple trophic levels, such as herbivores, secondary consumers, and even predators have directly consumed or unknowingly ingested microplastics throughout their lifespan (Rochman et al. 2015, Naidu et al. 2018). In fact, predation has proven to be an effective form of inadvertent ingestion by means of trophic transfer (Naidu et al. 2018). Once consumed, this contaminant can induce neurotoxicity and genotoxicity, even hindering feeding and reproductive success (Zhang et al. 2019a). These toxicokinetic reactions could pose significant impacts not only on organismal survival, but population and community success (Lusher, A. 2015).

Of the aquatic organisms impacted by this emerging contaminant, filter feeders have been shown to be some of the most vulnerable. In fact, documented cases of filter feeders ingesting various particle sizes and shape warrants the concern of potential bioaccumulation and community-wide impact (Moore 2008, Eerkes-Medrana et al. 2015, Pedersen et al. 2020). Considering these findings, Pedersen et al. conducted an acute 24-hour exposure, where quagga mussel mortality, filtration rate, oxygen consumption, and accumulation were tracked when exposed to red HDPE microbeads. This polymer type was chosen due to its incorporation within water bottles and corrosive resistant piping (Pedersen et al. 2020). The study found that bioaccumulation was directly correlated with microplastic concentration, while oxygen

consumption was inversely proportional. Despite observed tissue damage, Quagga mortality nor spawning was affected by the exposure, corresponding with past studies (Von Moos et al. 2012, Watts et al. 2016).

Mentioned previously, due to the proximity of anthropogenic infrastructure concentrations of microplastics, among other environmental contaminants are far worse in freshwater systems (Nava et al. 2023). For instance, Chinese research institutions have found that freshwater systems throughout China harbor alarming concentrations of microplastics and biphenol compounds, commonly found in epoxy resins (Mu et al. 2022, Yan et al. 2017, Wang et al. 2013, Jin and Zhu 2016, Yamazaki et al. 2015). To assess the effect of the co-contaminant exposure experienced by freshwater taxa, an acute exposure and response toxicokinetic experiment was implemented on zebra fish embryos and larva (Mu et al. 2022). The study found that zebra fish embryos were shielded from the toxicological effects of this co-contaminant exposure, due to the protective barrier provided by the chorion, while larva exhibited a toxicological response (Mu et al. 2022).

A similar study was conducted to measure the toxicological impact on *Daphnia magna* when co-exposed to microplastic fibers as well as silver nanoparticles (AgNPs) and silver nitrate (AgNO_3) (Tourinho et al. 2021). Throughout the study, physical immobilization and cellular energy allocation (CEA) were tracked to assess the impact of these contaminants. After a standard 48-hour exposure, microplastic fibers showed no impact on immobilization, CEA, or mortality. However, *Daphnia* mortality was affected after increasing exposure length beyond 72-hours. These findings support previous studies where *Daphnia* mortality was unaltered after the standard 48-hour exposure, but toxicity increased with prolonged exposure (Rehse et al. 2016, Jemec et al. 2016, Horton et al. 2018, Kokalj et al. 2018, 2019, Eltemsah and Bohn 2019, Tourinho et al. 2021). Although no evidence of acute toxicity was observed when co-exposed to

microplastics, AgNPs, and AgNO₃, the subcellular impacts found in the study could potentially be explained by the considerable amounts of microplastic fibers ingested. In fact, the digestion of microplastics has been attributed to a significant proportion of the adverse impacts experienced by aquatic organisms (Canniff and Hoang 2018, Cole et al. 2013, Tourinho et al. 2012).

Before entering a marine environment, organisms and pollutants transition into an estuary. These brackish environments may act as sinks for riverine plastic debris (Lopez et al. 2021). Commonly found within estuaries, grass shrimp heavily occupy vegetated areas of these habitats (Gray and Weinstein 2017). Considering the abundance and ubiquity of microplastics within these inhabitants, Gray and Weinstein conducted an acute exposure assay, followed by a 96-hour depuration period. The grass shrimp were exposed to differing size fractions of plastic microbeads, fragments, and fibers to better understand residence time within the gut and gill chambers (Gray and Weinstein 2017). The use of grass shrimp in the study provided valuable ecological insight due to their inherent sensitivity to alternative contaminants (Nimmo et al. 1977, Key et al. 1998, Spehar et al. 1999, DeLorenzo et al. 2006, Key et al. 2006, 2008, Yonkos et al. 2014). Although the residence and depuration times varied, the most interesting finding was the heightened mortality rate, primarily caused by fibrous plastic particles. Specifically, fibers of <50 µm imposed the most notable toxicological effect. Past studies have found that ingested fibers may cause gastrointestinal entanglement, tissue damage, and even death (Nimmo et al. 1977). In fact, similar toxicological responses when exposed to microplastic fibers have been documented in lobsters, amphipods, and *Daphnia* (Au et al. 2015, Jemec et al. 2016, Murray et al. 2011).

Due to microplastics toxicological effects compounded by its ubiquitous nature, this contaminant could impose detrimental stress on the trophic pyramid affecting not only other

organisms, but the interactions they facilitate. Until recently, studies focusing on the toxicological impact of microplastics have primarily been focused on the individual taxa, rather than the interactions they facilitate (Nizzetto et al. 2016, Lamb et al. 2018, van den Berg et al. 2020, Leifheit et al. 2021). This knowledge gap creates an intellectual blind spot as some organismal partnerships have been shown to increase organismal adaptability and survivability during period of stress and disturbance (Li et al. 2006, Maher et al. 2020, Prada et al. 2022, Chen et al. 2023).

Symbiosis

One of the most fascinating, naturally occurring, evolutionary adaptations is that of symbiotic relationships. These relationships can be found throughout every biome within the ecosphere of Earth. Spanning from organismal, molecular, and even cellular scales, these relationships are common in nature, and are at the core of all eukaryotic life (Sapp et al. 2016). The organisms that partake in this survival strategy have evolved mechanisms to exploit other organisms sub-lethally, to ultimately increase their fitness and survival (Boucher et al. 1982).

Symbiosis is an overarching concept that can be broken down into the four distinct symbiotic interactions; mutualism, commensalism, parasitism, and amensalism (Beckrich et al. 2015). These interactions can range from mutually beneficial or antagonistic depending on the cost and benefit felt by each taxon (Ewald 1987, Bronstein 1994). Moreover, biotic and abiotic factors within the environment can play an integral role in determining the type of organismal relationship (Bronstein 1994). In fact, these interactions, such as symbiosis, lie on a continuum where such factors can shift the context dependency of these interactions (Silknetter et al. 2020).

Symbiosis as an evolutionary adaptation is believed to have significant influence on the distribution of species across the planet (Douglas 2010, Chomicki et al. 2019, 2022). On a global

scale, symbiosis is most understood to have influenced biodiversity by bolstering metabolic capabilities between interacting organisms. Such increases of productivity are seen in deep ocean settings, where inhabitants benefit from the microbial communities increasing access to community-wide energy (Osman and Weinnig et al. 2022). On an evolutionary timescale, the adaptation of symbiosis is credited with mediating the creation of organs, and subsequent organ systems, allowing taxa to explore novel habitats (Douglas et al. 2020, Dimijian G.G., 2000) As the adaptation of symbiosis continued, host life cycles became increasingly intertwined, and in some cases, dependent. Across multitudes of species, organismal development and lifecycles became increasingly intertwined by symbiotic interactions (Sieber et al. 2021). In fact, some marine species often completely rely on symbiotic relationships during larval development. But most importantly, symbiosis has been credited with influencing global biodiversity, as increased resiliency is experienced by the beneficiary during periods of stress or disturbance (Prada et al. 2022).

The Impact of Microplastics on Symbiosis

A recent study tested the resiliency of dinoflagellate coral symbionts during high and low disturbance events (Burmester et al. 2017). Environmental disturbances were represented by mechanical damage, such as lesions and breaks, paired with varying ranges of increased water temperature. The coral structures that contained a full symbiont community had a 2.4 times higher probability to initiate natural healing processes, and a 5.8 times higher likelihood to fully complete that healing process. The same study was repeated, across three temperature gradients of increasing water temperature. Coral with symbionts had a 3.2 times higher likelihood of completing the healing process than that of coral without (Burmester et al. 2017).

In terrestrial systems, a well-known symbiosis that bolsters plant resiliency is that of the mycorrhizal symbiosis. This ancient fungal symbiotic relationship is credited with the expansion and evolution of woody plants around the world (Bueno et al. 2022). In fact, mycorrhizal fungi are known to create complex underground systems where the exchange of nutrients, water, and even antimicrobial effects can increase the overall health and adaptability of woodland ecosystems (Bueno et al. 2022). Furthermore, its estimated 340,000 species of vascular plants rely on this fungal relationship due to the inherent resiliency this partnership provides (Genre et al. 2020). The benefits of this terrestrial symbiosis are so well-known the implementation of mycorrhizal fungi has become a common practice within agricultural efforts. In fact, when mycorrhizal fungi are supplemented within crop plots, a dramatic increase of phosphorous uptake is observed. A recent study determined that experimental crops showed an increase in root and shoot biomass, as phosphorous uptake exceeded normal amounts by 50% (Li et al. 2006, Chen et al. 2023). Just as the mycorrhizal symbiosis increases overall stability and growth of terrestrial plants, the intricate relationship between dinoflagellates and coral reefs have shown resiliency when faced with climatic and anthropogenic stress (Maher et al. 2020). Despite both examples of symbiosis spanning across completely different environments, the influence of host and symbiont relationship impose cascading effects within the surrounding environment. Ultimately, these relationships have been documented to increase overall adaptability to external stressors (Maher et al. 2020). However, how resilient are symbiotic interactions when faced with an anthropogenic pollutant such as microplastics?

Although the dinoflagellate-coral symbiosis showed impressive robustness when subjected to mechanical and environmental stressors, an estimated 11 billion plastic items are currently entangled around coral structures throughout the Asia-Pacific (Lamb et al. 2018). As

coral structures become littered by plastic debris, the likelihood of diseases increase 20-fold. Once enveloped by plastic, multiple stressors in the form of light deprivation, anoxia, and even toxin-release begin to set in (Lamb et al. 2018). In fact, in some cases, microplastic fragments have even been documented within the gastric cavity and mesenterial tissues, further stressing the coral-dinoflagellate relationship. On land, due to plastic-based agricultural techniques, microplastic contamination continues to encroach on the mycorrhizal-plant symbiosis (Nizzetto et al. 2016, van den Berg et al. 2020, Leifheit et al. 2021). As plastic fragments find themselves within these exposed environments, fragmentation becomes inevitable. Over time, these plastic particles begin leaching breakdown-products, or chemicals utilized during the initial manufacturing process. For instance, dodecanal, an intermediate chemical used in the manufacturing process of plastics, has been shown to accumulate within the rhizosphere of the plant-fungi symbiosis, negatively impacting overall plant and fungal growth (Qi et al. 2020). With microplastic contamination predicted to only worsen, commercial agricultural entities are desperately searching for potential alternatives and remediation strategies (Qi et al. 2020).

Despite these examples representing very different ecosystems, they both show how these relationships react when exposed to microplastics contamination. These examples continue the overarching discussion of microplastic toxicity and the impact on symbiotic partnerships. Although the effects of microplastics on symbiotic interactions have been addressed in marine and terrestrial habitats, no empirical research has been conducted to understand the effect of MPs on a freshwater symbiosis. This knowledge gap is what makes my experiment truly exciting as it will be the first scientific endeavor to assess the impact of microplastics on a freshwater symbiosis. The relationship in question is known as the crayfish-branchiobdellidan cleaning symbiosis and has previously been used in multiple empirical studies (Brown et al. 2002, Brown

and Creed 2004, Lee et al. 2009, Brown et al. 2012, Skelton et al. 2013, Farrell et al. 2014, Skelton et al. 2014, Creed et al. 2015, Thomas et al. 2016, Skelton et al. 2017, Creed and Brown 2018, Creed et al. 2021, Creed et al. 2022). Not only does my study system facilitate a symbiotic relationship, but the host of this relationship fills the ecological roles of an ecosystem engineer and a keystone species (Creed 1994, Creed and Reed 2004, Usio and Townsend 2004). Given the severity of microplastic contamination in freshwater habitats, the environmental relevance of our study system stands to further the understanding of the impact of microplastics in freshwater ecosystems (Mani et al. 2015, Horton et al. 2017a, Horton et al. 2017b, Hurley, Woodward, and Rothwell et al. 2018, Nava et al. 2023).

The Crayfish-Branchiobdellida Symbiosis

The crayfish-branchiobdellidan symbiosis is a freshwater relationship that can influence its surrounding habitat while also maintaining experimental amenability. Crayfish not only facilitate a symbiotic relationship but fill the ecological role of ecosystem engineer and keystone species in many aquatic systems. (Creed 1994, Creed and Reed 2004, Usio and Townsend 2004). As a generalist omnivore, crayfish commonly impose downward trophic pressures on primary and secondary producers found in freshwater environments (Lodge et al. 1994, Perry et al. 2000, Twardochleb et al. 2013). Crayfish are regarded as ecosystem engineers, due to their influence on leaf-litter breakdown and nutrient cycling within stream systems (Charlebois and Lamberti 1996, Bobeldyk and Lamberti 2008). On a community-wide scale, the abundance of invertebrates, amphibians, algal and macrophyte species are heavily influenced by crayfish predation pressures (Gamradt and Kats 1996, Gamradt et al. 1997). Additionally, the presence of this taxa influences freshwater biogeochemistry and organismal abundance. Previous studies found that symbionts can have a significant indirect effect on freshwater communities if they affect taxa that function

as keystone species or ecosystem engineers (Creed et al. 2020). Mentioned previously, crayfish fill both of these ecological roles (Creed 1994, Creed and Reed 2004, Usio and Townsend 2004).

Many species of crayfish are engaged in symbiotic interactions with multiple species of branchiobdellidan annelids that rely indefinitely on the crayfish host for a microhabitat to feed, develop, and reproduce (Young 1966, Brinkhurst and Gelder 2001, Creed et al. 2015, Holt 1964, Gelder 1991, Gelder and Hall 1990, and Brown and Creed 2004). Classified in the Phylum: Annelida, Class: Clitellata, and Order: Branchiobdellida, these worms are a sister taxon to leeches (Phillips et al. 2020). In terms of range, branchiobdellidan worms, are holartically distributed and are known to span from Costa Rica to Southern Canada within the western hemisphere (Holt 1964, Gelder 1991, Gelder and Hall 1990).

Previous research focusing on the crayfish-branchiobdellidan symbiosis has found that this relationship is a context-dependent symbiosis that can range from a mutualistic cleaning interaction to a commensal relationship, and even a parasitism (Brown et al. 2002, Lee et al. 2009, Brown et al. 2012). The context of this organismal interaction depends on the species of both host and symbiont, as well as other biotic and abiotic factors such as symbiont density and environmental variability (Young 1966, Bishop 1968, Keller 1992, Rosewarne et al. 2012, Brown et al. 2002, Lee et al. 2009, Brown et al. 2012, Chamberlain, Bronstein, & Rudgers, 2014).

As a cleaning symbiosis, the branchiobdellidan worms commonly graze on ambient organic matter gathered on the crayfish hosts exoskeleton, or even material fouling the gills. In fact, the beneficial response of this cleaning symbiosis is presumed to be the result of increased gas exchange and nitrogen excretion rates across the gill epithelial tissue (Jennings and Gelder 1979, Brown et al. 2002, Creed et al. In Review). In an experimental setting, environmental

fouling was manipulated to elicit possible changes in the species interaction. At low fouling levels, the crayfish-branchiobdellida symbiosis shifted to commensalism where the annelids were the sole beneficiary (Lee, Kim, & Choe, 2009). However, at high fouling levels, the context of this relationship shifted to mutualism as the cleaning symbiosis elicited a mutually beneficial effect (Lee et al. 2009). Additionally, the abiotic context of high fouling levels also increased the reproductivity rates of the branchiobdellidan worms (Thomas, Creed, Brown et al. 2013).

When symbiont densities reach a moderate level, crayfish experience heightened growth and improved survivability compared to those without symbionts (Brown et al. 2002). In a 58-day study, the impact of varying symbiont densities on crayfish growth and mortality was examined, revealing that intermediate worm densities correlated with increased growth and decreased mortality rates (Brown et al. 2002). Additionally, if this mutualistic context persists, this facilitation has even been documented to significantly downregulate immune-related genes within the crayfish host (Brown et al. 2016).

If the environmental context shifts to a point at which resources are limited, and branchiobdellidan densities are high, a mutualistic or commensal symbiosis can shift to parasitism as the branchiobdellidan worms will turn to the host to satisfy metabolic needs (Brown et al. 2012, Creed and Brown et al. 2018). These antagonistic behaviors have been documented to take the form of gill tissue and hemolymph consumption, resulting in gill scarring (Creed and Brown et al. 2018). At high levels, gill scarring has been observed to significantly impact crayfish energetic reserves and gas exchange, subsequently affecting physical development (Brown et al. 2012). However, a contradictory study suggests the opposite, as parasitic behavior, in the form of gill scarring, resulted in no net reduction in crayfish development (Thomas et al 2016).

If the context of a symbiosis is antagonistic, hosts often take action that can control for this over-exploitation. In the crayfish-branchiobdellidan symbiosis, crayfish use grooming behaviors to maintain symbiont densities before they become parasitic (Farrell et al. 2014, Skelton et al. 2014). In fact, across differing sizes of crayfish hosts this grooming strategy was seen to influence overall symbiont density. Moreover, the grooming effectiveness of smaller crayfish hosts were notably focused on larger symbiont species like *Cambarincola ingens*, when compared to that of the smaller symbiont species like, *Cambarincola philadelphicus*. This specificity of grooming is most likely due to the size and metabolic demand of the larger species of worms, resulting in the increased grooming susceptibility. On the other hand, when this trend of grooming preference is compared to the larger crayfish hosts, the population decline of *Cambarincola philadelphicus* can be attributed to the intraguild predation imposed by *Cambarincola ingens*, rather than grooming itself. Overall, the microhabitat of crayfish harbors an intricate dual control by crayfish grooming and succession of different species of branchiobdellidan worms, dictated by a competition-colonization tradeoff. Such mechanisms are known to influence symbiont heterogeneity and composition within freshwater systems. However, if effective grooming strategies fail, the annelid symbionts will naturally turn to gill tissue for sustenance (Farrell et al. 2014, Skelton et al. 2014).

It is well known that crayfish are omnivores that prey on lower-level producers and consumers, within a given habitat. This symbiosis can impose direct effects on crayfish predation pressures, through imposed parasitic interactions. Furthermore, the ecological influence of bioturbation acts as an additional influence within ecosystem composition and abundance. Despite the notable environmental manipulation crayfish impose on their surrounding habitat, they still represent prey species to higher trophic level predators. This aspect is essential when

considering the potential trophic transfer crayfish could facilitate as vectors of microplastic contamination.

A previous study found that branchiobdellidan density on crayfish hosts can significantly influence macroinvertebrate abundance and overall community structure (Creed et al. 2021, Brown et al. 2012). Across symbiont densities ranging from zero-worms to twelve-worms, it was the crayfish host with moderate symbiont densities that had overall greater growth and impact to sediment volume and macroinvertebrate abundance. However, more importantly, it was the twelve-worm treatment that resulted in the lowest macroinvertebrate abundance and sediment volume. A likely reason for the impact on the surrounding environment is due to the parasitic behaviors imposed by the symbionts. High levels of gill scarring were observed within this treatment group, which is believed to highly impact crayfish energetic reserves (Brown et al. 2012). Lack of such reserves resulted in increased sediment displacement, as predation demands heighten to maintain metabolic needs. Naturally, due to this parasitic effect, the ambient macroinvertebrate abundance began to drop due to increased predation. Notably, this environmental influence, induced by the crayfish-branchiobdellidan symbiosis, was documented across two, separate freshwater systems proving the replicability of these symbiotic effects on ecological dynamics (Clemson, SC and Boone, NC) (Creed et al. 2021).

Given the amenability to laboratory monitoring and manipulation, as well as the ecological importance and influence of this taxon, the crayfish-branchiobdellidan symbiosis is a viable study-system to observe microplastic toxicity on freshwater symbiosis and the potential community-wide impacts.

Summary

Environmental habits around the globe are now inundated with plastic pollution, due to surges in infrastructural development and advancement (van der Wal et al. 2015, Gonzalez et al. 2016, Horton et al. 2017a, Horton et al. 2017b). The mitigation of plastic waste, as an environmental contaminant, has become increasingly more difficult as degradation processes proliferate miniscule plastic particulates, known as microplastics, throughout our environment (Lebreton et al. 2019, Mihai et al. 2022). Due to their miniscule size, microplastics are now ubiquitous throughout ecosystems around the globe and are even found within organisms through the process of bioaccumulation (Weiss et al. 2006, Arthur et al. 2009, Corcoran et al. 2009, Lusher et al. 2013, Cauwenberghe and Janssen 2014, Desforges et al. 2015, GESAMP 2016, Corcoran et al 2020, Linggi et al. 2021, Hao et al. 2022).

Although the field of microplastic research continues to broaden in terrestrial and marine setting, similar research within freshwater settings is still in its infancy (Sadri and Thompson 2014, Eerkes-Medrano et al. 2015, Wagner et al. 2014, Gallagher et al. 2016, Vendel et al. 2017, Li et al., 2020, Meijer et al. 2021, Nava et al. 2023). This knowledge gap poses a serious concern, as freshwater systems are understood to have higher rates of plastic pollution due to the proximity of anthropogenic infrastructure (Mani et al. 2015, Hurley and Woodward et al. 2018, Li et al. 2020). Moreover, due to the natural hydrology and vegetation found in these habitats, plastic debris become entangled, hindering downstream movement until the next storm event (Free et al. 2014, Jiang et al. 2019, Meijer et al. 2021). Due to this entanglement effect, plastic debris experiences a heightened intensity of degradation processes, which is why freshwater habitats are known to harbor more microplastics than any other aquatic habitat (Blettler et al. 2018).

Despite the severity of this emerging contaminant, microplastic research in freshwater ecosystems is still in its infancy (Sadri and Thompson 2014, Eerkes-Medrano et al. 2015, Wagner et al. 2014, Gallagher et al. 2016, Vendel et al. 2017, Li et al. 2020, Meijer et al. 2021, Nava et al. 2023). My thesis aims to not only assess the toxicokinetic impact of microplastics on freshwater organisms, but more importantly, the organismal interactions facilitated by freshwater taxa. Only focusing on the toxicological impact of an individual organism turns a blind eye on the community interactions that maintain community structure and ecosystems processes. As such, my experiment focuses on a freshwater symbiotic interaction found throughout freshwater ecosystems around the globe.

Symbiosis as an evolutionary adaptation has granted countless species a metabolic advantage, increasing the fitness and adaptability of species worldwide (Zook, D.P. 2001, Prada et al. 2022). Furthermore, symbiosis spans across marine, terrestrial, and freshwater habitats, but also spans physical scales from organismal to cellular (Douglas 2010, Sapp et al. 2016, Chomicki et al. 2019, 2022). Thus, the intellectual progression of how microplastic toxicity may affect symbiosis warrants increased exploration. Moreover, there is currently no previous empirical studies that have assessed the influence of microplastics on a freshwater symbiosis.

As such, we used the crayfish-branchiobdellidan cleaning symbiosis to investigate the influence of microplastics by conducting a 180-day standardized chronic exposure assay. Thirty-six crayfish replicates, with varying symbiont densities, were exposed to microplastics (MPs) to assess the influence on host physical growth, symbiont behavior, and organismal survivability. Microplastics used within the experiment mimicked environmentally relevant levels as well as morphologies and polymers commonly found in freshwater habitats (Buss et al. 2022)

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Chapter 2

Symbiotic Shift: Microplastics Induce Parasitism in a Freshwater Symbiosis

Abstract

Annually, it is estimated that 82 million tons of global plastic waste is either mismanaged or littered, bypassing waste management practices. This mismanagement causes the permeation of plastic debris into the environment, which then undergoes natural degradation processes. These degradation processes result in the proliferation of miniscule plastic particles known as microplastics. Due to the inherent proximity to sources of anthropogenic waste, concerns of microplastic pollution and its impact on freshwater ecosystems have recently increased. Until recently, microplastic research has primarily been focused on the toxicological effects on an individual organism rather than the interactions that occur between taxa. Only focusing on the toxicological impact experienced by an individual organism turns a blind eye to the communities that maintain ecosystem health and stability. To that end, our experiment is the first study to assess the impact of microplastics on a freshwater symbiosis. Until recently, symbioses in the scope of toxicokinetic studies have primarily been dominated by that of terrestrial and marine relationships. This knowledge gap poses a concern as previous literature suggests freshwater systems are more contaminated than that of any other aquatic habitat. To address this knowledge gap, we conducted mesocosm-based exposure-response assays, exposing a crayfish-branchiobdellidan symbiosis to microplastics while varying symbiont densities. We used the crayfish-branchiobdellidan model system in our study due to its amenability to laboratory monitoring and manipulation. The crayfish *Cambarus appalachiensis*, common in the New River Basin of Virginia, USA, served as host to obligate ectosymbiotic annelids in the order Branchiobdellida. Previous research using the crayfish-branchiobdellidan symbiosis demonstrated that the interaction is a cleaning symbiosis, where hosts benefit from reduced gill fouling while symbionts benefit from increased resource availability. We observed the physical and behavioral changes of the crayfish-branchiobdellidan symbiosis over a 172-day chronic exposure assay. Our results show that crayfish hosts with higher symbiont densities experienced decreased physical growth when exposed to microplastics compared to the control. This alteration in host growth was the result of increased antagonistic symbiont behavior in the form of gill tissue consumption. Our results suggest microplastics caused a reduction in epibiont abundance, thus decreasing symbiotic resource availability. This reduction in resources resulted in a shift in context dependency, resulting in increased parasitic symbiont behavior. This study demonstrates as freshwater ecosystems continue to be inundated with plastic pollution, the presence of microplastics have the capability to shift symbiotic context from a mutualism to a parasitism.

Introduction

Despite current waste-management practices, terrestrial and aquatic ecosystems have been inundated with millions of metric tons (MMT) of plastic pollution (Mihai et al. 2022). Although plastic contamination within terrestrial habitats poses as a serious ecological concern (Nizzetto et al. 2016, Van den Berg et al. 2020, Leifheit et al. 2021), it is estimated 80% of aquatic plastic debris originates from land-based sources and is now prevalent in both oceanic and freshwater environments around the globe (Mendenhall et al 2018, Ostle et al. 2019).

As plastic debris permeates into these aquatic environments, degradation processes cause the breakdown of this waste, such as grocery bags, water bottles, and fishing nets into smaller, more ubiquitous plastic particles known as microplastics. (Arthur et al. 2009, Corcoran et al. 2009, GESAMP 2016, Corcoran et al 2021). These plastic fragments are classified as microplastics once they reach a diameter of less than 5 millimeters. (Arthur et al. 2009).

Due to the proximity of municipal, industrial, and agricultural infrastructure, freshwater systems are now understood to contain the highest concentration of microplastics than any other form of aquatic systems (Mani et al. 2015, Hurley, Woodward, and Ruthwell et al. 2018, Li et al. 2020). At an ecosystem level, the understanding of microplastics' impact on freshwater organisms and ecosystem dynamics is still in its beginning stages compared to that of marine environments (Wagner et al. 2014, Eerkes-Medrano et al. 2015, Meijer et al. 2021). In fact, a bibliometric analysis of previous microplastic research found that 87% of cumulative scientific literature between 1980 and 2015 was focused on marine ecosystems, while only 13% was focused on freshwater ecosystems (Blettler et al. 2018). Moreover, microplastic research has primarily been focused on the toxicological affects felt by an individual organism, rather than the intricate interactions that occur between these organisms. Both knowledge gaps pose as major

ecological concerns as freshwater ecosystems, which harbor a disproportionate amount of biodiversity compared to their size, are among the most heavily polluted aquatic environments in the world (Nyingi et al. 2013, Wagner et al. 2014, Erkes-Medrano et al. 2015, Reid et al. 2020, Kumari and Sarika et al. 2022).

To assess this knowledge gap, our experiment focuses on symbiosis, an interaction known to bolster environmental and organismal adaptation. (Dimijian G.G. 2000, Li et al. 2006, Douglas et al. 2020, Maher et al. 2020, Sieber et al. 2021, Prada et al. 2022, Chen et al. 2023). Symbiosis, as an organismal interaction, is known as a close, prolonged association between two different biological species, where the type of relationship depends on the cost and benefit felt by both organisms. Symbiosis occurs in all eukaryotic organisms and can be found in every biome on earth (Sapp et al. 2016). Moreover, symbiosis has been credited with increasing organismal adaptation and survivability during periods of stress and disturbance (Li et al. 2006, Maher et al. 2020, Prada et al. 2022, Chen et al. 2023). However, recent studies suggest microplastics, as an emerging contaminant, can cause notable negative effects to not only the individual organism, but the symbiotic relationships they support (Nizzetto et al. 2016, Lamb et al. 2018, van den Berg et al. 2020, Leifheit et al. 2021). Although previous research has addressed the effects of microplastics on symbiotic interactions in marine and terrestrial habitats, no empirical research has been conducted to understand the effect of microplastics on a freshwater symbiosis. To address this knowledge gap, we studied a freshwater cleaning symbiosis consisting of crayfish and their branchiobdellidan worms, to assess the effects of microplastics on a freshwater symbiosis.

Crayfish participate in a symbiotic relationship with annelids in the order Branchiobdellida, commonly referred to as crayfish worms (Holt 1964, Gelder 1991, Gelder and

Hall 1990, Brown and Creed 2004, Lee et al. 2009, Brown et al. 2012, Creed et al. 2020, 2021). For many studied species, this partnership is a cleaning symbiosis in which the worms graze on organic matter and epibionts adhered to the hosts exoskeleton and gills (Jennings and Gelder 1979, Lee et al. 2009, Gale and Proctor 2011, Farrell et al. 2012). Removal or dislodging of the material found within the gill chamber has proven to have positive effects to crayfish growth and survivability (Brown et al. 2002, Lee et al. 2009, Brown et al. 2020). This beneficial physiological response is presumed to be the result of increased gas exchange and nitrogen excretion rates across the gill epithelial tissue (Jennings and Gelder 1979, Brown et al. 2002, Creed et al. In Review). Unlike many other cleaning symbioses, most of these annelids live directly on the host and rely indefinitely on the crayfish to feed, develop, and reproduce (Young 1966, Brinkhurst and Gelder 2001, Creed et al. 2015).

However, species interactions, such as the crayfish-branchiobdellidan cleaning symbiosis, lie on a natural continuum where external influences can shift the context of this interaction. Depending on symbiont density, host sex, and environmental conditions, this symbiosis can shift between mutualism, commensalism, and parasitism (Berry and Holt 1959, Young 1966, Bishop 1968, Keller 1992, Lee et al. 2009, Brown et al. 2002, 2012, Rosewarne et al. 2012, Skelton et al. 2013, Farrell et al. 2014). In an experimental setting, environmental fouling was manipulated to elicit possible changes in the crayfish-branchiobdellidan interaction. At low fouling levels, the crayfish-branchiobdellidan symbiosis shifted to commensalism where the annelids were the sole beneficiary (Lee et al. 2009). However, at high fouling levels, the context of this relationship shifted to a mutualism as the cleaning symbiosis elicited a mutually beneficial effect. (Lee et al. 2009). In this example, the mutualistic context benefitted both partners by removing material fouling the gills of the host, while also increasing resource availability to the symbionts (Thomas

et al. 2013). However, If the environmental context shifts to a point at which resources are limited, and branchiobdellidan densities are high, a mutualistic or commensal symbiosis can shift to parasitism as the branchiobdellidan worms will consume gill tissue and hemolymph to satisfy energetic needs (Brown et al. 2012, Creed and Brown et al. 2018). This antagonistic behavior results in gill scarring, which has been determined to significantly impact crayfish energetic reserves and subsequent physical development (Brown et al. 2012, Creed and Brown et al. 2018).

In most context-dependent symbioses, hosts are able to exert some control over symbiont density or activity (Berry and Holt 1959, Young 1966, Bishop 1968, Keller 1992, Lee et al. 2009, Brown et al. 2002, 2012, Rosewarne et al. 2012, Skelton et al. 2013). Crayfish use a grooming strategy to maintain symbiont densities at lower levels, before they become parasitic (Farrell et al. 2014, Skelton et al. 2014). If these grooming strategies fail, the symbiont density will naturally rise, thereby increasing energetic demands and subsequent antagonistic behavior of symbionts (Brown et al. 2012). Across differing sizes of crayfish, this grooming strategy was seen to influence overall symbiont colonization dynamics (Farrell et al. 2014, Skelton et al. 2014, Thomas et al. 2016). The grooming effectiveness of smaller crayfish hosts were notably focused on larger symbiont species like *C. ingens*, when compared to that of a smaller symbiont species, *C. philadelphicus* (Thomas et al. 2016). This specificity of grooming is most likely due to the size and metabolic demand of the larger species of worms, resulting in the increased grooming susceptibility (Thomas et al. 2016). On the other hand, when this trend of grooming preference is compared to the larger crayfish hosts, the population decline of *C. philadelphicus* can be attributed to the intraguild predation imposed by *C. ingens*, rather than grooming itself (Thomas et al. 2016). Overall, the microhabitat of crayfish harbors an intricate dual control by crayfish grooming and succession of different species of branchiobdellidan worms, dictated by a

competition-colonization tradeoff. Such mechanisms are known to influence symbiont heterogeneity and composition within freshwater systems (Skelton et al. 2014, Thomas et al. 2016).

Due to their impact on leaf-litter breakdown, nutrient cycling, and sediment accumulation within stream systems, crayfish function as both ecosystem engineers and keystone species. Because crayfish assume both ecological roles, branchiobdellidan worms have the capability to induce indirect effects on their surrounding environment (Creed 1994, Creed and Reed 2004, Usio and Townsend 2004, Charlebois and Lamberti 1996, Bobeldyk and Lamberti 2008). In fact, the composition and density of symbionts found on the crayfish host can have considerable indirect effects on sediment accumulation, macroinvertebrate abundance, and even freshwater community structure (Brown et al. 2012, Creed et al. 2021).

Due to the ecological importance, experimental amenability, and plasticity of the symbiotic interaction, this freshwater symbiosis has been utilized in multiple ecological studies (Brown et al. 2002, Brown and Creed 2004, Lee et al. 2009, Brown et al. 2012, Skelton et al. 2013, Farrell et al. 2014, Skelton et al. 2014, Creed et al. 2015, Thomas et al. 2016, Skelton et al. 2017, Creed and Brown 2018, Creed et al. 2021, Creed et al. 2022). As such, the crayfish-branchiobdellidan symbiosis is an effective study-system to observe microplastic toxicity not only on freshwater symbioses, but the potential community-wide impacts they exude (Brown et al. 2002).

Using a standardized chronic exposure assay, we exposed the crayfish-branchiobdellidan cleaning symbiosis to an environmentally relevant dosage of microplastics, while observing alterations in host-symbiont interactions. Our approach was grounded by the growing body of evidence suggesting microplastics may have a detrimental effect on symbiotic partnerships

(Okubo et al. 2018, Machado et al. 2019, Zhang et al. 2023). We formulated two competing hypotheses; 1) When exposed to microplastics, the crayfish-branchiobdellidan interaction would be disrupted, leading to a decrease in host benefit. This hypothesis addresses the possible shifts in organismal interaction stemming from factors such as resource limitation due to decreased microbial abundance or even behavioral alterations experienced by the symbionts when in the presence of microplastics. Alternatively, our second hypothesis theorized 2) The cleaning symbiosis facilitated by the worms would result in a mutualistic outcome in the presence of microplastics by dislodging plastic particles found within the gill chamber. This hypothesis primarily focused on the host, where we predicted an increased bioaccumulation of microplastics would lead to reduced host growth. This decrease in growth could occur either due to an artificial sense of satiation or as a physiological response triggered by an underlying toxicokinetic effect.

In addition to testing these competing hypotheses, we also evaluated several potential mechanisms that may have explained the different treatment effects observed during the exposure. For example, we also assessed the influence of host sex difference, symbiont density, and disease on host growth and symbiont behavior. By including these additional mechanisms, we conducted a throughout evaluation of how microplastics influence the crayfish-branchiobdellidan symbiosis during our experiment.

Materials and Methods

Study System

The crayfish-host, *Cambarus appalachiensis*, was used in our experiment due to their environmental relevance and availability in the surrounding regions (Creed 1994, Creed and Reed 2004, Usio and Townsend 2004, Charlebois and Lamberti 1996, Bobeldyk and Lamberti

2008). This recently described species of crayfish is endemic to the New River Basin and found within Virginia and West Virginia (Loughman et al. 2017, USGS). The symbiont used within our experiment is known as *Cambarincola ingens* and can be found in the Phylum: Annelida, Class: Clitellata, Order: Branchiobdellida. In terms of range, branchiobdellidan worms, are holartically distributed and are known to span from Costa Rica to Southern Canada within the western hemisphere (Holt 1964, Gelder 1991, Gelder and Hall 1990). Branchiobdellidan worms have been used in a number of empirical studies, especially in conjunction with crayfish hosts (Brown et al. 2002, Brown and Creed 2004, Lee et al. 2009, Brown et al. 2012, Skelton et al. 2013, Farrell et al. 2014, Skelton et al. 2014, Creed et al. 2015, Thomas et al. 2016, Skelton et al. 2017, Creed and Brown 2018, Creed et al. 2021, Creed et al. 2022).

The crayfish-host, *C. appalachiensis*, and the symbionts, *Cambarincola ingens*, were collected from an 800-meter span of Sinking Creek (37.310841N, -80.516956W), within Newport County, Virginia, USA. Crayfish collections were completed using dipnets, and collection efforts were specifically designed to target adolescent hosts. Since the main pursuit of our study was tracking growth, we used adolescent crayfish due to their increased growth and molting rates (Thomas et al. 2016). To ensure this age-range, our experiment used crayfish with carapace lengths between 28 and 34 mm.

Experimental Design

We conducted a standardized chronic exposure assay where the crayfish-branchiobdellidan symbiosis was exposed to microplastics (MPs) to assess the impact on crayfish host physical growth, behavior, and survivability. Thirty-six crayfish replicates were randomly sorted into control and experimental treatments, and further split into three staggered symbiont loads of 0-worms, 3-worms, and 6-worms. Replicates within the microplastic treatment

were exposed to an environmentally relevant dose of microplastics (15mg/L) (Buss et al. 2022). Each dose consisted of 5 mg of microbeads, tire wear particles, and marine rope, to best mimic microplastics currently found in the environment (Schwarz et al. 2019, Leads and Weinstein et al. 2019, Ertel et al. 2023). Crayfish were individually housed in 4L mesocosms where they were observed and measured over a span of 180 days (spanning from February 28th, 2023, until August 25th, 2023). Each mesocosm contained 1000 g of sediment, sourced from Stroubles Creek, found within Montgomery County, Virginia, USA. In addition to the sediment, the mesocosms were filled with 2L of water sourced from the host collection site. Aeration and subsequent oxygen concentration were maintained by *Tetra Whisper 60* air pumps. Finally, MPs influence on the crayfish-branchiobdellidan symbiosis was tracked by documenting crayfish-host growth and mortality, as well as symbiont density and location overtime.

Due to the natural decline in worm densities either from grooming behaviors or symbiont mortality, we maintained a consistent symbiont density throughout the experiment to replicate the natural symbiont abundances found within the field (Brown et al. 2013). Worm density treatments of 6 (High) and 3 (Low) were replenished biweekly, and symbiont loss was documented to track possible alteration in grooming behaviors. Replenishment efforts closely mimicked field behavior where symbiont densities are regularly replenished by crayfish-to-crayfish dispersal. Symbionts for replenishments were collected by deworming *C. appalachensis* and *C. chasmodactylus* found within the same section of Sinking Creek. Using soft-touch dissection tweezers, *C. ingens* were detached from the recently collected crayfish and transferred to the crayfish-hosts experiencing symbiont deficit (Brown et al. 2002).

To best mimic the plastic polymers currently contaminating freshwater habitats, our dosage consisted of three environmentally relevant plastic polymers: polypropylene (PP),

polyethylene (PE), and styrene butadiene (SB) (Schwarz et al. 2019, Leads and Weinstein et al. 2019, Ertel et al. 2023). These plastic polymers mimicked plastic debris commonly found within freshwater environments such as aquatic rope (PP), microbeads (PE), and tire-wear particles (SB). Each portion of the dosages was sourced from commercial origin. The dosage portion representing primary microplastics (microbeads) was sourced from *Cospheric*, the tire-wear particles were purchased from *NorthernWestStuff* and finally, secondary microplastics consisted of incised marine rope fragments were sourced from *ULINE*. To ensure the size of the fragments were of uniform microplastic size (<5 mm), *Fisher Scientific* sieves were used to separate plastic particles. Each (15 mg) dosage consisted of (5mg) of each plastic morphology. Based on the New Jersey Department of Environmental Protection Science Advisory Board, we used an environmentally relevant dosage of (15 mg/L), to best mimic current aquatic microplastic levels (Buss et al. 2022).

Due to the natural buoyancy of the plastic polymers used, we treated each dosage with (2 mL) of Tween 80 surfactant. After the plastic polymers were weighed, the surfactant was used to cover the surface area of the microplastics within the dosage. The surfactant induced a change in buoyancy, allowing for full water column dispersal, ensuring our dosage effectively spread throughout the mesocosm (Kaewtatip and Tanrattanakul, 2008, Adhikari et al. 2021). To maintain consistency across treatments, 2 mL of Tween 80 was also added to the control mesocosms.

To reduce potential ammonia build-up, weekly water changes were conducted to maintain ideal water chemistry in the mesocosms. To ensure homogeneity and ideal water chemistry throughout the experiment, water was collected from the same section of Sinking Creek from which the crayfish were originally collected. On a weekly basis, mesocosms were drained of water by a *Hygger* hand pump and replaced with freshly collected Sinking Creek water.

Transportation of Sinking Creek water was conducted using four 20L Nalgene carboys. To ensure retention of microplastics during water changes, a *Dabpress* 37-micron filter was attached to the hand pump allowing for effective water siphoning and nominal microplastic loss. Any microplastics that adhered to the surface of the filters during water replacements were rinsed off with ambient Sinking Creek water.

Measurements: Percent Carapace Length and Blotted Wet Mass Change

To evaluate crayfish growth throughout the experiment, we performed growth assessments on a biweekly basis to collect and document changes in carapace length and blotted wet mass, two standard measures of crayfish size (Brown et al. 2002 and 2012). For blotted wet mass, we used a digital balance to measure the biomass of the crayfish host. After removing the crayfish out of their mesocosm, we would lightly pat the crayfish dry, then place it into a tared beaker and document the replicate's mass. To collect carapace length, we used calipers to measure the length from the tip of the rostrum to the opposite end of the carapace. Similar to pMC, carapace growth was calculated via percent carapace length change (pCLC) over time to elicit reliable comparison across replicates. During the exposure period, eight of our female crayfish hosts became gravid. To assure no discrepancies arose, we compared the pMC and pCLC of the normal dataset and a dataset without gravid females.

To evaluate a potential shift in context dependency from mutualism to parasitism, we conducted a gill scarring assessment at the conclusion of the study (Brown et al. 2012). Gill scarring manifests itself through the presence of melanized spots on the sides of the gill filaments and the absence of filament tips (Thomas et al. 2016). After the crayfish hosts were sacrificed, a portion of the carapace was surgically removed revealing the branchial chamber (Burggren et al. 1974). By exposing the branchial chamber, we were able to adequately access and inspect each

podobranch and subsequent gill filaments. To maintain consistency, we would only remove and quantify the right gill chamber of each replicate (Brown et al. 2012). Utilizing a digital-capable microscope, we quantified the total gill scarring count for each replicate and compared by treatment, worm density, and sex.

To estimate microbial abundance found on the crayfish exoskeleton, bacterial DNA (ng/ml) and chlorophyll a ($\mu\text{g}/\text{cm}^2$) samples were collected and quantified using qPCR and spectrophotometry. These analyses were conducted to quantify the amount of resources available to the symbionts, to better understand the mechanisms driving a shift in context dependency. Bacterial load, in the form of DNA (ng/ml), was quantified using qPCR through the DNeasy PowerSoil Pro Kit (Qiagen, Valencia, CA, United States). Chlorophyll a density ($\mu\text{g}/\text{cm}^2$) was determined by measuring the absorbance using Spectrophotometry, following the chlorophyll a extraction protocol found in Steinman and Lamberti (1996).

Using the swabbing technique of Skelton et al. 2017, we used sterile cotton swabs to collect algal (Chl a) and bacterial (DNA) samples from the hosts' exoskeletons. Immediately afterwards, the heads of the cotton swabs were placed within microcentrifuge tubes and stored in the freezer. Algal (Chl a) samples were submerged in boiling ethanol and centrifuged to effectively separate any remaining particulates. The chlorophyll a pigment was then isolated and transferred to a cuvette, where absorbance was calculated across wavelengths of 750 nm and 664 nm before and after an acetone acidification. Before chlorophyll a density could be calculated; the average surface area of the crayfish carapace was deduced using a modified open cylinder equation. Absorbance values were then compared against blanks, to determine respective sample absorbance. Chlorophyll a density was then calculated using formula 14.4 (Equation 1) from Steinman and Lamberti (1996).

Bacterial DNA was isolated from the collection swabs using the DNeasy Power Soil Pro Kit (Qiagen, Valencia, CA, United States) to obtain a proxy for microbial biomass growth. After the DNA extractions were completed, DNA yield was determined using a Qubit 2.0 (Thermo Fisher Inc., Waltham, MA, United States) for fluorometric quantification.

Bioaccumulation: The Physical Absorption of Microplastics

To quantify the bioaccumulation of microplastics within the crayfish hosts, systematic dissections were performed to excise the hosts' muscle tissue. Immediately after dissecting, the muscle tissue was stored in Falcon Tubes and preserved in the freezer. Similar to Weinstein and Gray et al. 2022, we conducted a tissue digestion using a 10% KOH solvent and a shaker table, where the muscle tissue was agitated and heated to 60° Celsius for 72 hours (Phuong et al. 2018, Dawson et al. 2020, Weinstein et al. 2022). The resulting solution of the tissue digestion process was then separated by vacuum filtration to stratify the organic material from the bioaccumulated microplastics. After letting the filters dry, plastic particles were counted and categorized by morphology and color using a bright-field microscope.

Crayfish Plague

During gill scarring quantification, darker, sub-cuticle markings were observed throughout the ventral and dorsal surfaces of the crayfish host. Ranging in size, these black marks were later discovered to be evidence of hyphae establishment of *Aphanomyces astaci*, also known as Crayfish Plaque (Edsman et al 2015 and Thomas et al. 2020). Crayfish replicates were observed under a bright-field microscope, and fungal discoloration marks were quantified and photographed for future analyses.

Data Analysis

We examined variation in host growth using a linear mixed model for a repeated measures design. This model included three fixed factors: microplastic treatment, symbiont density, and time, defined as the day of the experiment. To account for the repeated measurements of growth, we incorporated a random intercept of the host. These analyses were conducted using the *lme()* function in the R package *nlme* (Pinheiro and Bates et al. 2000).

We assessed gill scarring trends across our replicates using a univariate linear regression to compare scar count across microplastic treatment, sex, and worm density. The linear regression models utilized the *lm()* function while the ANOVA models used the *aov()* function, both of which are integrated into the base platform of R (R Core Team 2021). We also used linear regression and ANOVA to assess the responses of crayfish plague, the microbial load, and bioaccumulation with microplastic treatment, sex, and worm count as predictor variables.

To assess the variation of symbiont location over time, I performed a permutational multivariate analysis of variance using a Euclidean distance matrix (McArdle & Anderson 2001) and visualized the response Principal Components Analysis (PCA) using the *princomp()* function found in the *factoextra* package (Pearson 1901, Goodall 1954, Kassambara and Mundt 2017, 2020). Since symbiont locations changed through time, we examined separate PCAs for each sampling date.

Results

Host mortality was recorded throughout the observation period and collected as count data. Strong evidence suggests treatment significantly influenced mortality, as experimental replicates showed a 31% mortality rate compared to 6% in control replicates. Throughout the

exposure period, six replicates exposed to microplastics died compared to one control replicate, which died 140 days into the experiment.

Host growth through time was measured as percent mass change (pMC) (Table 1) and percent carapace length change (pCLC) (Table 2) and showed strong effects of all three predictors: microplastics, symbiont density, and time (Figure 2). When comparing pMC across worm densities there was strong evidence suggesting a considerable influence of treatment and initial worm count ($p = 0.0159$). Moreover, very strong evidence was found when considering the interaction of treatment, initial worm count, and time ($p = 0.0002$). For pCLC the interaction of treatment, initial worm count, and time suggested an absence of influence ($p = 0.3783$).

The pMC of the non-gravid dataset (Table 3) suggested strong evidence of the treatment and initial worm count interaction ($p = 0.0053$) and very strong evidence of the treatment, initial worm count, and time interaction ($p = 0.0001$). Likewise, even when gravid females were extracted from the dataset, pCLC (Table 4) lacked any evidence of influence in terms of the interaction between treatment, worm count, and time ($p = 0.2185$).

The results of our simple linear regression suggested treatment ($p = 0.2013$), sex ($p = 0.1541$), and initial worm count ($p = 0.3003$) had no influence on gill scarring. The results of our multiple linear regression suggested the interaction of treatment and sex, were somewhat influential ($p = 0.05551$). While, based on the two-way ANOVA, gill scarring was highly influenced by sex ($p = 0.03036$). However, when we isolated the replicates by sex, then compared scarring count across treatment and symbiont density, we found strong evidence of a female-biased parasitism within the experimental treatment ($p = 0.0448$), when compared to the male replicates ($p = 0.9764$) Our results demonstrated that female gill scarring increased with larger symbiont densities when exposed to microplastics (Figure 3).

When a univariate linear regression was used to assess fungal discoloration, or hyphae establishment on crayfish, strong evidence was found that initial worm count had a considerable influence ($p = 0.04538$) (Figure 4). However, when comparing hyphae establishment across microplastic treatment ($p = 0.3923$) and sex ($p = 0.9546$), there is no evidence to suggest that these variables have an effect.

To evaluate the potential for resource limitation created by microplastics, we evaluated algal and microbial biomass on the hosts. A univariate linear regression of Chlorophyll a ($\mu\text{g}/\text{cm}^2$) density showed that the microplastic treatment had no influence on algal abundance ($p = 0.3279$). Our results suggests neither sex ($p = 0.2027$) nor initial worm count ($p = 0.5979$) had a strong influence on chlorophyll a ($\mu\text{g}/\text{cm}^2$) density.

Evidence was found, using a univariate linear regression to suggest bacterial DNA density was influenced by the interaction of treatment and sex ($p = 0.03021$). In the control treatment, female exoskeletons hosted an average of approximately 40% less bacterial DNA than that of the male exoskeletons. Moreover, our findings suggest microbial load was influenced by sex alone ($p = 0.01372$) (Figure 7). When averaged across both treatments, female exoskeletons contained approximately 30% less bacterial DNA than male exoskeletons. In contrast, when the same statistical model was used to assess the influence of initial worm count on microbial load, no significant influence was found ($p = 0.8237$) (Figure 8).

Using a PCA, *C. ingens* were primarily found on the ventral margins of the carapace, ventral abdomen, and cephalothorax - all of which surround the branchial chamber. The prevalence of attachment site selection determined host sex ($p = 0.589$) (Table 5) and treatment ($p = 0.981$) (Table 6) had no influence on symbiont microhabitat selection, while initial worm count ($p = 0.001$) (Table 7) (Figure 9) did.

Bioaccumulation was compared across treatment ($p = 0.2694$), host sex ($p = 0.3228$), and initial worm count ($p = 0.1132$) and suggested none of the predictor variables showed an influence on MP uptake (Figure 10). When compared, plastic fragments that were purposely added to our mesocosms were bioaccumulated dramatically less ($p = 0.8684$) than that of background plastic ($p = 2.2e-16$). In fact, only one microplastic fragment that was purposely exposed to the replicates were recovered, while a total of one hundred and thirty-two background fragments were found concluding the tissue digestion. Therefore, the majority of bioaccumulation observed within our replicates originated from a background source in the field prior to the collection of crayfish hosts for our experiment. Moreover, in support of previous literature, the majority of plastic debris found within are replicates were that of fibrous morphology (75.2%) which bioaccumulated three times more than that of particle morphology (24.8%) (Figure 11) (Lozano-Hernandez et al. 2021, Li et al. 2018).

Discussion

Exposure to microplastics altered the outcome of the crayfish-branchiobdellidan cleaning symbiosis, shifting it from a mutualism to a parasitism. When comparing percent mass change (pMC) across treatment and symbiont densities, a context-dependent shift became increasingly evident, and in this case, the context was the presence or absence of microplastics. While this shift in organismal interaction was observed with mass as a response variable, the same trends were not evident in percent carapace length change (pCLC). One reason why these growth parameters differed could be due to the fluctuation of biomass that naturally occurs within the exoskeleton, like the chelae and abdominal muscle tissue. These regions of the body sometimes accrue biomass at higher rates than other portions and can lead to the internal fluctuation of tissue relative to the exoskeleton (Simon and Stewart et al. 2014, Anderson and Simon 2015).

Since carapace length is simply a measure of the length of the cephalothorax, it doesn't capture these sorts of differential tissue accumulation. Thus, by itself, pCLC as a growth parameter was not used to explain the influence MPs had on our study system.

The changes in biomass of the control replicates showed evidence of a mutualistic symbiosis, where increased symbiont densities resulted in a gradient of elevated pMC. Based on previous literature, the mutualistic response is likely due to a cleaning symbiosis, and that the influence of symbionts on host growth are similar to those seen in previous studies (Brown et al. 2002, Lee et al. 2009, Brown et al. 2012, Skelton et al. 2013, Farrell et al. 2014, Skelton et al. 2016, Creed et al. 2018, Creed et al. 2021). However, when we compared the change in biomass of the control replicates to that of the microplastic treatment, the effect was reversed. When exposed to microplastics, symbiont context dependency shifts from increased mutualistic effects with increased symbiont density, to symbiont density negatively affecting host growth. From this evidence, we can deduce that microplastics, as an anthropogenic pollutant, can alter the context of the crayfish-branchiobdellidan symbiosis, shifting it from a mutualism to a parasitism.

Within any symbiotic relationship, a shift in context can take many forms of behavioral alteration. In fact, organismal interactions, such as symbioses, lie on a continuum where a myriad of external influences can result in a change of interaction, and thus symbiotic context (Skelton et al. 2016, Silknetter et al. 2020 and Bronstein 1994). Our findings support previous literature that shifts in this symbiosis are often dependent on environmental factors such as habitat and resource availability (Berry and Holt 1959, Young 1966, Bishop 1968, Keller 1992, Rosewarne et al. 2012, Brown et al. 2002, 2012, Lee et al. 2009, Skelton et al. 2013, Creed and Brown et al. 2018). In our case, the presence of MPs acted as the environmental factor driving the contextual shift to parasitism.

Gill scarring within the host's branchial chamber is a reliable indicator of symbiont parasitism (Brown et al. 2012). A previous study using the crayfish-branchiobdellidan symbiosis found that increased gill scarring was attributed to decreased gill efficiency, ultimately reducing host growth (Brown et al. 2012, Thomas et al. 2016, Creed et al. In Review). Although our control replicates experienced gill scarring, there was no evidence suggesting this antagonistic behavior, as a mechanism, had a direct influence on growth. However, when exposed to MPs, female hosts experienced heightened parasitic behavior compared to their male counterparts (Figure 3). Such female-preferred antagonistic behaviors have been documented in other organismal models due to differences in susceptibility, morphology, and even hormonal levels. (Morales-Montor et al. 2004, Duneau et al. 2012, Stephenson 2019) Our results suggest the presence of MPs lowered bacterial abundance on female replicates, thus inducing a sex-biased parasitism within the crayfish-branchiobdellidan symbiosis. However, the mechanism driving the reduction in bacterial load is unknown and warrants further investigation.

We hypothesized that the increased antagonistic behavior experienced by our crayfish hosts was due to resource limitation induced by the presence of MPs. Multiple studies have suggested MPs have the potential to disrupt aquatic food webs across multiple trophic levels (Zhang et al. 2017, Guschina et al. 2020). For instance, previous studies have demonstrated the effects of pollutants on pivotal aquatic primary producers, such as epibionts, could cause an ecosystem-wide disruption (Huang et al. 2016, Pham 2019). For example, recent findings suggest microplastics have been found to not only bioaccumulate with epibiotic diatoms, but to adversely affect the growth and physiology of such taxa (Linggi et al. 2021, Hao et al. 2022). These primary producers are well-known prey for the annelids and are integral in determining the resource-based context by which these symbionts thrive (Brinkhurst and Gelder 2001), Creed

and Brown et al. 2018). In fact, in food-limited scenarios, branchiobdellidan worms have been documented to shift to parasitic behaviors such as gill tissue and hemolymph consumption (Creed and Brown et al. 2018, Skelton et al. 2014, Cheney and Cote 2005), whereas, in food-stable conditions, symbionts facilitate a cleaning symbiosis by grazing on microalgal growth and detritus build up within the branchial chamber (Farrell et al. 2012).

The results of our microbial assay lack definitive evidence that MPs had an effect on bacterial load. According to Figure 7, MPs seemed to have increased the disparity of bacteria between male and female hosts. Interestingly though, when bacterial load is compared across worm densities, a unimodal pattern emerges, suggesting an intermediate disturbance effect (Gao et al. 2019). Across both treatments, crayfish harboring an intermediate worm density (3-worms) were found to have the highest bacterial load.

Additionally, results of our microbial assay suggest a sex-based discrepancy of microbial load. Across both algal and bacterial densities, female hosts had overall lower abundances of microbes, potentially explaining the sex-biased parasitism (Morales-Montor et al. 2004, Duneau et al. 2012, Stephenson 2019). However, when gill scarring alone is compared across treatment, MPs further incited the antagonistic behavior of the symbionts. These findings suggest female hosts are inherently parasitized more due to their lower microbial load when compared to that of their male counterparts. It stands to reason that, when exposed to MPs, this resource discrepancy is further exacerbated increasing parasitism experienced by female hosts, although the mechanism driving this resource reduction is still unknown (Brown et al. 2012, Creed and Brown et al. 2018).

Based on past research, alteration of environment and host behavior can have profound influence on the behavior of symbionts on their hosts (Rio et al. 2005, Mieog et al. 2009, Brown

et al. 2012, Brown et al. 2018). In addition to MPs effect on symbiotic context, we assessed the influence this toxicant imposed on worm location on the host. By dividing the surface area of the host into designated quadrants (Brown et al. 2002), and tracking worm movement and density overtime, we determined the presence of MPs had no influence on worm microhabitat selection nor density. However, when we compared microhabitat selection across initial symbiont load, we found evidence of notable variation, although, this result was expected as increased worm density will naturally displace other symbionts, thus increasing microhabitat variation. While our results indicated MPs had a considerable influence on the context dependency of the crayfish-branchiobdellidan partnership but found no evidence of direct influence on the branchiobdellidans themselves as measured by their preferred positions on their hosts.

The intake or ingestion of plastic debris can be passive or purposeful and may lead to the bioaccumulation of this contaminant within the tissue of the organism (Weiss et al. 2006, Lusher et al. 2013, Cauwenberghe and Janssen 2014, Desforges et al. 2015, Linggi et al. 2021, Hao et al. 2022). By utilizing a standard KOH tissue degradation protocol (Phuong et al. 2018, Lewis, A. 2021, Weinstein et al. 2022, Minder et al. 2023), we determined a vast majority of the MPs found within our replicates were of fibrous morphology. These findings support previous literature, as plastic fibers are notoriously known to bioaccumulate more than any other form of MP (Lozano-Hernandez et al. 2021, Patterson et al. 2019, and Li et al. 2018). However, the most interesting finding of our bioaccumulation results is the significant discrepancy between background MPs and the MPs intentionally exposed to our study system. In fact, among the 133 MP particles collected, only 1 green florescent microsphere was successfully recovered from the original (15 mg/L) dosage. Based on this evidence, we can conclude bioaccumulation primarily occurs over a longer period of time than that of our experiment, or the MPs used were too large for absorption.

Despite the quantity of MPs found within our replicates, there was no grounds to suggest the bioaccumulation of MPs influence our crayfish host growth or behavior.

In addition to MP pollution, crayfish populations are also commonly exposed to a variety of diseases. These ailments can range from viral (Stentiford et al. 2009), bacterial (Quaglio et al. 2006), and even fungal (Cerenius et al. 1988, Oidtmann et al. 2002) origins. Major concern has been raised concerning the spread of *A. astaci*, also known as crayfish plague, within European crayfish populations (Kozubikova et al. 2007 and Longshaw et al. 2011). Despite its deadly nature in European populations, it is believed North American crayfish have an inherent resilience to *A. astaci* (Unestam et al. 1969, Cerenius et al. 2003). Moreover, it is suggested the spread of *A. astaci* originated from an invasive North American Signal Crayfish. Due to its presence within North American waterways, our replicates began showing consistent evidence of *A. astaci* infection during the exposure period. Though a serendipitous result, our findings suggest increased symbiont load lessens the severity of crayfish plague and supports the claim that *C. ingens* play a role in a cleaning symbiosis that may not only improve host gill function but may also reduce the severity of a common crayfish disease (Brown et al. 2002 and Brown et al. 2012).

Conclusion

Our study demonstrated MPs, as an anthropogenic pollutant, can shift the context of a symbiotic relationship in freshwater systems. In our case, MPs shifted the symbiosis of crayfish and their symbiont branchiobdellidans from a mutualism to a parasitism. Evidence suggests the shift in context dependency originated from the reduction in resource availability which resulted in increased parasitic behavior. As such, our replicates exposed to microplastics experienced heightened antagonistic behavior in the form of gill tissue consumption. We the increased

parasitic pressures posed by the symbionts induced a reduction in host energetic reserves, translating to decrease host growth.

Until recently, MP research has primarily been focused on the toxicological effects felt by an individual organism, rather than the intricate interactions that occur between taxa. In fact, only focusing on the individual toxicological impact ignores the communities that maintain ecosystem processes. Our experiment is unique in that our study focuses on a pivotal organismal interaction that heavily influences freshwater community structure and habitat dynamics. Moreover, our experiment will be the first study assessing the impact of a freshwater symbiosis, as symbioses in the scope of toxicokinetic studies have primarily been dominated by that of terrestrial and marine relationships.

Objectively, this knowledge gap poses a major concern for freshwater habitats, not only due to the infrastructural reliance we've placed on freshwater ecosystems (Green et al. 2015, Apostolaki et al. 2019), but for the disproportionate rate of disturbance and extinction that occurs within such habitats (Dobson et al. 1997, Ricciardi and Rasmussen 1999, Sanders et al. 2024). Based on the results within this study, as the prevalence of microplastics continue to worsen within freshwater habitats, we can expect crayfish populations to decline inducing community wide impacts. We can also expect the context of the crayfish-branchiobdellidan symbiosis to become increasingly more antagonistic. Specifically, if a parasitic context becomes more prevalent among female crayfish, reproduction rates will naturally decline, further affecting overall crayfish populations.

If this population decline were to occur, the significant reduction of a keystone species and ecosystem engineer, would inflect ripples throughout the freshwater community structure, destabilizing not only the surrounding food web but also the physical ecosystem they dwell in

(Power et al. 1996, Delibes-Mateos et al. 2011). However, as an intermediate prey species the propensity of bioaccumulation, and subsequent trophic transfer, will increase the MP quantities found within the taxa comprising the surrounding food web (Rochman et al. 2015, Hammer et al. 2016).

Crayfish are but one of many organisms documented to harbor and facilitate the trophic transfer of MPs (Eriksson and Burton et al. 2003, Farrell and Nelson 2013, Hammer et al. 2016, Rochman et al. 2015, Lusher et al. 2016). For instance, the multi-billion-dollar aquaculture industry, opens an avenue by which commerce can result in increased human bioaccumulation through the consumption of contaminated aquatic organisms (Van Cauwenberghe and Janssen 2014, Rochman et al. 2015, Seltnerich et al. 2015).

Overall, as environmental microplastic contamination continues to worsen over time, how will organismal interactions adapt to this pollutant (Okubo et al. 2018, Zhang et al. 2023, and Machado et al. 2019)? Organismal partnerships, such as symbioses, can be found in every eukaryotic organism on earth and are largely responsible for the incredible diversity and stability found throughout our biosphere (Douglas et al. 2010, Chomicki et al. 2019, 2022, Prada et al. 2022). How will the intensifying presence of MPs, among other anthropogenic pollutants, affect the organismal dynamics interconnecting the natural world around us?

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Tables

Table 1: Crayfish Percent Mass Change: Linear Mixed Effect Model

	numDF	denDF	F-value	P-value
(Intercept)	1	354	143.7335	<.0001*
Treatment	1	32	0.0125	0.9795
Initial Worm Count	1	32	0.4307	0.4979
Days	1	354	624.1753	<.0001*
Treatment:Initial Worm Count	1	32	6.8184	0.0159
Treatment:Days	1	354	2.9406	0.0538
Initial Worm Count:Days	1	354	4.0865	0.0486
Treatment:	1	354	13.2450	0.0002

Table 2: Crayfish Percent Carapace Length Change: Linear Mixed Effect Model

	numDF	denDF	F-value	P-value
(Intercept)	1	354	99.2952	<.0001*
Treatment	1	32	0.0003	0.9871
Initial Worm Count	1	32	0.0023	0.9622
Days	1	354	558.5260	<.0001*
Treatment:Initial Worm Count	1	32	0.1509	0.7003
Treatment:Days	1	354	4.3297	0.0382*
Initial Worm Count:Days	1	354	1.0203	0.3131
Treatment:	1	354	0.7783	0.3783

Table 3: Non-Gravid Crayfish Percent Mass Change: Linear Mixed Effect Model

	numDF	denDF	F-value	P-value
(Intercept)	1	266	136.7356	<.0001*
Treatment	1	24	0.1032	0.7508
Initial Worm Count	1	24	0.1126	0.7401
Days	1	266	587.8468	<.0001*
Treatment:Initial Worm Count	1	24	9.3930	0.0053
Treatment:Days	1	266	2.9749	0.0857
Initial Worm Count:Days	1	266	4.3600	0.0377*
Treatment:	1	266	15.1731	<.0001*

Table 4: Non-Gravid Crayfish Percent Carapace Length Change: Linear Mixed Effect Model

	numDF	denDF	F-value	P-value
(Intercept)	1	354	99.2952	<.0001*
Treatment	1	32	0.0003	0.8652
Initial Worm Count	1	32	0.0023	0.7757
Days	1	354	588.5260	<.0001*
Treatment:Initial Worm Count	1	32	0.1509	0.5489
Treatment:Days	1	354	4.3297	0.0374*
Initial Worm Count:Days	1	354	1.0203	0.2443
Treatment:	1	354	0.7783	0.2185

Table 5: Symbiont Location Across Sex: Principal Component Analysis

	DF	Sum of Sqs	R2	F	p-value
Minimum	1.0	0.1098	0.002259	0.4754	0.589
1 st Quadrant	105.5	24.3007	0.500000	0.4754	0.589
Median	210.0	48.4917	0.997741	0.4754	0.589
Mean	140.7	32.4010	0.666667	0.4754	0.589
3 rd Quadrant	210.5	48.5466	0.998871	0.4754	0.589
Maximum	211.0	48.6014	1.000000	0.4754	0.589
NA's				2	2

Table 6: Symbiont Location Across Treatment: Principal Component Analysis

	DF	Sum of Sqs	R2	F	p-value
Minimum	1.0	0.00469	0.0000965	0.02028	0.985
1 st Quadrant	105.5	24.30072	0.5000000	0.02028	0.985
Median	210.0	48.59675	0.9999035	0.02028	0.985
Mean	140.7	32.40096	0.6666667	0.02028	0.985
3 rd Quadrant	210.5	48.59909	0.9999517	0.02028	0.985
Maximum	211.0	48.60144	1.000000	0.02028	0.985
NA's				2	2

Table 7: Symbiont Location Across Worm Count: Principal Component Analysis

	DF	Sum of Sqs	R2	F	p-value
Minimum	1.0	2.282	0.04694	10.34	0.001
1 st Quadrant	105.5	24.301	0.50000	10.34	0.001
Median	210.0	46.320	0.95306	10.34	0.001
Mean	140.7	32.401	0.66667	10.34	0.001
3 rd Quadrant	210.5	47.461	0.97653	10.34	0.001
Maximum	211.0	48.601	1.00000	10.34	0.001
NA's				2	2

Figures

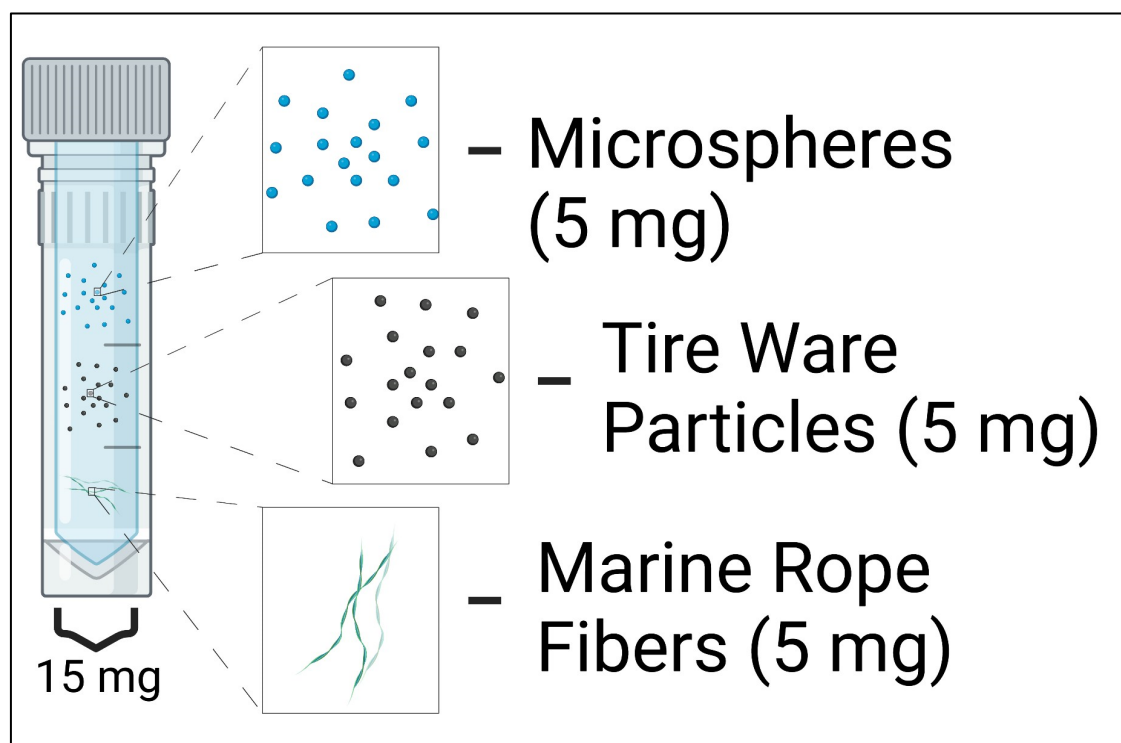


Figure 1 Caption: The Microplastic Dosage Breakdown by Plastic Morphology

Example of the microplastic dosage used within the study, and dosed within the experimental mesocosms. Within each dosage, there were three plastic morphologies that contributed (5 mg) to the total (15 mg) total volume. The polymers used were polypropylene (PP), polyethylene (PE), and styrene butadiene (SB). Each polymer represented a different morphology: marine rope (PP), microbeads (PE), and tire-wear particles (SB). Moreover, these polymers and subsequent morphologies were picked due to their prevalence within environmental plastic pollution (Grillo et al. 2021 and Ivar do Sul 2021).

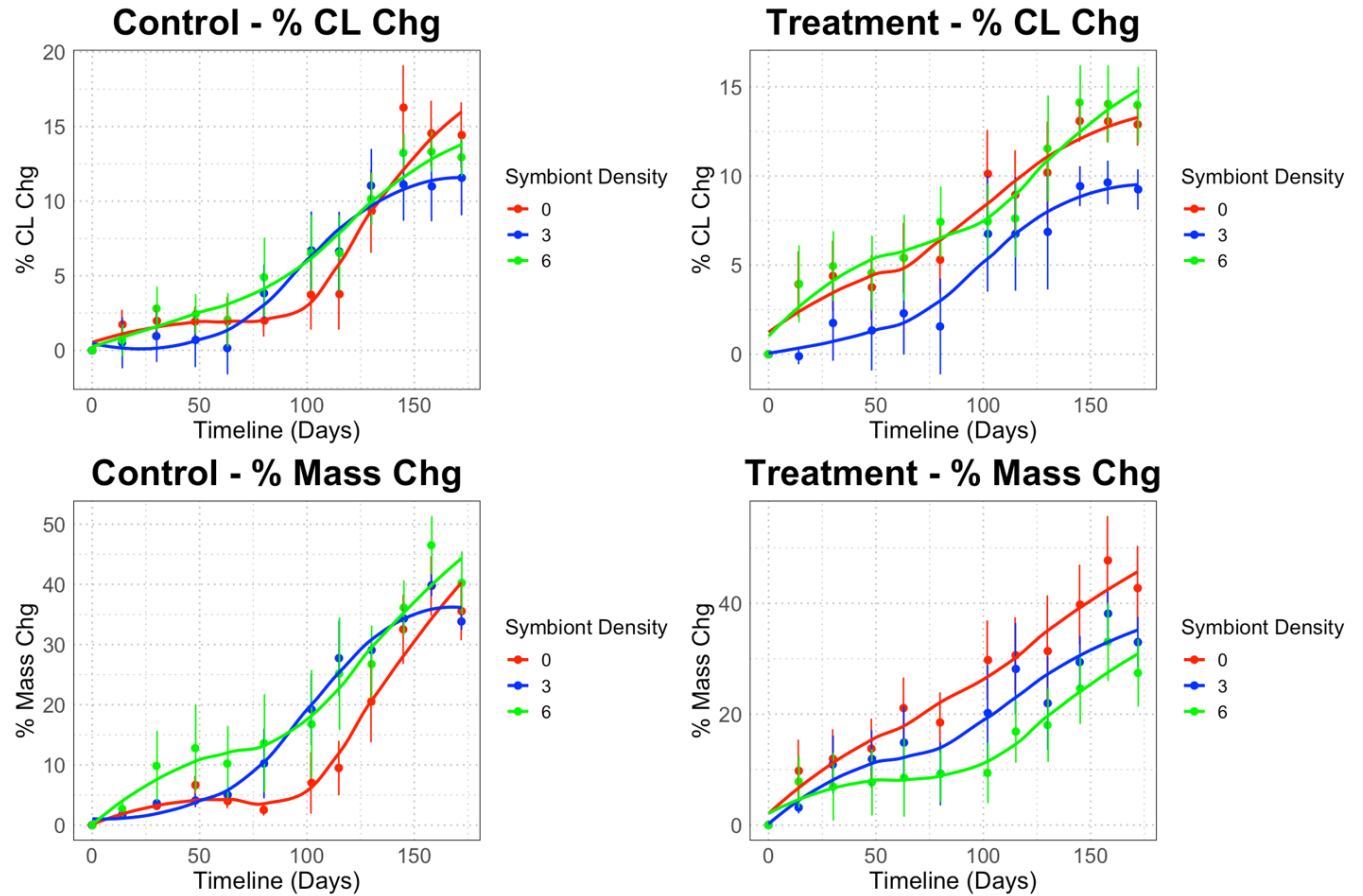


Figure 2 Caption: Crayfish Growth Trends Incorporating Percent Mass Change and Percent Carapace Length Change

A comparison of crayfish growth rate with varying symbiont densities when exposed to microplastics. Treatment replicates were exposed to microplastics, while Control replicates were not. Symbiont densities vary from 0 worms (red), 3 worms (blue), and 6 worms (green). Time series growth is calculated by percent mass change (PMC) and percent carapace length change (PCLC). In both PMC graphs, there was a highly significant effect of the interaction between treatment, worm count, and time based on a linear mixed model effect (p-value: 0.0002). Error bars represent 1 SE.

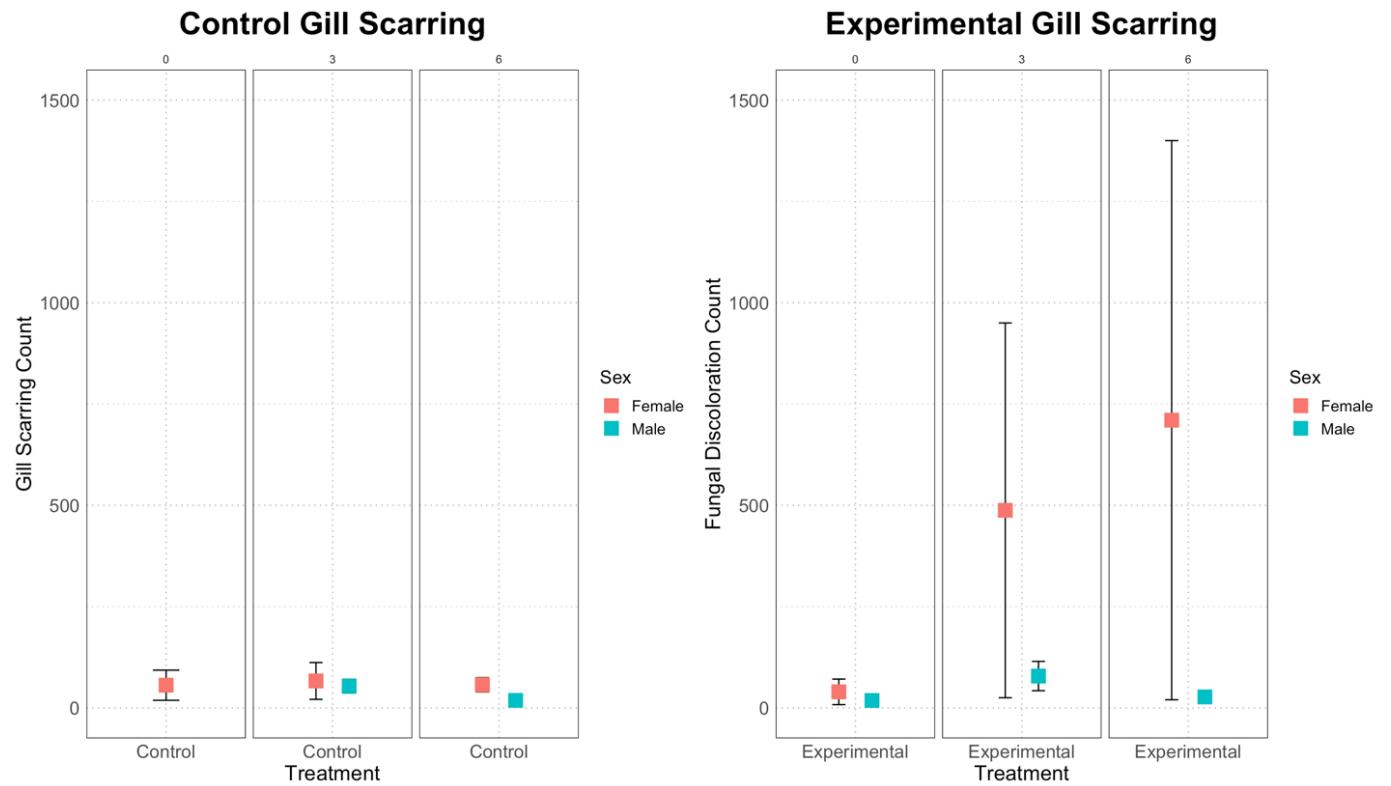


Figure 3 Caption: Crayfish Gill Scarring Count Across Sex, Treatment, and Worm Count

A comparison of crayfish gill scarring count with varying symbiont densities when exposed to microplastics. Treatment replicates were exposed to microplastics, while Control replicates were not. Symbiont densities vary from 0 worms (left), 3 worms (middle), and 6 worms (right). There was a significant effect on the interaction between the experimental treatment and worm count, based on a multiple linear regression (p-value: 0.0233). Error bars represent 1 SE.

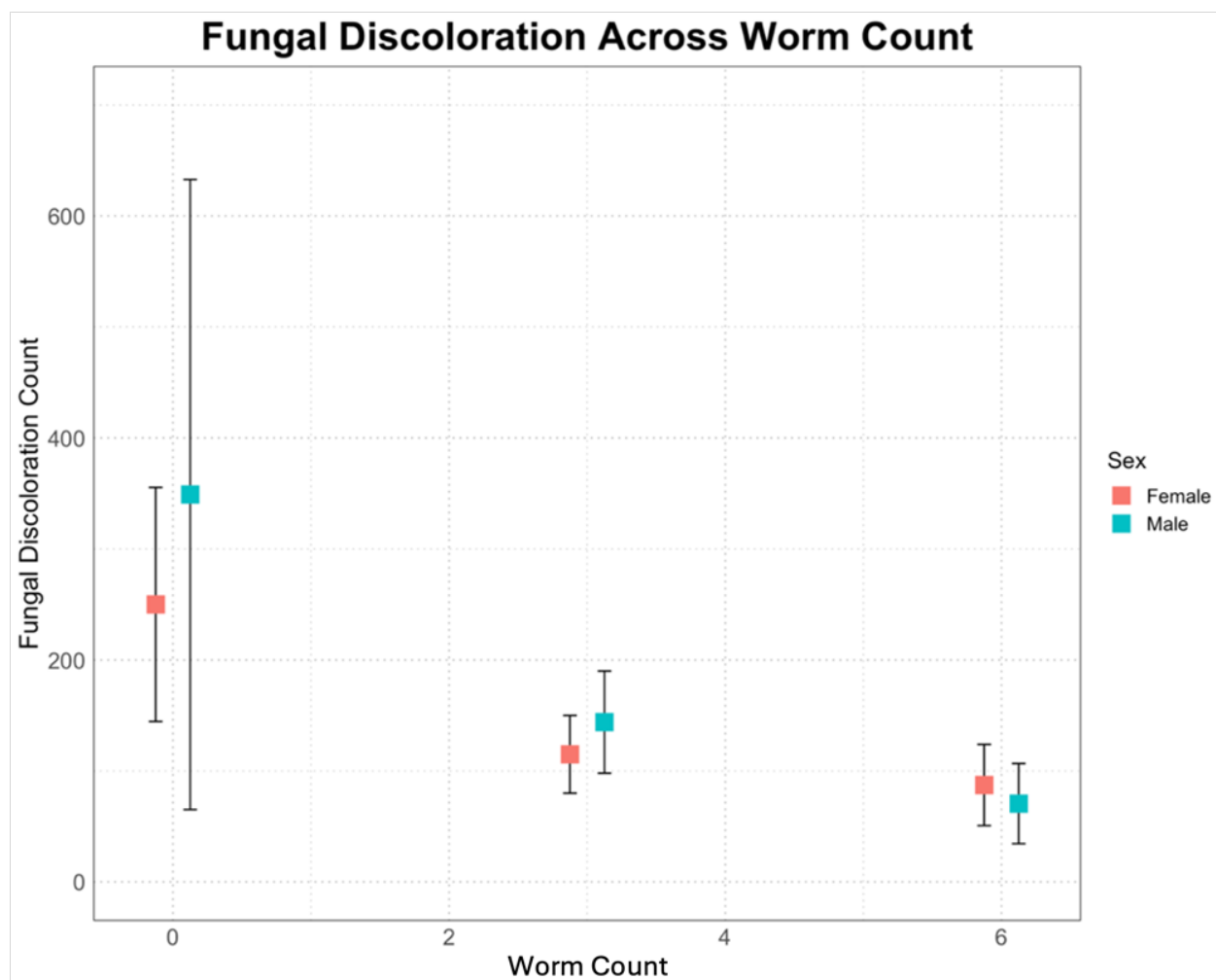


Figure 4 Caption: Fungal Discoloration Counts Across Sex and Worm Count

A comparison of total fungal discoloration across sex and worm count. Using a simple linear regression, worm count was found to have a significant influence on fungal discoloration (p-value: 0.04538). Replicates were divided by worm count: 0-worms, 3-worms, and 6-worms (designated by the x-axis), and further divided by sex (females: red, males: blue). Error bars represent 1 SE.

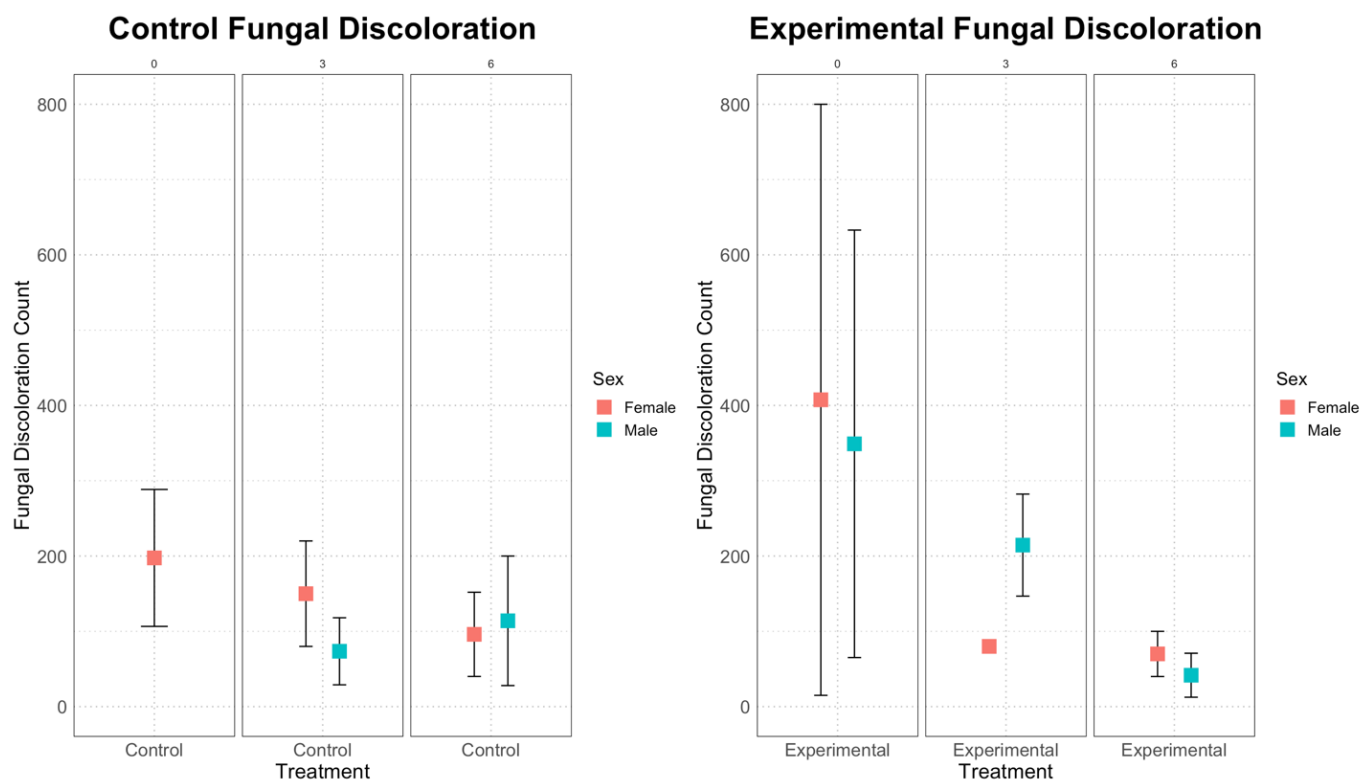


Figure 5 Caption: Fungal Discoloration Count Across Treatment, Sex, and Worm Count

A comparison of fungal discoloration across treatment, sex, and worm count. Using a simple linear regression, worm count was found to have a significant influence on fungal discoloration (p -value: 0.04538). The control (left) and the treatment (right) separate the replicates by experimental treatment. Replicates were divided by worm count: 0-worms, 3-worms, and 6-worms (designated by the x-axis), and further divided by sex (females: red, males: blue). Error bars represent 1 SE.

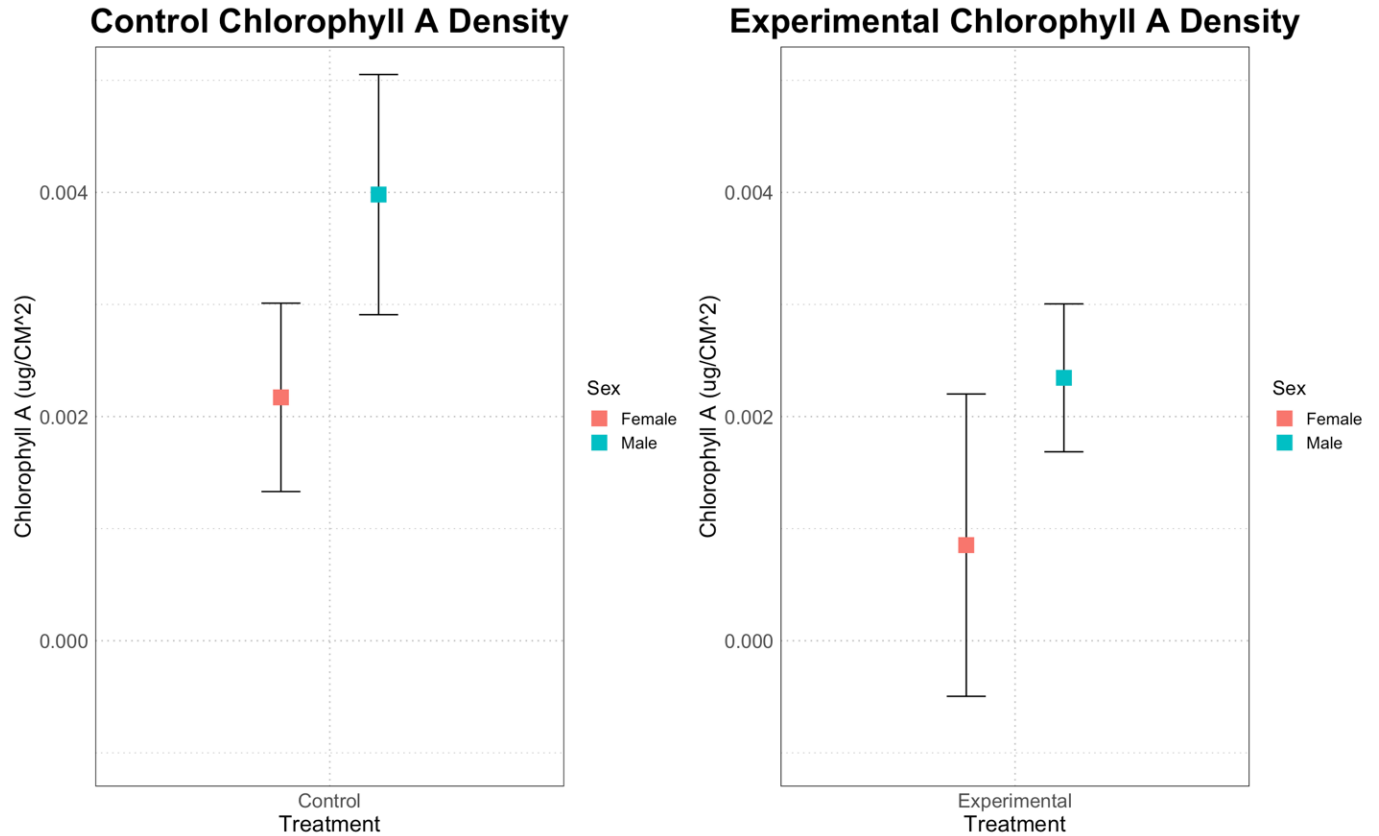


Figure 6 Caption: Chlorophyll A Density Across Sex and Treatment

A comparison of Chl a density (ug/cm²) found on the carapace of our replicates across treatment and sex. Using a simple linear regression, variables such as treatment, sex, and worm count lacked any statistically significant findings. The control (left) and the treatment (right) separate the replicates by experimental treatment, while female (red) and male (blue) are further designated within such plots. Error bars represent 1 SE.

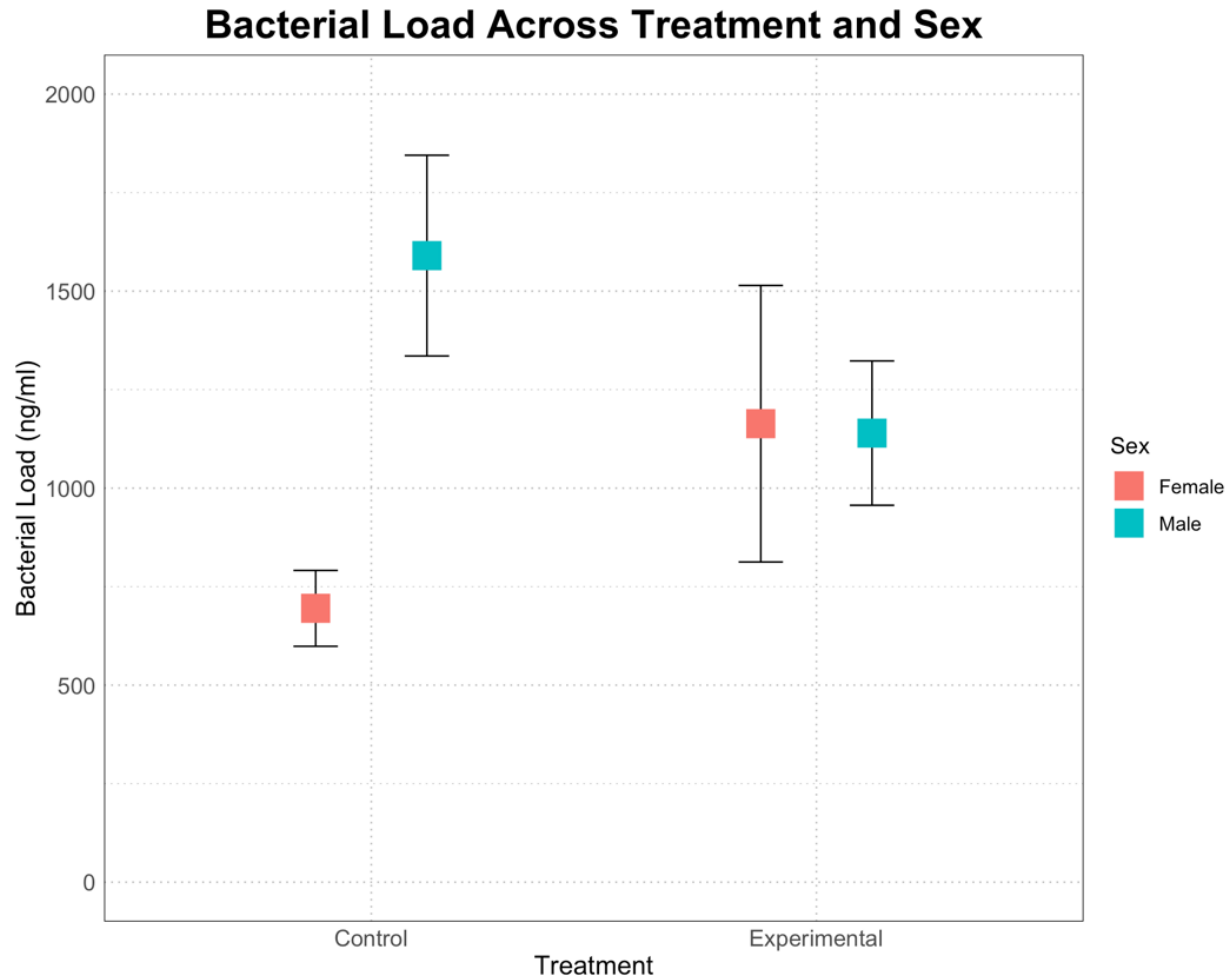


Figure 7 Caption: Bacterial DNA Density Across Sex and Treatment

Comparison of bacterial DNA (ng/ml) found on the replicates' carapace across treatment and sex. Based on a multiple linear regression, the interaction of treatment and sex had a significant influence on bacterial prevalence (p-value: 0.03021), while sex as a lone variable, had an even more significant influence (p-value: 0.01372). The control (left) and the treatment (right) separate the replicates by experimental treatment, while female (red) and male (blue) are further designated within such plots. Error bars represent 1 SE.

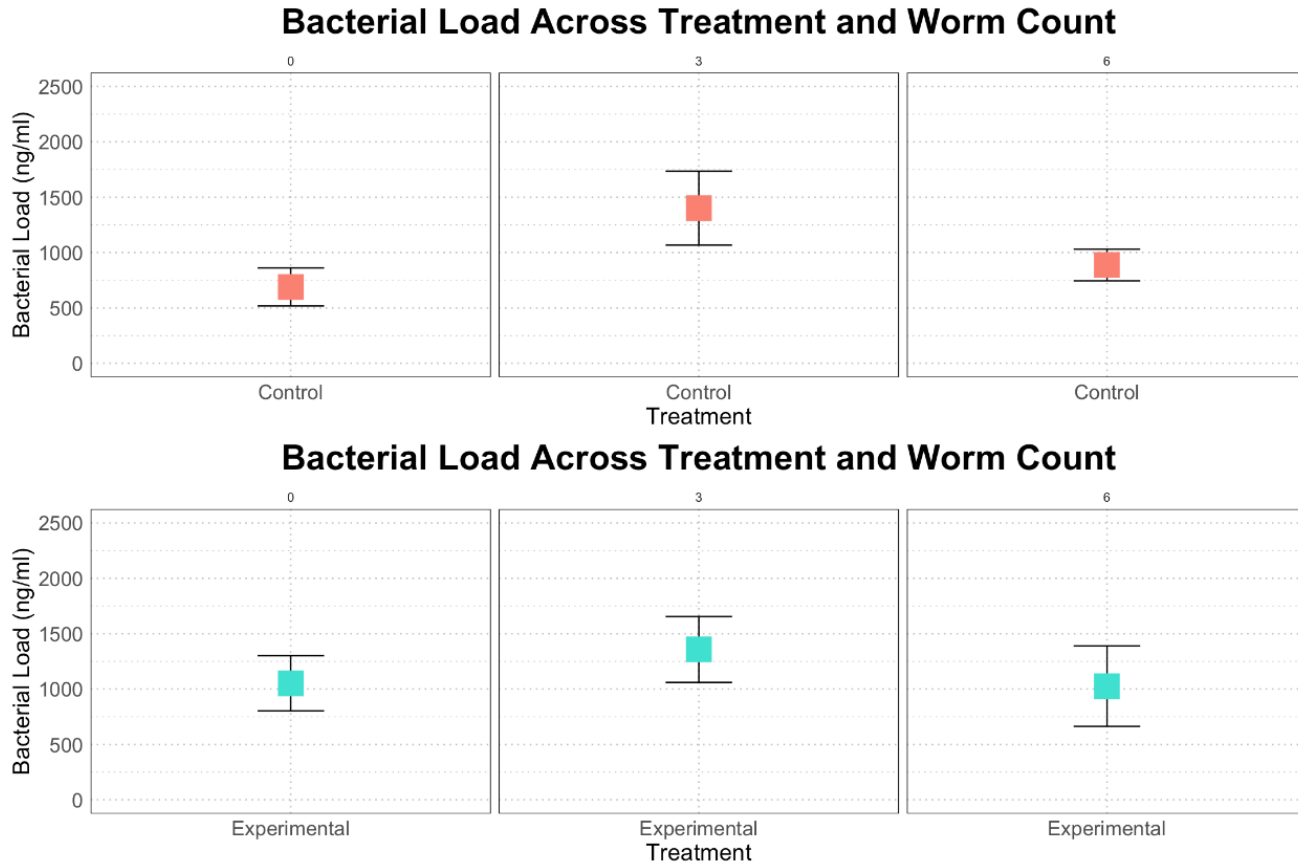


Figure 8 Caption: Bacterial DNA Density Across Treatment and Worm Count

Comparison of bacterial DNA (ng/ml) found on the replicates' carapace across treatment and Worm Count. Based on a multiple linear regression, the interaction of treatment and worm count had a complete lack of influence on bacterial prevalence (p-value: 0.03021). The control (top) and the treatment (bottom) separate the replicates by experimental replicates, each window represent worm densities; 0, 3, and 6, respectively. Error bars represent 1 SE.

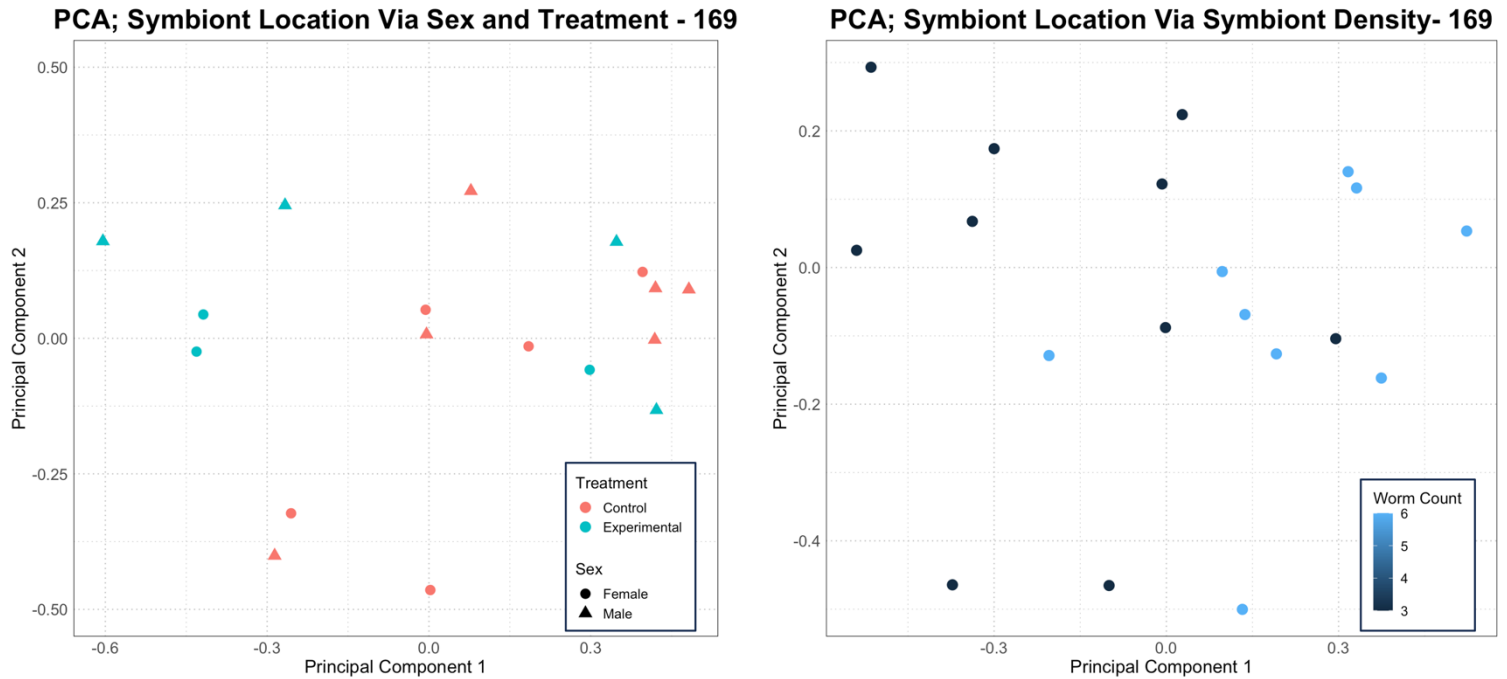


Figure 9 Caption: Principal Component Analysis Quantifying Symbiont Microhabitat Location

A permutational multivariate analysis (PERMANOVA) of symbiont microhabitat selection. Variation of symbiont location overtime was assessed using a Principal Component Analysis (PCA) and compared using a PERMANOVA through *adonis2()*. On the left, Replicates are designated by experimental treatment (Control: red and Treatment: blue) and sex (Male: triangles and Female: circles). The treatment and sex variables were found to have no significant influence on symbiont location; however, using the same statistical test, initial worm count proved to have a highly significant influence (p-value: 0.001).

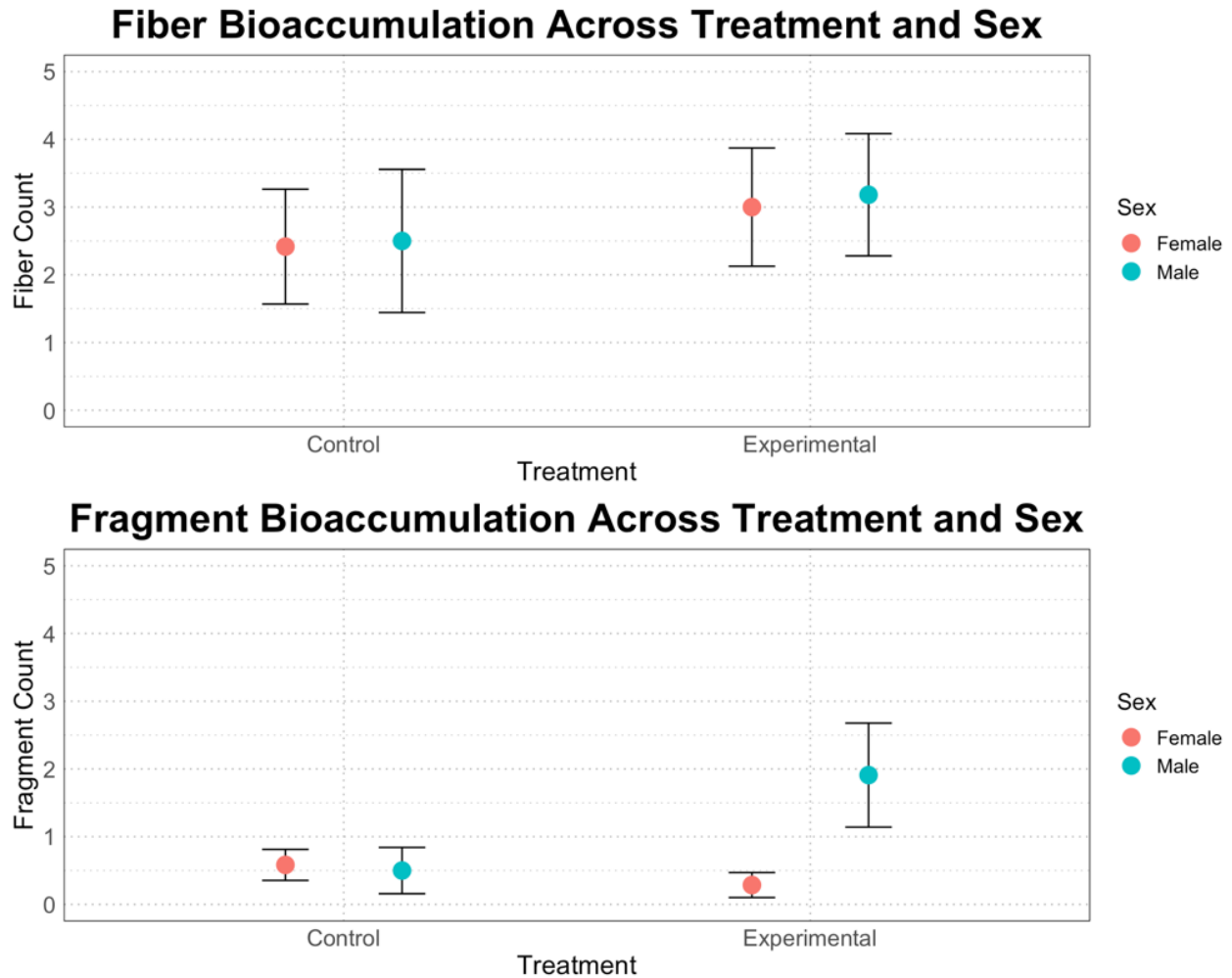


Figure 10 Caption: Difference of Microplastic Bioaccumulation Across Sex and Treatment

Comparison of plastic bioaccumulation within crayfish hosts across treatment and sex. Using a simple linear regression, the bioaccumulation of plastic particles was determined to lack any influence from treatment, sex, or worm count.

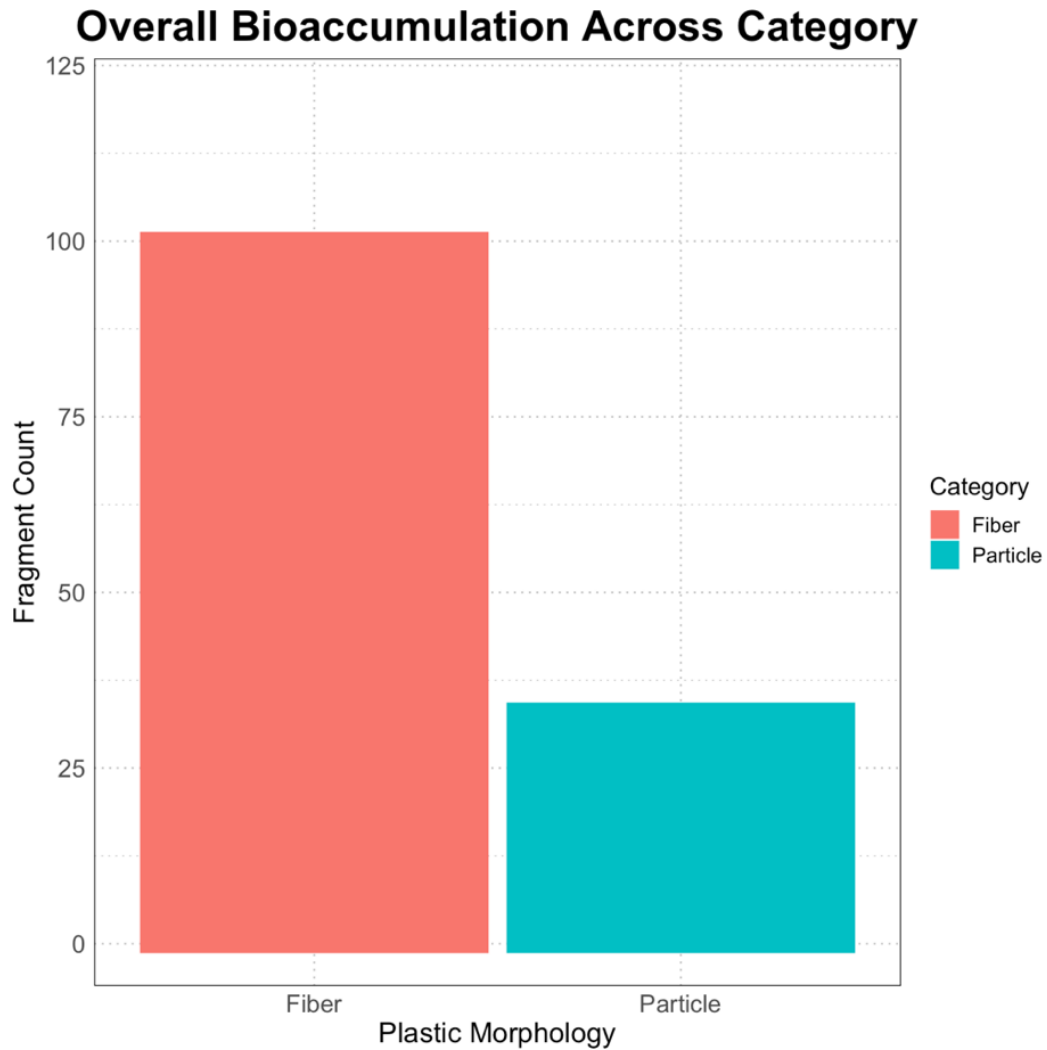


Figure 11 Caption: Difference of Microplastic Bioaccumulation Across Different Morphologies

Comparison of plastic bioaccumulation within crayfish hosts across plastic morphologies. Using count data, we determined plastic with fibrous morphology made up 75.2% of the total plastic bioaccumulation, while plastic with particle-like morphology made up the remaining 24.8%. Our findings support previous evidence suggesting fibrous plastic bioaccumulate more than that of particle (Lozano-Hernandez et al. 2021, Li et al. 2018). Error bars represent 1 SE.

Equations

Equation 1: Chlorophyll A Concentration

$$\text{Chlorophyll } a \left(\frac{\mu g}{cm} \right) = \frac{26.7(E_{664b} - E_{665a}) \times V_{ext}}{\text{carapace } (cm^2) \times L}$$

