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Effects of Environmental Aging on the Acute Toxicity and Chemical Composition of Various Microplastic Leachates

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By

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Master's Thesis

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Allie M. Johnson
November 26, 2021

Effects of Environmental Aging on the Acute Toxicity and Chemical Composition of Various Microplastic Leachates

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

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Allie Johnson
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Abstract

Microplastics have become ubiquitous in the environment and have been intensively studied in recent years. Researchers have documented several toxic effects to aquatic organisms, but the role of different microplastic properties in the toxic responses is not well understood. Toxic effects can be altered by the microplastic pieces themselves, by chemicals from the microplastics, and by sorbed environmental organic or metal pollutants, which microplastics concentrate and transport. I decided to focus on the chemical aspect of microplastic toxicity by observing responses of a marine invertebrate when exposed to several types of leachate solutions, created by soaking microplastics in seawater for 48 hours. Juvenile mysids (*Americamysis bahia*) were exposed to various types of polystyrene (PS), polyethylene terephthalate (PET), polycarbonate (PC), polyester, and polyacrylonitrile microplastic leachates for 4 days and mortality was recorded. Toxicity tests were also performed on environmentally aged versions of each leachate, which were created using microplastics deployed in Bellingham Bay for 70-76 days. I used log-logistic models to model concentration-response relationships and determined the concentration of leachate that results in 50% mortality (the LC_{50}). LC_{50} values were compared with log-likelihood ratios to determine significant differences between leachate types and aging conditions. Non-aged and aged versions of the PS, PET, and PC leachates caused no significant mortality at concentrations as high as 100 grams per liter. All types of non-aged microplastic fiber leachates caused mysid mortality, with red polyacrylonitrile being the most toxic, followed by green polyester and white polyester. In contrast, all three of the same fiber leachates that were aged caused no mortality at concentrations as high as 50 grams per liter, suggesting that the acute toxicity of microplastic fibers decreases after being subject to environmental processes. Chemical analysis of the fiber leachate types was performed with LC-QTOF-MS, and it shows unique chemical features differentiating the toxic leachates (created from non-aged fibers) and the non-toxic leachates (created from aged fibers). My results have important implications for future microplastic toxicity studies and regulations on plastic debris, suggesting more study of microplastic fibers is warranted, and the role of chemicals in microplastic toxicity needs consideration in addition to physical hazards of microplastic ingestion.

Keywords: Microplastics, environmental aging, microplastic leachate, microplastic fibers, mysid shrimp

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1. Introduction

1.1 Background

1.1.1 Plastic Pollution

Since advent of the first fully synthetic plastic in the early twentieth century, plastic use has become commonplace in virtually every aspect of our lives, and methods to curb and recycle plastic waste are unable to keep up. Mismanaged plastic waste (defined as plastics not currently in use, fully contained in landfills, or put towards other post-consumer applications) is projected to triple from 2019 to 2060 (Lebreton and Andrady, 2019), and the amount of plastic waste that entered the oceans from land in 2010 is between 20 and 2,000 times more than the amount of plastic we have measured in the oceans (Jambeck et al. 2015).

Due to insufficient management of plastic waste across the globe, waterborne plastic debris has become ubiquitous in the environment. Eriksen et al. (2014) estimated that there are more than 5 trillion plastic pieces in the ocean, collectively weighing hundreds of thousands of tons – and this was a conservative estimate. Another estimate puts the number of pieces present between 15 and 51 trillion and this massive reserve of plastic in the oceans is projected to continually increase over the years (Van Sebille et al. 2015). In addition to being an eyesore in ocean garbage patches or when washing up on beaches, large pieces of plastic debris have detrimental physical effects on sea life upon ingestion or entanglement (Gregory, 2009).

Researchers studying the issue of plastic pollution tend to agree that an urgent improvement of plastic pollution mitigation efforts should be a high priority (e.g. Jambeck et al. 2015; Borrelle et al. 2020; Lau et al. 2020; MacLeod et al. 2021), rather than a focus on cleaning up what is

already in the ocean. To highlight the importance of reducing and recycling plastic waste, it is useful to study how detrimental plastic pollution is to organisms and to the environment. Even if releases of plastic debris to the environment were to be immediately stopped, the issue of plastic pollution in the environment will persist well into the foreseeable future. This is because plastic litter can persist in oceans for hundreds to thousands of years (Barnes et al. 2009), slowly breaking into the small pieces that are the subject of study in this thesis.

1.1.2 Microplastics and Associated Chemicals

As plastic debris fragments and degrades into smaller pieces, it becomes readily transported throughout the environment and more bioavailable to organisms. Tiny plastic pieces, termed microplastics, are generally defined as less than 5 mm in diameter (Syberg et al. 2015). Microplastics are transported through all environmental compartments, including air (Sommer et al. 2018), water (Cole et al. 2011), sediments (Burns and Boxall, 2018), and from organism to organism (Setälä et al. 2014). They have been found deep in Arctic oceans (Lusher et al. 2015), high in mountain glaciers (Ambrosini et al. 2019), and in virtually every region in between. Over 220 species are reported to have consumed microplastics (Lusher et al. 2017), which can be mistaken for food and cause false satiety or disrupt filter feeding (Teuten et al. 2009). Humans also consume thousands to tens of thousands of microplastics every year by eating seafood, drinking, and breathing (Cox et al. 2019).

Since a microplastic can be any small plastic particle, more specific terms exist for different kinds. Primary microplastics are plastics that were manufactured to be small, such as pre-production plastic pellets or microbeads in personal care products. Secondary microplastics result from environmental degradation of larger plastics, such as fibers from discarded fishing nets, or

fragments of soda bottle caps (Syberg et al. 2015). Microplastics can be further differentiated into several categories by source, shape, age, color, polymer, and more (Hidalgo-Ruz et al. 2012). This thesis focuses on two stages of production, three shapes, five polymers, and the impact of weathering, which are described in detail in section 1.2.

The number of studies investigating the detrimental effects that microplastics cause in aquatic organisms is rapidly growing alongside published papers about ocean plastics, which increased from 46 papers in 2011 to 853 papers in 2019 (UNESCO, 2021). So far, microplastics have been shown to bioaccumulate in bivalves (Von Moos et al. 2012; Bour et al. 2018) as well as induce many toxic effects, including endocrine disruption, reproductive and feeding changes (Sussarellu et al. 2016), decreased energy reserves (Bour et al. 2018), and inflammation and histopathological changes (Von Moos et al. 2012; Optiz et al. 2021), all due to microplastic ingestion. Several of these effects (in addition to mortality) have also been observed in cladocerans (Lithner et al. 2012; Jemec et al. 2016; Kim et al. 2017; Ziajahromi et al. 2017; Kokalj et al. 2019), barnacle larvae (Li et al. 2015), amphipods (Au et al. 2015), copepods (Bejgarn et al. 2015), shrimps (Gray and Weinstein, 2017; Wang et al. 2020), mysids (Wang et al. 2017; Lee et al. 2021) and fish (Oliveira et al. 2013; Luís et al. 2015; Kokalj et al. 2019; Qiao et al. 2019), with some detrimental effects occurring even if the microplastic pieces were not ingested (e.g. Lithner et al. 2012; Bejgarn et al. 2015; Li et al. 2015).

Though many plastic polymers that make up microplastics are biologically inert, microplastics can still be associated with biologically active chemicals. During plastic production, it is common to include chemical additives (e.g. phthalates, bisphenol A, chemical dyes) to customize properties of the resulting products (Hahladakis et al. 2018), such as increasing flexibility or changing color. Microplastics release these additives into the environment upon

degradation in water or sediments (Cole et al. 2011; Bandow et al. 2017), upon ingestion by organisms (Bakir et al. 2014), and upon formation of biofilms on the plastic surface (Rummel et al. 2017). Many of these additives are toxic to aquatic organisms (Lithner et al. 2012; Bejgarn et al. 2015; Li et al. 2015; Groh et al. 2019), so they can be partially responsible for toxic responses to microplastic exposure.

Microplastics may also facilitate bioaccumulation of other environmental contaminants, such as organic pollutants (Rochman et al. 2012; Wardrop et al. 2016; Müller et al. 2018; Guo and Wang, 2019; Zhou et al. 2020) and dissolved metals (Luís et al. 2015; Turner and Holmes, 2015; Kim et al. 2017; Munier and Bendell, 2018) already present in water because these contaminants concentrate on plastic surfaces. Organisms can then ingest the chemical-laden microplastics, which may release sorbed chemicals into the organism and introduce a new route of internal chemical exposure separate from contaminated prey ingestion and water-borne chemicals. However, the significance of the role of microplastics as vectors for contaminants into organisms is disputed (Koelmans et al. 2016) and conflicting study results have made it difficult to form a scientific consensus on whether microplastics increase or decrease toxicity of these substances (Rodriguez et al. 2019). Laboratory studies have reported both enhanced and mitigated toxicity of contaminants when exposed to organisms in combination with microplastics (Oliveira et al. 2013; Kim et al. 2017; Guilhermino et al. 2018; Zocchi et al. 2019), with results depending on the types of contaminants, microplastics, and test organisms. For example, more hydrophobic (nonpolar) contaminants, such as PCBs, might tend to stay sorbed to a similarly hydrophobic plastic surface, while more hydrophilic (polar) contaminants, such as ionic metals, could be more readily released from a plastic surface into the acidic gut environment of a test organism. If there is anything the studies agree on, it is that the vector role of microplastics warrants further study.

1.1.3 The Many Facets of Microplastic Toxicity and Research Implications

Due to the vast possibilities and combinations of types of microplastics, their additives, and contaminants from the environment sorbed on the plastics, there is still much to be discovered about toxic effects they can cause to aquatic organisms. Assessing the toxicity of individual chemicals is already complicated; assessing the toxicity of microplastics as a whole involves assessing the toxicity of potentially hundreds of associated chemicals, with the additional caveat that the microplastics themselves do not behave like chemicals; they are physical pieces that can be ingested, but also can be a source and sink for chemicals.

As discussed in the previous section, the term microplastics covers all tiny plastic pieces, which widely vary in size, shape, density, crystallinity, degree of degradation and biofouling, and chemical composition (Lambert et al. 2017). Any of these properties can potentially influence how toxic microplastics are to organisms. To date, studies show that microplastic polymer (Lithner et al. 2012; Au et al. 2015; Bejgarn et al. 2015), shape (Gray and Weinstein, 2017; Ziajahromi et al. 2017; Qiao et al. 2019), size (Gray and Weinstein, 2017; Bour et al. 2018), and environmental aging (Kokalj et al. 2019) all cause significant changes in toxic response. Clearly, it is ill-advised to consider microplastics as a single emerging toxicant – rather, findings to date show that toxicity studies should be conducted on many different kinds of microplastics to better understand the range of potential detrimental effects on organisms and ecosystems.

Microplastic studies tend to use brand new, additive-free (termed virgin microplastics in this thesis) plastic microspheres of one plastic polymer type (e.g. Oliveira et al. 2013; Luís et al. 2015; Sussarellu et al. 2016; Wardrop et al. 2016; Opitz et al. 2021), which are not representative of the countless types, shapes, and degradation states of plastic pieces found in the environment.

Therefore, the applicability of these study results is limited, since results from one type of plastic cannot be used to discern possible toxic effects from other plastic types.

In addition, microplastic toxicity studies often use only one or a small number of exposure concentrations (e.g. Von Moos et al. 2012; Sussarellu et al. 2016), but it is necessary to have many (five or more) concentrations to elucidate statistically significant concentration-response relationships and EC_x/LC_x values (Environment Canada, 2005). The use of concentration and concentration-response curves is a fundamental practice of ecotoxicology, and the usefulness of these curves extends to environmental regulation and ecological risk assessment. For example, the United States Environmental Protection Agency requires sufficient dose response data to set applicable Water Quality Criteria for aquatic life (Stephen et al. 1985).

To inform future regulation and risk assessment for microplastics, more comprehensive, usable, and comparable toxicity data needs to be generated. As of now, the scientific community is far from having a complete understanding of how different microplastic properties, additives, or other sorbed contaminants affect toxicity, and this thesis is intended to aid in filling the many knowledge gaps surrounding how variable microplastic toxicity can be.

1.1.4 Microplastic Leachate Toxicity

For purposes of this thesis, I was particularly interested in plastic additives within, and other chemicals sorbed to, microplastics. I wanted to determine whether the release of these chemicals from microplastics contributed to toxic responses seen in marine organisms. Few existing microplastic toxicity studies are able to distinguish between physical (resulting from ingestion, such as false satiety or internal organ damage) and chemical (resulting from exposure to monomers, degradation products, chemical additives, or sorbed environmental contaminants)

toxicity of microplastics (Zimmermann et al. 2020); I chose to focus on only the chemical aspects of toxicity.

One way to determine the role of plastic additives and sorbed contaminants from the environment on microplastic toxicity is to perform toxicity testing with leachates, instead of the microplastics themselves. Even if organisms do not ingest microplastic particles, they have the potential to be exposed to any chemicals released from the microplastics into the surrounding environment.

Several recent studies have investigated the toxicity of macro and microplastic leachates to aquatic organisms. These leachates have been found to induce many types of detrimental effects on a few species and life stages. Reported sublethal effects in freshwater organisms include decreased reproductive output in daphnids (Zimmermann et al. 2020), increased fathead minnow larvae deformities (Bucci et al. 2021), increased DNA fragmentation in apoptotic germ cells of nematodes (Ficociello et al. 2021), and inhibition of photosynthesis in microalgae (Luo et al. 2019; Luo et al. 2020). Reported sublethal effects in saltwater organisms include mussel embryo abnormalities (Silva et al. 2016), sea urchin embryo and larvae abnormalities and inhibition of development (Oliviero et al. 2019; Rendell-Bhatti et al. 2021), decrease in growth of sea urchin larvae (Cormier et al. 2021), decreased settlement of barnacle cyprids (Li et al. 2015), and decreased predator avoidance in aquatic snails (Seuront, 2018). Plastic leachates have also been found to cause mortality in freshwater daphnids (Lithner et al. 2009; Dave and Aspegren, 2010; Lithner et al. 2012; Zimmermann et al. 2020), as well as saltwater copepods (Bejgarn et al. 2016), barnacle nauplii (Li et al. 2015), and mussel embryos (Silva et al. 2016).

Results from these studies almost always show that toxic effects differ depending on the polymer type, additive content, environmental aging, and artificial aging of plastic used to create the leachates. For example, Lithner et al. (2009) investigated the toxicity of leachates generated from consumer products and found that polyvinyl chloride (PVC) and polyurethane (PUR) plastic leachates caused mortality in *D. magna*, but leachates from polyethylene, polypropylene (PP), and four other common plastic polymer types did not. Oliviero et al. (2019) found that sea urchin (*Paracentrotus lividus*) larval development was inhibited by microplastic leachate created from PVC consumer items containing additives, while it was not inhibited by leachate created from virgin (also known as pre-consumer or pre-production) microplastics of the same polymer type. Overall, leachate studies using plastics with little to no additives tend to find sublethal or no effects (e.g. Langlet et al. 2020; Rendell-Bhatti et al. 2021; Seuront et al. 2021) while studies using plastics with additives tend to find greater sublethal effects and/or mortality (e.g. Lithner et al. 2012; Li et al. 2015; Bejgarn et al. 2016), indicating that toxic chemicals associated with microplastics can migrate into surrounding water, especially when microplastics generated from consumer plastics, which include additives, are involved.

There is less of a consensus on how leachates from aged (in the environment or artificially) versus unaged microplastics differ. For example, Rummel et al. (2019) found that in cell-based bioassays, more oxidative stress response was observed if leachates from artificially aged microplastics were used in the bioassays, compared to leachates from the same versions of those microplastic types that did not undergo the artificial ultraviolet light aging process. However, Bejgarn et al. (2016) found that depending on the type of consumer microplastics they used to create their leachates, aging with artificial sunlight either increased, decreased, or had no effect on the toxicity of the resulting leachates to a marine copepod (*Nitocra spinipes*). Rendell-Bhatti et al.

(2021) found that leachate made from environmentally aged polyethylene nurdles caused developmental abnormalities in sea urchin (*Paracentrotus lividus*) embryos, but leachate from virgin low-density PE nurdles did not; they noted that the aged nurdles contained polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs). Silva et al. (2016) similarly found that leachate made from environmentally aged pellets caused more mortality and abnormalities in brown mussel (*Perna perna*) embryos than leachate made from unaged polypropylene (PP) pellets, and Bucci et al. (2021) found that leachates from environmentally aged plastic fragments caused more deformities in fathead minnow (*Pimephales promelas*) larvae than leachates from unaged PE and PP. Though most studies so far show that leachate toxicity tends to increase with microplastic aging (either in the environment or artificially), findings are not completely consistent, and more microplastic types and organisms should be considered. Aging clearly affects toxicity of microplastic leachates, and therefore warrants further consideration.

Many studies investigating microplastic leachates are underway or have been published recently, but it is difficult to compare results among them. Though existing studies wisely compare between microplastic leachates created from different polymer types and aging states, they often do not compare between microplastic sizes – and different studies use different sizes. The smaller microplastics are, the more surface area is available for leaching, and surface area is an important aspect to consider when assessing the risks of microplastics (Hidalgo-Ruz et al. 2012; Syberg et al. 2015; Lambert et al. 2017). The leachates themselves are created in the lab at different temperatures, exposure times, salinities, pH values, mixing speeds, lighting regimes, and artificial aging scenarios; using different leachate preparation methods affects the amounts and types of chemicals present in the leachates (Luo et al. 2019) and leachate toxicity (Lithner et al. 2012; Bejgarn et al. 2016; Luo et al. 2020; Rendell-Bhatti et al. 2021). Concentrations of microplastics

in leachates are mostly measured by mass per unit volume (Lithner et al. 2009; Lithner et al. 2012; Bejgarn et al. 2016; Oliviero et al. 2019; Rummel et al. 2019; Pflugmacher et al. 2020), but also volume per unit volume (Seuront, 2018; Langlet et al. 2020; Rendell-Bhatti et al. 2021; Seuront et al. 2021), and sometimes even surface area per unit volume (Li et al. 2015), making toxicity values generated from some studies impossible to compare with others. Sometimes the particles are not removed from the water prior to the beginning of toxicity tests (Dave and Aspegren, 2010; Silva et al. 2016), which means physical damage and ongoing leaching may be important. These inconsistent methods introduce uncertainty when comparing study results. Forming a scientific consensus on the hazard and risk of these chemicals to aquatic life will remain challenging without more consistency in plastic leachate study methodologies.

1.2 Study Design & Objectives

Understanding the facets of microplastic toxicity to marine organisms is incomplete. With this work, I intended to find concentration-response data for microplastics to better understand microplastic-associated chemical toxicity to help fill gaps in our current knowledge of the role of additives and other sorbed chemicals in microplastic toxicity and to allow decision makers to predict effects at environmentally expected concentrations. By monitoring the responses of a marine mysid (*Americamysis bahia*) exposed to leachates created from unaged microplastic and microplastics environmentally aged in a marine site, and at a wide range of concentrations and plastic types, I wanted to address three hypotheses.

My first null hypothesis was that toxic effects of microplastic leachates on mysids do not differ between plastic types. Different kinds of plastics contain different chemicals and additives,

which in other studies, has led to different types and degrees of toxic effects. To test this hypothesis, I included eight different microplastic types in this study. Within these eight, there are five represented polymer types (polystyrene [PS], polyethylene terephthalate [PET], polycarbonate [PC], polyester, and polyacrylonitrile) and two represented production types (virgin and consumer). Three types of microplastic fibers, which are underrepresented in toxicity studies despite often being reported in monitoring studies to be a commonly found microplastic type (Hidalgo-Ruz et al. 2012; Burns and Boxall 2018; Cox et al. 2019), are also included within my eight types. Fibers have also been reported as more toxic than particles for multiple aquatic species (Gray and Weinstein, 2017; Ziajahromi et al. 2017; Qiao et al. 2019).

My second null hypothesis was that microplastic leachate toxicity to mysids did not differ between environmentally aged and non-aged plastics. For each of the eight microplastic types in my study, I also included versions that were deployed in the environment for 76 days, for a total of sixteen treatment types. This was done to assess if toxicity changes when the plastics are exposed to environmental aging processes, which may cause plastic additives to be released and result in contaminants sorbing from the environment (which may then desorb in surrounding waters or inside organisms when ingested). The aging aspect of my work is more environmentally relevant and realistic compared to most tests in the literature.

My final null hypothesis was that toxic responses were not attributable to chemicals released into leachates by the microplastics. To test this, I set aside leachate samples of all sixteen microplastic treatment types and analyzed them for trace organic contaminants and dissolved metals. Then, I determined which contaminants and metals were only present in the toxic leachates. Many existing studies do not determine what chemicals are present in their leachates, and I wanted

to investigate and characterize the contaminants in my leachates that were responsible for observed toxicity.

To help answer these questions and be able to test so many plastic types, I chose mysids as my model toxicity organism. Mysids are an example of organisms reported to have consumed microplastics (Setälä et al. 2014; Lee et al. 2021). They are shrimp-like crustaceans, also known as opossum shrimp, that inhabit estuarine and marine waters. Their sensitivity to microplastic exposure is not well-known, though their use as test organisms in toxicology is widespread due to the convenience, short test duration, and the mortality endpoint (USEPA 2016). There are only two toxicity tests with microplastics and mysids documented in the literature, and neither used leachates. In the first study, mysids (*Neomysis japonica*) were exposed to virgin 5 μm fluorescent PS microspheres for 72 hours using only 3 test concentrations of 50, 500, and 1000 μg/L, and 30% mortality was found at the highest concentration (Wang et al. 2017). The authors suggested that toxicity was due to physical effects from excessive ingestion of plastics and did not consider chemical toxicity of MPs that may occur because of plastic degradation and chemical release. In the second study, juvenile and adult mysids (*Neomysis awatschensis*) were exposed to two sizes of PS microbeads (1 and 10 μm) in both acute and chronic tests using six test concentrations of 1,000, 5,000, 10,000, 50,000, 100,000, and 500,000 microplastic particles per mL (Lee et al. 2021). Juvenile mysids were more sensitive to microplastics, though both the adults and juveniles died and had decreased feeding rates. Like the first study, there was no consideration of the chemical aspect of microplastic toxicity. My thesis is the first known study investigating the chemicals in microplastic leachates to that may be toxic to mysids.

2. Materials and Methods

2.1 Microplastic creation

2.1.1 Microplastic particles

Polystyrene (PS) nurdles were obtained from Pinwheel Blankets (Plymouth, MA), while polyethylene terephthalate (PET, in the form of white pellets) and polycarbonate (PC; LEXAN 141R-111) nurdles were obtained from wholesale vendors on eBay. Two "consumer" plastics, in the form of heavy-duty black PS forks (YR Foods) and PET disposable cup lids (Dart Container Corp, Mason, MI), were purchased from a Smart Foodservice Warehouse store located in Bellingham, WA. These five plastic types were used to create 5 types of microplastic particles: virgin PS, consumer PS, virgin PET, consumer PET, and virgin PC (**Figure 1**).

A Blendtec® Total Classic Blender was used to grind plastic nurdles into smaller pieces, in a similar process as Bejgarn et al. (2015). Prior to blending, plastic nurdles were scooped into ice cube trays filled with Barnstead Nanopure™, hereafter referred to as Nanopure water. Filled trays were stored for at least 12 hours at -20 °C so the water would freeze, making the plastics more brittle; this was done in light of findings by Eitzen et al. (2019), who showed that yield of smaller microplastics increases with decreasing plastic temperature and increased pre-cooling times. Plastic-water ice cubes were placed in the blender 6 at a time with approximately 1 cup of Nanopure water and then blended for a total of 2 minutes and 10 seconds with the "Frozen Treats" cycle. The resulting solution from the blender was poured through 1000 μm and 63 μm sieves to separate microplastics by size. This process differed slightly for the consumer plastics, which first needed to be blended enough to fit into the ice cube trays; 6-8 forks or 8-10 lids were placed in the

Figure 1. Five plastic types prior to grinding into microplastic particles in a Blendtec blender. The top row includes (from left to right) virgin polystyrene, virgin polyethylene terephthalate, and virgin polycarbonate. The bottom row includes (from left to right) consumer polystyrene as black disposable forks, and consumer polyethylene terephthalate as disposable cup lids.

blender at a time, blended for 2 minutes and 10 seconds, and then added to the ice cube trays, after which they were frozen and blended as described above.

Once wet sieved, the microplastic particles were spread onto aluminum foil lined trays and dried for up to 12 hours 80 °C in a VWR drying oven. Dry microplastic particles were sieved again, and the 63-1000 μm size fraction was added to amber jars and stored at room temperature in a dark place until use.

The impact-resistant properties of PC made it impossible to break down its plastic nurdles in the blender. These nurdles, which in all dimensions ranged from 0.2 – 0.5 cm, were used as is for leachate creation and toxicity testing.

2.1.2 Microplastic fibers

Three microplastic fiber types (red polyacrylonitrile, white polyester, and green polyester) were created from red 100% acrylic yarn (Caron® One Pound™ Scarlet), white 100% polyester felt, and green 100% polyester felt, respectively. All fabrics and yarn were purchased in August 2019 from Jo-Ann Fabrics and Crafts in Bellingham, WA (**Figure 2**).

The Blendtec blender was used to separate the yarn and felt into small fibers. Use of a small ball mill was also attempted to grind fibers, but the process was ultimately not scalable to produce the several pounds of fibers needed for experiments. Prior to blending, felt was cut into squares with maximum dimensions of approximately 1 x 1 centimeters. Yarn was also cut into lengths of about 1 centimeter to avoid tangling of fibers around blender blades. Felt squares and yarn clippings were placed into gallon size plastic bags, filled with Nanopure water, and stored at 4 °C for up to 12 hours to cool, which helped prevent blender overheating. One handful of wet felt

Figure 2. Red acrylic yarn and white polyester felt prior to separation of fibers in a Blendtec blender. The green polyester felt (not pictured) looked similar to the white felt, except for the difference in color.

squares or yarn clippings was blended at a time with enough Nanopure water to submerge them, using the "Batter" setting once for a total of 25 seconds. The blended fibers were squeezed with clean gloved hands to remove as much water as possible.

Fibers were dried on aluminum foil lined trays for 8-12 hours at 80 °C in a VWR drying oven. Dry microplastic fibers were wrapped in foil, placed in plastic bags, and stored in a dark place at room temperature until use.

2.2 Field deployment

I used a subset of the microplastic particles and fibers I created in a field deployment, located near Boulevard Park, Bellingham, WA (**Figure 3**). Approximately 630 grams of each of the eight microplastic types were placed in nylon monofilament filter bags (Duda Energy, Decatur, AL) which were sewn closed afterwards. The filter bags had a 10 μm mesh size, which allowed seawater in, but kept sea life and large sediments out while also preventing the contained microplastics from escaping (**Figure 4**).

On September 27th, 2019 at morning low tide (-0.24 feet), the microplastic deployment bags were rolled up and attached to an anchor with steel cable and zip ties. The anchor was buried, and a cable was attached so that when the bags of microplastics were submerged, they were suspended 1-2 feet above sediments. Green polyester and consumer PET deployment was done in the same place on October 3rd.

Weather and tidal conditions in the vicinity of the deployment site are summarized in **Table 1**. Eleven days prior to microplastic deployment, a major rainfall event of 1.32 inches occurred in

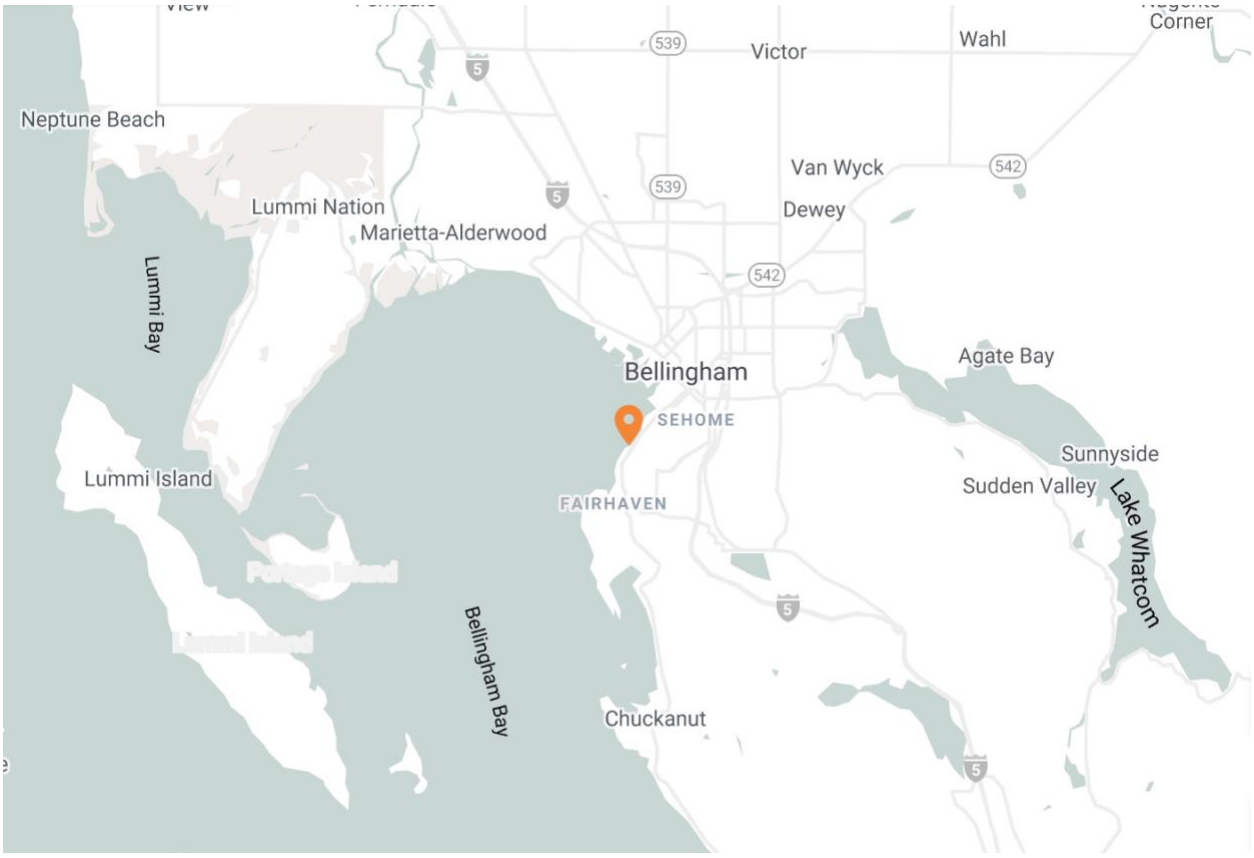


Figure 3. Location of nearshore deployment site in Bellingham Bay, Washington, United States (48.733058, -122.500650). Eight semipermeable nylon bags containing five types of microplastic particles and three types of microplastic fibers were anchored here 1-2 feet above sediments at low tide from Sept 27 - Dec 11, 2019.

Figure 4. Microplastic particles and fibers contained in 10 μm nylon monofilament filter bags immediately before deployment in Bellingham Bay, WA. Bags were sewn closed at the top and fabric loops included to allow attachment to an anchor setup.

Bellingham Temperature and Precipitation, September 27th – December 11th, 2019				
	Mean	Standard Deviation	Maximum	Minimum
Air Temperature (°C)	8.1	3.2	13.5	-0.1
Water Temperature (°C)	9.7	1.6	14.1	7.2
Precipitation (inches)	0.10	0.24	1.82	0.00
Bellingham Bay Tidal Conditions, September 27th – December 11th, 2019				
	Mean Lower Low Water	Mean Higher High Water	Lowest Tide	Highest Tide
Tidal Elevation (feet)	0.44	8.53	-2.06	9.49

Table 1. Summary of weather and tidal conditions in Bellingham, WA, United States during a 76-day microplastic deployment period near Boulevard Park, Bellingham, WA. Air temperature and precipitation measurements obtained from Bellingham International Airport Weather Station. Water temperature readings obtained for Bellingham at seatemperature.info. Tidal conditions were obtained for the Bellingham Bay region at tides.net.

Bellingham. Tidal conditions at the deployment site varied widely, with a minimum of -2.06 feet and a maximum of 9.49 feet throughout the deployment period (Data source: TIDES.net). Due to low winter tides, sometimes the bags were not submerged in seawater. Since they were deployed at -0.2 feet and suspended an approximate average of 1.5 feet above sediments, they were likely exposed (out of water and sitting on top of sediments) at tides lower than -1.7; this occurred 3 times throughout the 76-day deployment period. It rained for 36 of the deployment days, with a median of 0.11 inches of rain per precipitation day (minimum 0.1 inches, maximum 1.82 inches). The average air temperature across the deployment period was 8.1 °C, and the average water temperature was 9.7 °C (Data sources: Bellingham International Airport Weather Station, seatemperature.info). Both air and water temperature decreased gradually during the deployment period, as was expected during the fall season.

On December 11th, 2019 at evening low tide (-1.44 feet), all eight bags of microplastics were retrieved from the deployment site. As bags were separated from the anchor, biofouling organisms were scraped off and sediments were washed off with site water. Bags were taken immediately back to the laboratory, where as much water as possible was squeezed out with clean gloved hands. Microplastics were kept in their nylon deployment bags, wrapped in aluminum foil, and stored at -20 °C. When needed for toxicity testing or chemical analysis, microplastics were removed from bags and allowed to air-dry at room temperature for 4 days prior to use.

2.3 Microplastic leachate preparation and characterization

2.3.1 Glassware cleaning

Glassware was used whenever possible in all the following procedures. Unless otherwise stated, any glassware I used for leachate preparation, chemical analysis, and toxicity testing was soaked in a 10% nitric acid bath for at least 24 hours to remove metals. Glassware was also acetone rinsed, then heated in a muffle furnace for at least 8 hours at 550 °C to remove organics.

2.3.2 Leachate preparation

To create leachates, I soaked microplastic particles and fibers in 25 ppt 0.2 μm filtered natural seawater from Shannon Point Marine Center (SPMC) in Anacortes, WA. The seawater-microplastic mixtures were left at 21 °C on a lab shaker at 100 rotations per minute in darkness for 48 hours. Leaching containers were covered with Parafilm® and clean foil to prevent evaporation of seawater during the leaching period.

After 48 hours, leachate water was separated from particles by pouring the mixture through a 25 μm stainless steel filter screen, followed by a 10 μm nylon filter screen. For the microplastic fibers, stainless steel potato ricers outfitted with the 25 μm and 10 μm filter screens were used to squeeze out seawater. Leachates were used in toxicity tests within 6 (all definitive tests) to 8 hours of separation from microplastics; if holding times extended past 6 hours, which only occurred during range finding tests, leachates were stored at 4 °C until use.

2.3.3 Chemical analysis

Leachates from all sixteen treatment types (at a concentration of 100 g/L for particles and 50 g/L for fibers, **Figure 5**), in addition to a Nanopure blank and SPMC seawater blank, were

Figure 5. All sixteen microplastic types that were used to create microplastic leachates for toxicity experiments. The top row includes non-deployed versions of each plastic type, and the bottom row includes versions of each of the above types that were deployed in Bellingham Bay for approximately 2.5 months. From left to right, the types are virgin polystyrene, consumer polystyrene (from disposable forks), virgin polyethylene terephthalate, consumer polyethylene terephthalate (from disposable cup lids), virgin polycarbonate, red polyacrylonitrile (from acrylic yarn), green polyester (from felt), and white polyester (from felt).

prepped and analyzed for organics via liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) at the University of Washington, Tacoma Center for Urban Waters (CUW) from December 18-25, 2019. Leachate samples were stored in a refrigerator or on ice until they were extracted, which occurred within 20 hours of leachate generation. 900 mL leachate samples were separated into three 300 mL replicates and extracted in pre-conditioned OASIS HLB Solid Phase Extraction (SPE) cartridges over vacuum. Elutions were performed with 10 mL of methanol (Fisher Chemical, Optima LC/MS grade) and sample volumes were decreased with a BioTage TurboVap LV. Concentrated samples were then transferred to autosampler vials, spiked with a QTOF Internal Standard Mix, and analyzed in ESI+ mode. Individual molecular weights and retention times were identified in samples via high-resolution mass spectrometry (HRMS) and chemical features were identified and hierarchically clustered with Euclidean distance by researchers at UW Tacoma. Clustering was done to show how similar the chemical compositions of the leachates were to each other, and to determine if the leachates grouped together by plastic polymer type, aging, or status as a particle or fiber. Results from the blanks were subtracted from the leachate sample results, so the leftover chemical features were those unique to the leachates.

Leachates from all sixteen treatment types (at a concentration of 100 g/L for particles and 50 g/L for fibers), along with four control seawater samples, were also analyzed for dissolved metals at Western Washington University in Bellingham, WA using inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7500ce). The seawater leachate samples were diluted 10x with Nanopure water to keep total dissolved solids below 0.5% and acidified to 5% trace metal grade nitric acid prior to ICP-MS analysis. Chemical analytes included the metals Be, Mg, Al, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo, Ag, Cd, Sb, Ba, Tl, Pb, Th, and U.

2.4 Toxicity testing

2.4.1 Test organisms

I obtained opossum shrimps of the species *A. bahia* (also known as *Mysidopsis bahia*) from Aquatic Biosystems, Inc. in Fort Collins, CO. These mysids were used to start an in-house laboratory culture, maintained as recommended by USEPA (2009) in 0.2 μm filtered natural seawater from Shannon Point Marine Center in Anacortes, WA (the same seawater used to create leachates). Mysid populations were maintained in 10-gallon tanks with an undergravel filter and CORALine crushed coral substrate (CaribSea, Fort Pierce, FL). Dissolved oxygen, pH, and salinity were measured daily in each tank, while nitrate, nitrite, and ammonia were measured occasionally. Mysid cultures were fed less than 24-hour old Artemia sp. nauplii (cysts obtained from Brine Shrimp Direct Inc., Ogden, UT) to excess twice per day. Gravid adults were separated from cultures and a juvenile mysid collection system was created to harvest young mysids for toxicity testing using methods described by Langdon et al. (1996).

When testing requirements for young mysids became too demanding on the cultures, <24 hour old mysids were overnight shipped directly from Aquatic Biosystems and then used in toxicity testing, so the age of the test organisms at test start was <48 hours. The limit test and definitive test results presented in this thesis are from <48 hour test organisms, and the range finding test results are from <24 hour test organisms sourced from the in-house cultures.

2.4.2 Test design

Due to lack of literature on chemical toxicity of the microplastics, environmental conditions, and test organism I used, I adopted a three-step toxicity testing plan for each leachate type. First, limit testing was performed at a single high concentration (50 grams per liter for fibers

and 100 grams per liter for particles), which is greater than I would expect organisms to be exposed to in the environment. If greater than 10% of mysids in any limit test condition died, I proceeded with multiple-concentration range-finding and definitive tests. All limit, range-finding, and definitive tests were conducted for 96 hours with adapted methods from USEPA (2016). Throughout each toxicity test, water temperature was maintained at 25 °C, pH was at 8 ± 0.8, salinity at 25 ppt ± 2 ppt, and dissolved oxygen saturation between 50% and 100%. These parameters are close to what is recommended by the cited EPA methods, which are a water temperature of 25 °C ± 1 °C, a starting pH of between 7.5 and 8.5 with less than a 1 unit change throughout the test, a salinity of 20 ppt ± 2 ppt, and dissolved oxygen saturation between 60% and 100% (see **Table A6**). Each test chamber was fed 5-8 Artemia sp. nauplii per mysid twice per day and test chambers were covered with petri dishes to minimize evaporation while still allowing oxygen exchange at the air-water interface. Immobile and unresponsive mysids were removed and remaining alive mysids were counted every 24 hours until the end of each toxicity test.

I conducted range-finding tests in 250 mL glass beakers containing 175 mL of test solution and 5 mysids per beaker. Mortality of the test organisms was assessed across 5 nominal test concentrations separated by a dilution factor of 10, with 2 replicates per test concentration. Limit and definitive tests were conducted in 400 mL glass beakers containing 350 mL of test solution and 10 mysids per beaker. In limit tests, mortality of the test organisms was assessed in the single nominal high concentration over 2 replicates. In definitive tests, mortality of the test organisms was assessed across 5 nominal test concentrations separated by a dilution factor of 2, with 3 replicates per test concentration. To monitor sensitivity of the test organisms, a reference test was conducted alongside each definitive test. Reference tests used cadmium chloride as the toxicant

and assessed mortality across 5 nominal test concentrations separated by a dilution factor of 2, with 2 replicates per test concentration.

2.5 Statistical analysis

Mysid mortality data from the definitive tests was analyzed in R (R Core Team, 2021). Within R, concentration response models, confidence intervals, and LC_{50} values were calculated with the drc package (Ritz et al, 2015). The 4-parameter log-logistic model was used for all toxicity datasets. The fourth parameter of each dose response model (equivalent to the inflection point of the curve, or the LC_{50}) was statistically compared to the equivalent parameters in other treatment types with likelihood ratio tests using the compParm function (Diedrich et al. 2015) within the drc package. Confidence intervals at the LC_{50} for each treatment were evaluated for precision (Environment Canada, 2005) by determining the ratios between the confidence limit and the LC_{50}, with ratios at 1.3 or below considered good, and ratios between 1.5 and 1.8 considered acceptable.

3. Results and Discussion

3.1 Toxicity of microplastic leachates

3.1.1 Limit tests

Out of the eight unaged plastic leachate types tested, three types killed enough mysids to proceed to range finding testing. These three types were the white polyester (WPE; 35% mortality at 50 g/L), green polyester (GPE; 100% mortality at 50 g/L), and red polyacrylonitrile (RA; 100% mortality at 50 g/L). For all other unaged leachate types tested, an average of 5% mortality or less was observed (**Table 2**). More detailed results can be seen in the appendix (**Table A1**, **Table A2**).

All aged microplastics had a noticeable yellowish-brown color change compared to their unaged versions and tended to sink in water rather than float (see **Figure 5**). None of the eight aged plastic leachate types killed more than 5% of the mysids in their limit tests (**Table 2**), including the same three fiber leachate types that produced toxic leachates when unaged. Unlike the unaged leachate test chambers, sediment particles (<10 μm) from the microplastic deployment area substrate made it into the unaged leachate test chambers (**Figure 6**). To determine if these sediments affected toxicity results, sediments collected from the deployment site (described in the section 2.2) in December 2020 were dried and used to create sediment leachates at a concentration of 5 grams per liter. These leachates were filtered using the same process as the microplastic leachates. Turbidities were measured and compared to the turbidities of the aged microplastic leachates (**Table 3**) and limit tests were performed. Across two toxicity tests, sediment leachates killed an average of 11.7% the mysids (**Table 2**). Since the average turbidity of the aged microplastic leachates (26.3 NTU) was less than the average turbidity of the lab-created sediment leachates (35.1 NTU), and none of the aged microplastic leachates produced significant toxicity

Plastic Leachate Type	Aged?	% Mortality at 96 hours	Toxicity Test Completion Date
Virgin Polystyrene	No	5	3/29/2021
	Yes	0	3/29/2021
Consumer Polystyrene	No	0	3/29/2021
	Yes	5	4/10/2021
Virgin Polyethylene Terephthalate	No	0	3/29/2021
	Yes	5	3/29/2021
Consumer Polyethylene Terephthalate	No	0	3/29/2021
	Yes	0	3/29/2021
Virgin Polycarbonate	No	0	3/29/2021
	Yes	5	3/29/2021
White Polyester	No	35	3/29/2021
	Yes	0	3/29/2021
Green Polyester	No	100	3/29/2021
	Yes	5	3/29/2021
Red Polyacrylonitrile	No	100	3/29/2021
	Yes	5	3/29/2021
Sediment (5 g/L)	/	6.7	5/4/2021
Sediment (5 g/L)	/	16.7	4/27/2021
Control	/	3.3	3/29/2021
Control	/	3.3	4/10/2021

Table 2. Summary of 96-hour *Americamysis bahia* limit test results. Virgin and consumer polystyrene, virgin and consumer polyethylene terephthalate, and virgin polycarbonate were tested at 100 grams per liter, while the white polyester, green polyester, and red polyacrylonitrile were tested at 50 grams per liter. Percent mortalities are the average of two replicate beakers, containing a total of 20 juvenile mysids (<48 hours old, *Americamysis bahia*). Control and sediment leachate mortalities are an average of 3 replicate beakers, containing a total of 30 mysids.

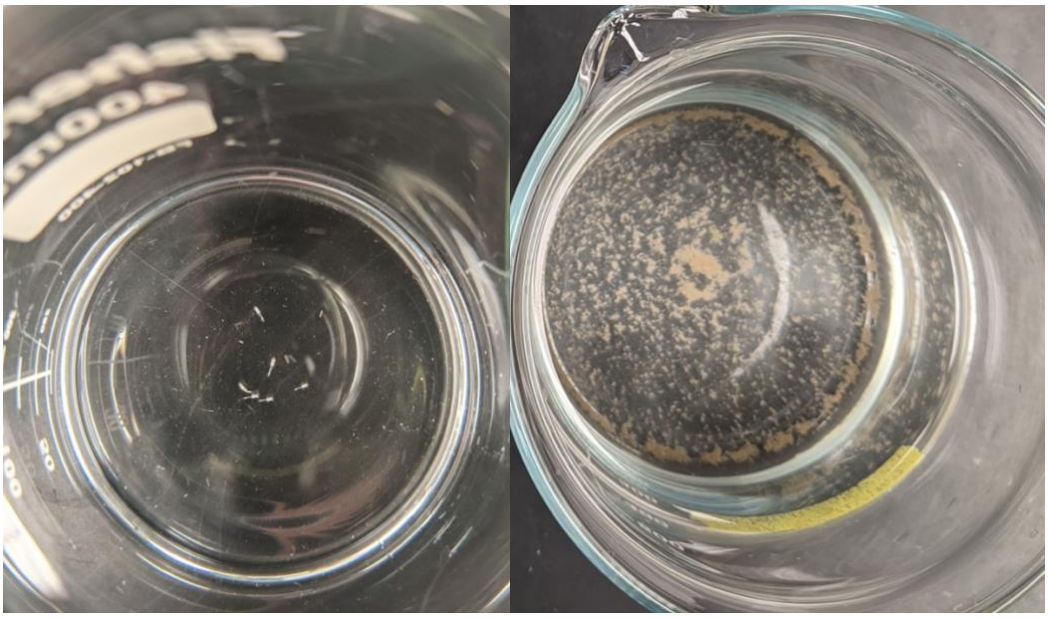


Figure 6. Photo of a toxicity test beaker filled with leachate from an unaged microplastic (left) versus a test beaker filled with aged microplastic leachate (right). Note the <10 μm sediments that have settled to the bottom of the right test chamber, which made it through the two filter screens used to process all leachates.

Aged Microplastic Leachate Type	Turbidity (NTU)
Virgin Polystyrene	36.5
Consumer Polystyrene	30.4
Virgin Polyethylene Terephthalate	45.6
Consumer Polyethylene Terephthalate	34.3
Virgin Polycarbonate	8.5
White Polyester	7.7
Green Polyester	12.5
Red Polyacrylonitrile	34.6
Sediment (5 g/L, 5/4/21)	27.7
Sediment (5 g/L, 4/27/21)	42.4

Table 3. Measured turbidity values of eight aged microplastic leachates (particles at 100 grams per liter, fibers at 50 grams per liter). Also included are turbidity values of two lab-created sediment leachates (without microplastics).

results, we concluded that the <10 μm sediments that made it into the aged microplastic leachate test chambers were unlikely to have caused additional toxicity to the mysids.

3.1.2 Range-finding tests

Range-finding tests were performed for the unaged WPE, GPE, and RA. The concentrations used for all three types were 25, 2.5, 0.25, 0.025, and 0.0025 grams of fibers per liter. Results are summarized in **Table 4**, and more detailed results can be seen in the appendix (**Table A3**, **Table A4**).

100% of mysids for RA, and 90% of mysids for GPE, died at the highest tested range finding concentration. Mysids didn't die in any other tested concentrations, so definitive tests were performed between 50 g/L and 2.5 g/L. No mysids died in the highest concentration of WPE, suggesting that mortality would only occur above 25 g/L for this type. One mysid died at 0.25 g/L WPE, but this was within an acceptable percent control mortality and there was no mortality at all other tested concentrations. Since WPE was much less toxic than GPE and RA, the concentrations used in definitive testing were increased.

3.1.3 Definitive tests

Definitive tests were performed for the unaged WPE, GPE, and RA. The concentrations used for unaged WPE were 60, 30, 15, 7.5, and 3.75 grams per liter. Concentrations used for unaged GPE and RA were 50, 25, 12.5, 6.25, and 3.125 grams per liter. Results are summarized in **Tables 5 and 6**, dose response curves are presented in **Figures 7-10**, and more detailed results can be seen in the appendix (**Table A5**, **Table A6**).

Plastic Leachate Type	Plastic Concentration (g/L)	% Mortality at 96 hours	Toxicity Test Completion Date
White Polyester	25	0	10/24/2020
	2.5	0	
	0.25	10	
	0.025	0	
	0.0025	0	
Green Polyester	25	100	10/24/2020
	2.5	0	
	0.25	0	
	0.025	0	
	0.0025	0	
Red Polyacrylonitrile	25	90	10/24/2020
	2.5	0	
	0.25	0	
	0.025	0	
	0.0025	0	
Control	0	0	10/24/2020

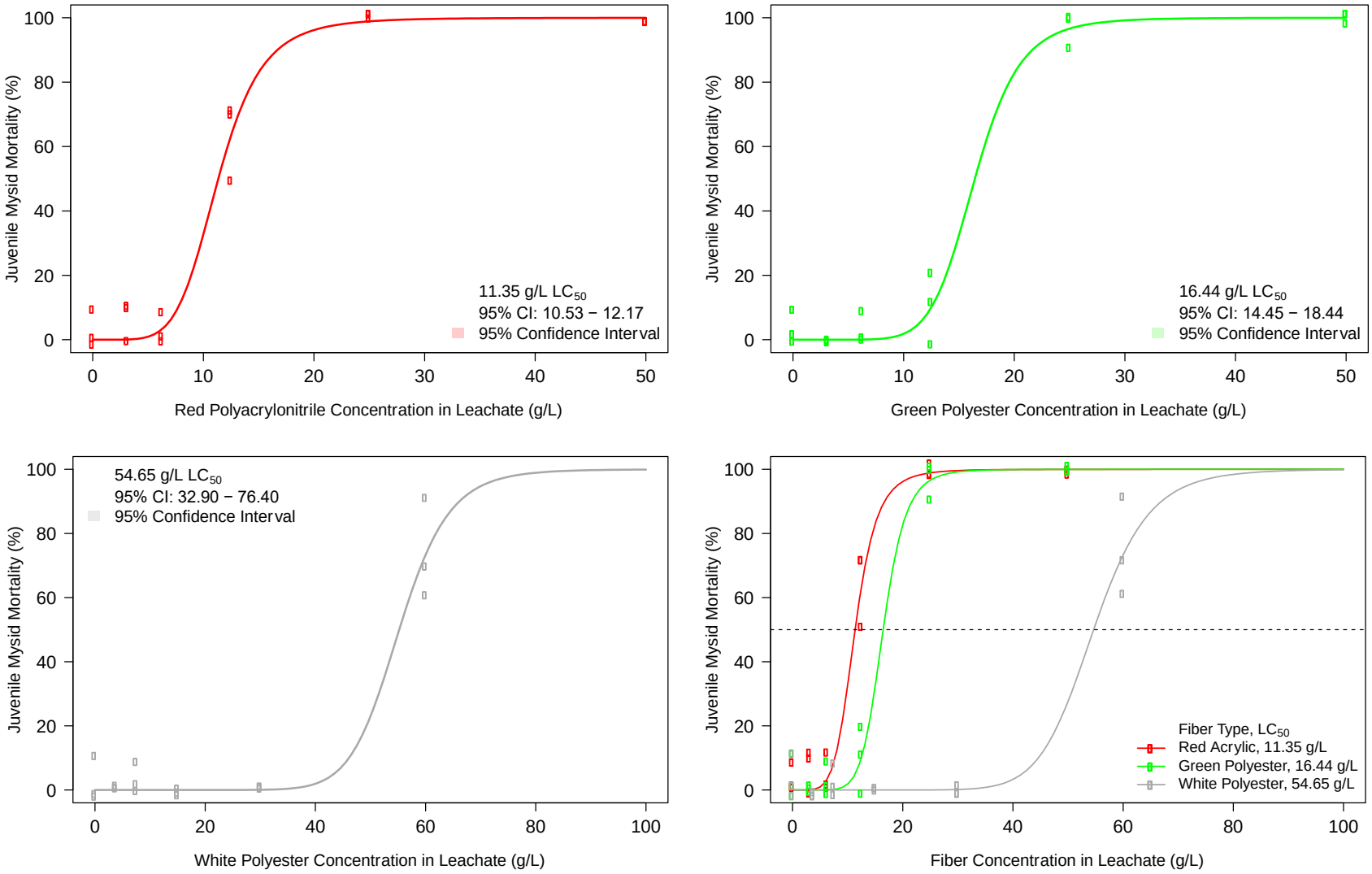
Table 4. Summary of 96-hour *Americamysis bahia* range finding test results. Percent mortalities are the average of two replicate beakers, containing a total of 10 juvenile mysids (<24 hours old, *Americamysis bahia*).

Plastic Leachate Type	Plastic Concentration (g/L)	% Mortality at 96 hours	Toxicity Test Completion Date
White Polyester	60	73.3	4/17/2021
	30	0	
	15	0	
	7.5	3.3	
	3.75	0	
Green Polyester	50	100	4/17/2021
	25	96.7	
	12.5	10	
	6.25	3.3	
	3.125	0	
Red Polyacrylonitrile	50	100	4/10/2021
	25	100	
	12.5	63.3	
	6.25	3.3	
	3.125	6.7	
Control	0	3.3	4/10/2021
Control	0	3.3	4/17/2021

Table 5. Summary of 96-hour *Americamysis bahia* definitive test results. All percent mortalities are the average of three replicate beakers, containing a total of 30 juvenile mysids (<48 hours old, *Americamysis bahia*).

Plastic Leachate Type	LC_{50} Value (g/L)	Lower 95% Confidence Interval (g/L)	Upper 95% Confidence Interval (g/L)	Dose Response Model Used
White Polyester	54.65	32.90	76.40	LL.4
Green Polyester	16.44	14.45	18.44	LL.4
Red Polyacrylonitrile	11.35	10.53	12.17	LL.4

Table 6. Summary of LC50 values, confidence intervals, and dose response models used for data obtained from 96-hour *Americamysis bahia* acute toxicity tests. LL.4 is the 4-parameter log-logistic model from the R drc package (Ritz et al. 2015).



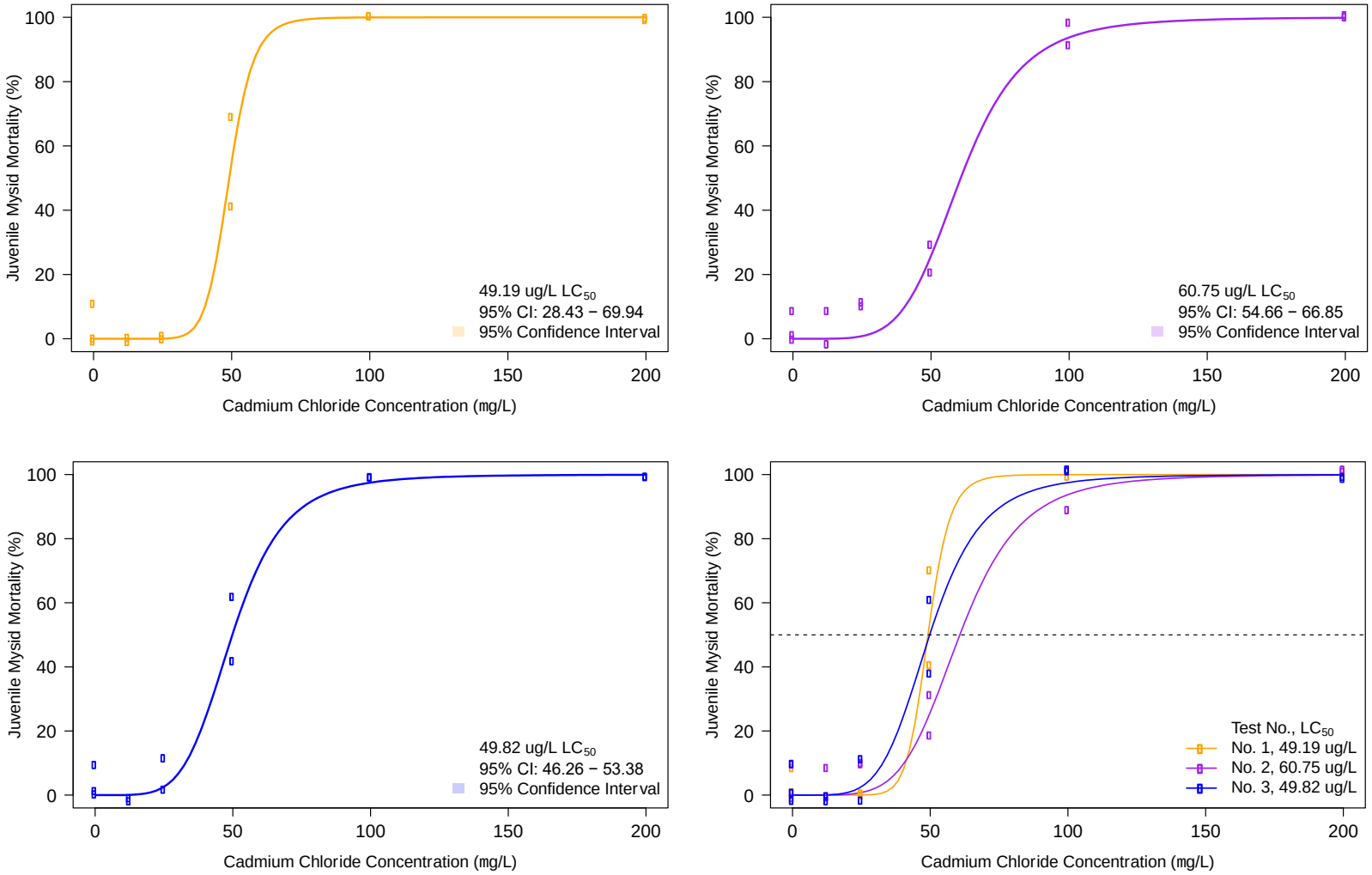
Figures 7-10. Dose response curves and confidence intervals for three 96-hour microplastic leachate toxicity tests performed with <48 hour old *Americamysis bahia* individuals. Each point indicates a percent mortality from one test beaker. The three leachate types can be ranked by toxicity as follows, from most toxic to least toxic: Red Polyacrylonitrile, Green Polyester, White Polyester.

The unaged WPE leachate was the least toxic of the three, with an LC_{50} value of 54.65 g/L ± 21.75 g/L (95% confidence interval). The unaged GPE had an LC_{50} value of 16.44 g/L ± 2.00 g/L (95% confidence interval). The RA leachate was the most toxic, with an LC_{50} value of 11.35 g/L ± 0.82 g/L (95% confidence interval). Likelihood ratio tests of the three LC_{50} values indicated that they were all significantly different from each other ($p < 0.0001$; **Table 7**).

Three reference tests were performed in total: one alongside the limit tests, and two with the definitive tests. Test one, finished on March 29th 2021, had an LC_{50} of 49.19 µg/L ± 20.75 µg/L cadmium chloride (95% confidence interval). Test two, finished on April 10th 2021, had an LC_{50} of 60.75 µg/L ± 6.10 µg/L cadmium chloride (95% confidence interval). Test three, finished on April 17th 2021, had an LC_{50} of 49.82 µg/L ± 3.56 µg/L cadmium chloride (95% confidence interval). These LC_{50} values are within 2 standard deviations of the mean of all LC_{50} values obtained for mysid cadmium chloride reference tests conducted in our lab (**Figure 15**), indicating the results of our reference tests were satisfactory (Environment Canada, 2005). Dose response curves for the reference tests are presented in **Figures 11-14** and more detailed results can be found in the appendix (**Table A7**). The LC_{50} values obtained in this study are larger than other values reported in the literature for similar cadmium chloride acute toxicity tests with juvenile *A. bahia*, which are 32.8 µg/L (Voyer and Modica, 1990) and 19.6 µg/L (Cripe, 1994). This difference may be explained in part by the use of mysids that are about 24 hours older than the mysids used in the other two studies.

LC_{50} Value Comparison	**Estimate**	**Standard Error**	**t-value**	**p-value**	**Significantly Different?**
RA/GPE	0.690274	0.048533	-6.3817	6.53×10^{-8}	**YES**
RA/WPE	0.207680	0.041780	-18.964	$<2.2 \times 10^{-16}$	**YES**
GPE/WPE	0.300867	0.062247	-11.232	4.91×10^{-15}	**YES**

Table 7. Statistical comparison of 96-hour *Americamysis bahia* toxicity values for three microplastic fiber leachates. This was done with likelihood ratio tests using the compParm function within the R drc package. All three LC_{50} values are significantly different from each other (α = 0.05).



Figures 11-14. Dose response curves and confidence intervals for three 96-hour cadmium chloride reference toxicity tests performed with <48 hour old *Americamysis bahia* individuals. Each point indicates a percent mortality from one test beaker. The top left curve is from a test performed on March 29th 2021, the top right curve is from April 10th 2021, and the bottom left curve is from April 17th 2021.

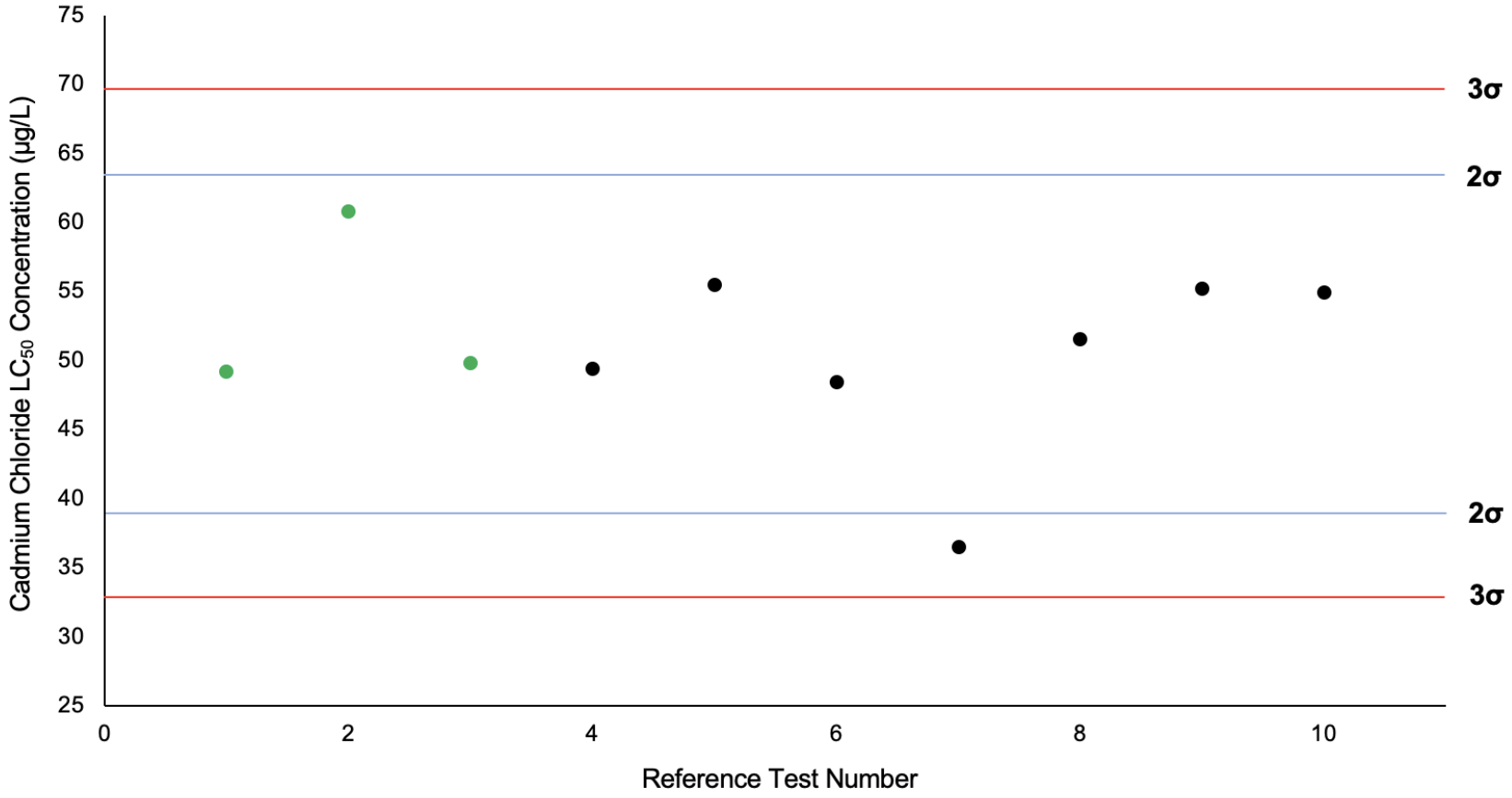


Figure 15. Control chart showing results of ten cadmium chloride *Americamysis bahia* acute reference tests conducted in the Sofield lab between March and June 2021. The first three reference tests (with LC_{50} values shown as green markers), which were the only ones conducted alongside he microplastic leachate tests conducted in this thesis, had LC_{50} values within 2 standard deviations of the mean LC_{50} across all tests, which was 51.15 µg/L.

3.2 Chemistry of microplastic leachates

3.2.1 Metals

Of the 24 metals analyzed via ICP-MS (see section 2.3.3 for a complete list), 23 were present at measurable levels in some or all sixteen microplastic leachate types used in this study (**Table A8**).

To help determine whether metals present in the microplastic leachates affected toxicity, concentrations found in leachates were compared to saltwater Criterion Maximum Concentration (CMC) values in the National Recommended Water Quality Criteria – Aquatic Life Criteria Table. These acute values are established by the US Environmental Protection Agency and are supposed to be the highest concentrations not expected to pose a significant risk to most aquatic species.

Out of nine metals analyzed that have established saltwater CMC values, three metals were present in some leachates at concentrations above CMCs (**Table A8**). Nickel was present in the aged GPE and aged WPE at levels above the CMC. Copper exceeded the saltwater CMC in several leachates: the unaged virgin PS, unaged RA, unaged WPE, aged consumer PS, and all three types of aged fibers (WPE, GPE, and RA). Finally, silver exceeded the saltwater CMC in the unaged virgin PET, unaged consumer PET, and unaged virgin PC. Despite these exceedances, it is important to note that significant mysid mortality was only observed in the three unaged fiber types, which had two exceedances of the copper CMC. In addition, these two copper exceedances were at concentrations lower than exceedances in most other nontoxic leachates, suggesting that the nine dissolved metals for which CMCs have been established did not contribute to observed acute toxicity.

One trend observed for the dissolved metals was higher concentrations in the fiber leachates compared to the particle leachates. For example, manganese was present in the particle leachates at an average of 0.4 μg/L, while in the fiber leachates, this average concentration was 15.1 μg/L. This pattern was also observed for nickel (avg. 0 μg/L versus 51.9 μg/L), zinc (avg. 0 μg/L versus 17.1 μg/L), and copper (avg. 3.5 μg/L versus 7.6 μg/L). One possible explanation for this is that consumer textiles are often treated with metals or organometallic compounds to add antimicrobial properties (Windler et al. 2013), though it is unknown if the felt and yarn used in this study were treated as such. Additionally, the fibers had a much larger surface area to release integrated or environmentally sorbed metals into surrounding water compared to the particles. Despite the higher concentrations used in the particle leachates (100 g/L) compared to the fiber leachates (50 g/L), the fibers had a much lower density and therefore filled much more space in the water column when leaching. It has been suggested by several authors that surface area is an important consideration when determining a microplastic's capacity to sorb and release chemicals (e.g. Hidalgo-Ruz et al. 2012; Turner and Holmes 2015; Luo et al. 2020).

3.2.3 Organics

Thousands of organic substances were present in the sixteen microplastic leachates analyzed via LC-QTOF-MS. When these chemical features were organized by hierarchical clustering, it became clear that different microplastic leachates had distinct chemical signatures and grouped together by type (particles versus fibers) and aging state (**Figure 16**). The leachates clustered into four groups: the unaged fibers, the aged fibers, the aged particles, and the unaged particles. Vertical comparison of the signatures between the aged and unaged versions shows that some chemicals are uniquely present in the unaged version of each leachate and vice versa. This indicates that during deployment, the microplastics could have released organic contaminants into

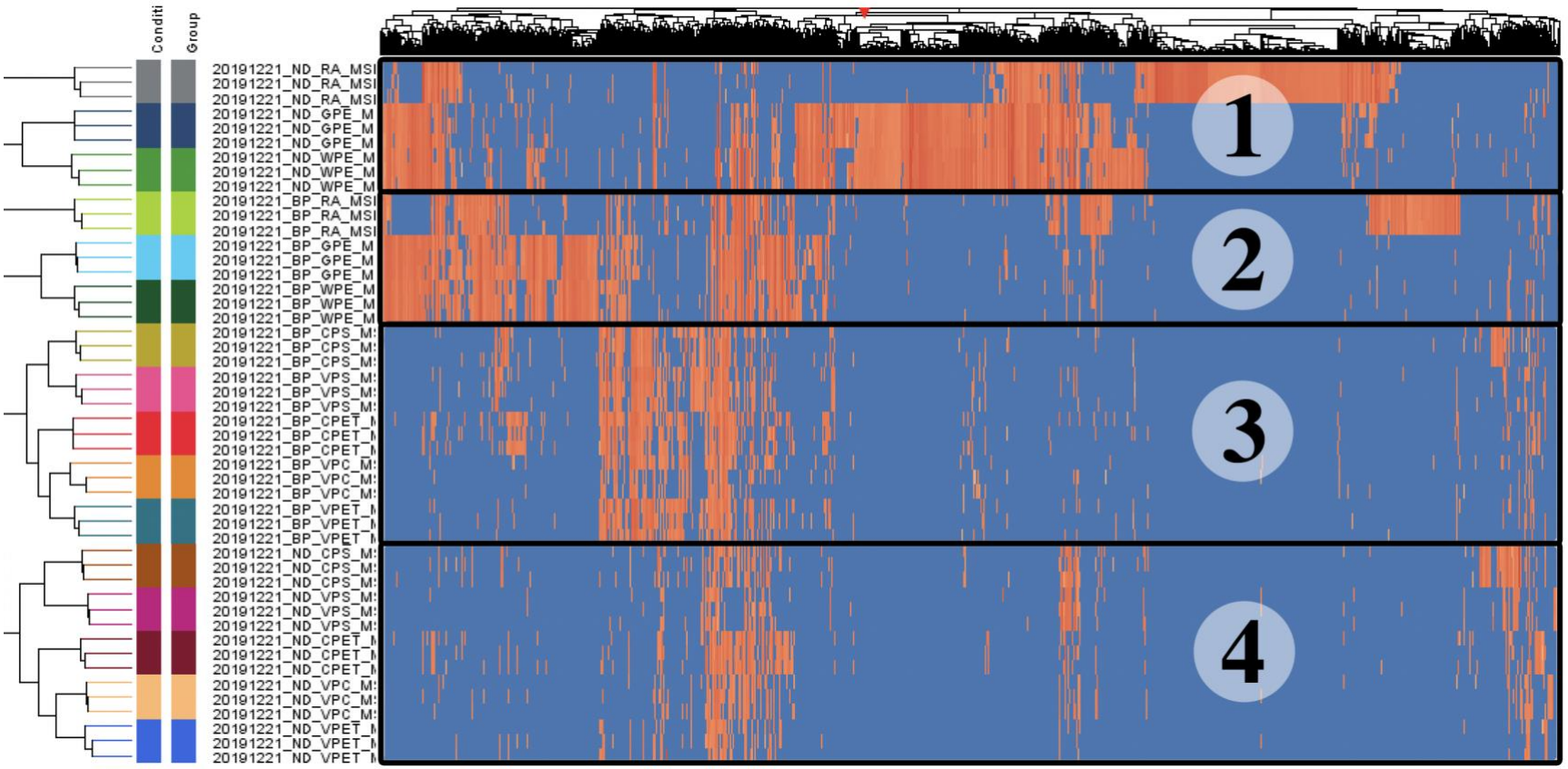


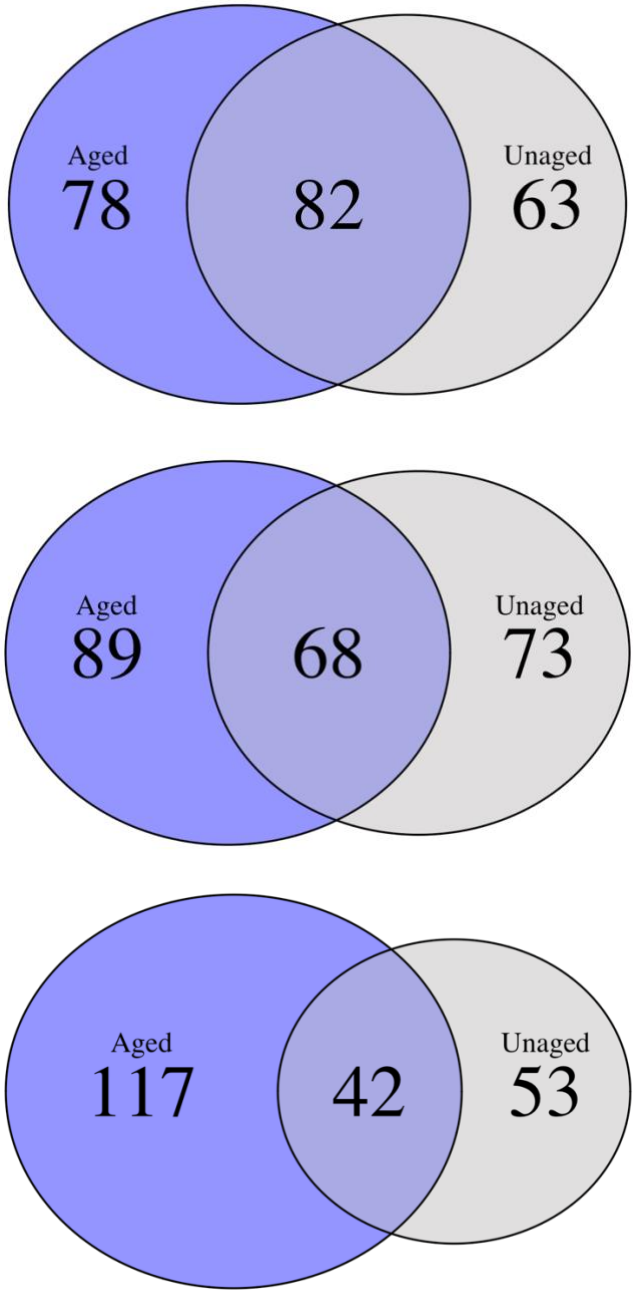
Figure 16. Hierarchical clustering of organic chemical features found in sixteen microplastic leachates, analyzed via LC-QTOF-MS. Immediately to the right of the dendrogram, each color represents one of the sixteen leachate types. To the right of each color are three named rows, indicating three replicate samples of each leachate type. BP stands for Boulevard Park (deployment location of aged samples), and ND stands for non-deployed. After each BP/ND, the particle sample names start with a V (virgin) or C (consumer), followed by the common plastic abbreviation (PS, PET, or PC). RA is red acrylic, GPE is green polyester, and WPE is white polyester. To the right of the names are the corresponding chemical signatures; each vertical line is a single chemical feature. Blue indicates absence of a chemical, while orange indicates a chemical is present, and the darker the orange the more of the chemical there is. The chemical signatures cluster into 4 distinct groups: 1) unaged fibers, 2) aged fibers, 3) aged particles and 4) unaged particles.

the environment, had their leachable additives or monomers altered during environmental aging, or sorbed new chemicals. Additionally, a much wider variety of chemicals are present in the microplastic fiber leachates than the microplastic particle leachates.

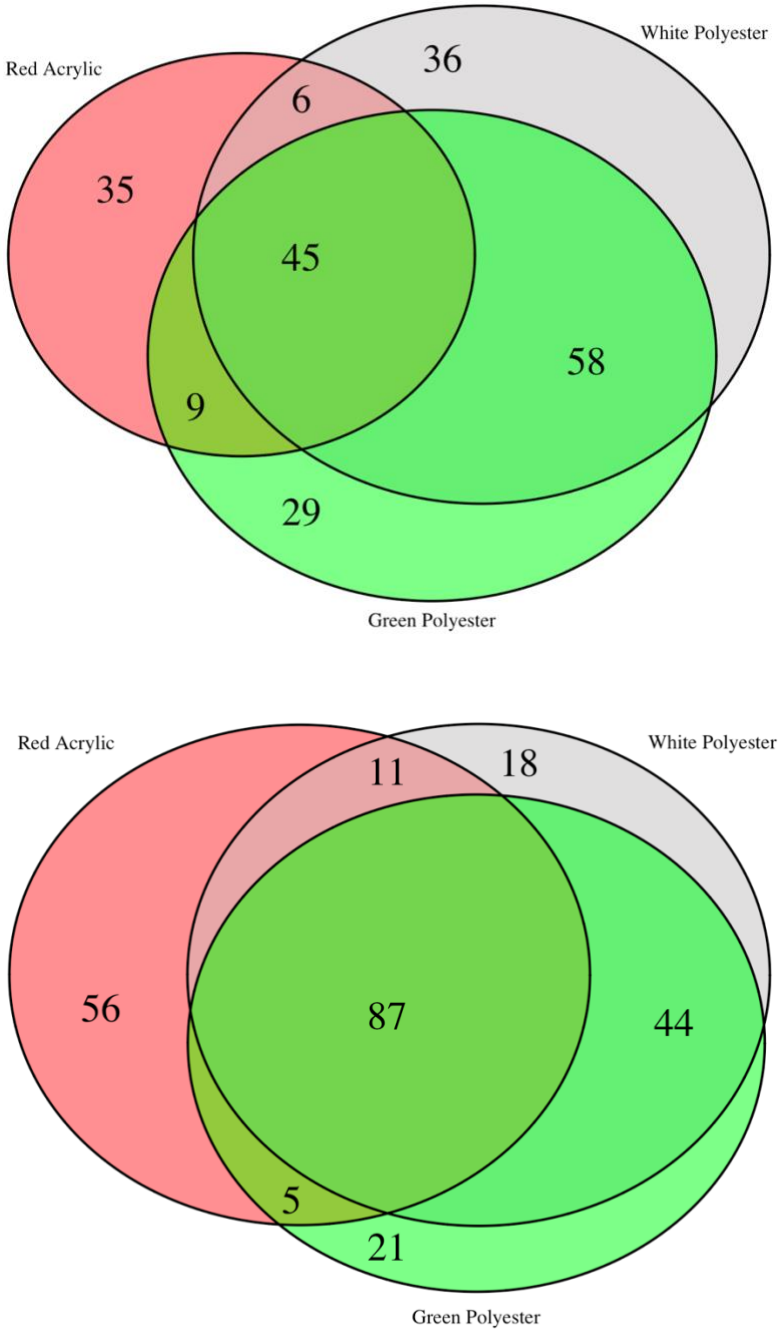
Because of the toxicity observed with the three unaged microplastic fiber leachates, I decided to focus on these, and their respective aged versions, for a more in-depth chemical analysis up to the MS1 stage (m/z and retention time pairs). A total of 333 unique chemical features were identified across all six fiber leachate types with confidence (match score of 80 or higher). Venn diagrams (**Figures 17-21**) highlight the similarities and differences between the fiber leachate types. Across aging states, the green polyester and white polyester were the most chemically similar, as was expected due to their shared plastic polymer type. The three fiber leachate types had many more chemicals in common after aging than before (87 versus 45; see **Figures 20-21**). Also, across all three types, there were more chemicals present in the aged versions of the leachates than the unaged versions (**Figures 17-19**), despite the much higher toxicity of the unaged versions. 32 chemical features that were only present in all three unaged fiber leachates have been prioritized for further identification (as in Du et al. 2017) since they possibly caused the mysid mortality observed in this study.

3.3 Comparison of results to other studies and implications

Results from the chemical analysis show that microplastic leachates can become more chemically similar after aging, but microplastics can also accumulate and release unique chemicals into the environment depending on the plastic type. These findings provide evidence for the difference in toxic effects observed between the different microplastic fiber leachates, and lend



Figures 17-19. Venn diagrams showing shared and unique chemical features present in six different microplastic fiber leachates, found during LC-QTOF-MS analysis. Venn diagrams only include the 333 chemical features identified in samples with confidence (match score greater than or equal to 80). Diagrams are separated by plastic type and compare features present in aged and unaged versions of each type. From top to bottom: white polyester (n = 223), green polyester (n = 230), red polyacrylonitrile (n = 212).



Figures 20-21. Venn diagrams showing shared and unique chemical features present in six types of microplastic fiber leachates, found during LC-QTOF-MS analysis. Venn diagrams only include the 333 chemical features identified in samples with confidence (match score greater than or equal to 80). Diagrams are separated by aging state and compare features present in leachates created from three different microplastic fiber types. From top to bottom: unaged (n = 218) and aged (n = 242).

support to the idea that different microplastic leachates are unique chemical cocktails. The microplastic fiber leachates are notable for containing the most organic chemical features by far, and often contained more metals than the particle leachates. Fibers are underrepresented in microplastic toxicity studies, despite often being the most common type found in monitoring studies (e.g. Lusher et al. 2015; Burns and Boxall 2018; Cox et al. 2019), and my toxicity and chemistry results further highlight the importance of including them in future research.

My toxicity results also highlight microplastic fibers, showing that unaged microplastic leachates can be more harmful than leachates created from similar aged microplastics. Though no other microplastic leachate study to date has investigated the effect of aging on fibers specifically, existing studies tend to find that aged microplastics produce more toxic leachates than unaged microplastics, which is the opposite of what I found for fibers (Silva et al. 2016; Rummel et al. 2019; Bucci et al. 2021; Rendell-Bhatti et al. 2021). Test conditions of these studies, along with one other relevant microplastic leachate study, are summarized in **Tables 8 & 9**. One thing that these four leachate studies have in common is that they used unaged *preconsumer* microplastic particles to make their leachates, not fibers or consumer particles and fibers, as I did. Consumer plastics (such as the microplastic fibers used in this study, or plasticized PVC) are much more likely to have leachable toxic additives present than preconsumer plastics, and multiple studies have found that unaged consumer microplastic leachates are toxic to aquatic organisms (Bejgarn et al. 2016; Oliviero et al. 2019; Rendell-Bhatti et al. 2021). When consumer microplastics are deployed in the environment, it follows that the leachable chemicals may change in both concentration and type. In the context of this study, it appears that enough leachable additives in the deployed fibers were lost, or chemically altered, during deployment so that they no longer caused mortality in mysids. It also appears that the aged fibers did not accumulate enough toxic

Author	Year	Test Organism Used	Test Conditions	Plastic Type(s)	Microplastic Aging Type	Leachate Generation Methods	Water Type & Quality	Other Notes
Silva et al.	2016	*Perna perna* (brown mussel) embryos	**Test length:** 48 hours **Endpoint(s):** Mortality, physical deformities. development **Temperature:** 25 °C **Lighting:** Unknown **Concentrations:** 3	Virgin PP pellets and beached pellets	**Environ-mental** (pellets collected from beaches)	**Concentration:** 0.5-2 mL MPs per 10 mL water **Time:** 24 hours, but left in test chambers throughout **Temperature:** 25 °C **Lighting:** Unknown **Mixing:** None	**Seawater**	Beached pellet leachates were much more toxic than virgin pellet leachates, though both caused toxic responses
Bejgarn et al.	2016	*Nitocra spinipes* (harpacticoid copepod) adults, 3-4 weeks old	**Test length:** 96 hours **Endpoint(s):** Mortality **Temperature:** 20 ± 1 °C **Lighting:** 24 h darkness **Concentrations:** 1	21 types of consumer product MPs, with polymer types including PP, PS, LDPE, HDPE, PVC, PET, and others	**Artificial** (Performed by irradiation with artificial sunlight for 96, 192, or 288 hours)	**Concentration:** 100 grams of MP per liter **Time:** 72 hours **Temperature:** Unknown **Lighting:** 24 h darkness **Mixing:** 6-21 rpm	**Brackish water** Salinity 7 ppt	8 out of 21 leachate types caused mortality. Some leachate types were more toxic, some were less toxic, and some had no toxicity change after artificial aging of the MPs.
Rummel et al.	2019	Cells (in various kinds of cell-based bioassays)	**Test length:** 24 hours **Endpoint(s):** xenobiotic metabolism activation, oxidative stress, endocrine disruption **Temperature:** Varies **Lighting:** Varies **Concentrations:** Vary	Additive-free pre-production PE, PET, PP, and PS	**Artificial** (Treated with ultraviolet light for 96 hrs while suspended in leaching water)	**Concentration:** 250 grams of MP per liter, concentrated by solid phase extraction **Time:** 96 hours, done alongside artificial aging **Temperature:** 20-30 °C **Lighting:** UV A+B light **Mixing:** horizontal rotation	**Seawater**	All leachates induced oxidative stress responses, more so with the UV treated samples. Few effects seen from non-aged pre-production leachates.

Table 8. Summary of existing studies that investigate the difference in toxicity between unaged microplastic (MP) leachates and aged MP leachates.

Author	Year	Test Organism Used	Test Conditions	Plastic Type(s)	Microplastic Aging Type	Leachate Generation Methods	Water Type & Quality	Other Notes
Bucci et al.	2021	*Pimephales promelas* (Fathead minnow) larvae	**Test length:** 14 days **Endpoint(s):** mortality, organism size, organism weight **Temperature:** 24 ± 1 °C **Lighting:** 16:8 light:dark **Concentrations:** 2	Preconsumer PE, preconsumer PP, and environmental MPs (mix of PE and PP)	**Environ-mental** (beached pellets collected near lake shoreline)	**Concentration:** 280 or 2800 MPs per mL **Time:** 24 hours **Temperature:** 24 °C **Lighting:** Unknown **Mixing:** None	**Freshwater** dechlorinated	Found increased deformities in leachate from environmental MPs, compared to leachate from unaged MPs.
Rendell-Bhatti et al.	2021	*Paracentrotus lividus* (purple sea urchin) embryos and larvae	**Test length:** 48 hours **Endpoint(s):** Mortality, physical abnormalities **Temperature:** 18 °C **Lighting:** 12:12 light:dark **Concentrations:** 3	Virgin LDPE pre-production nurdles, Plasticized PVC nurdles, beached nurdles (mostly PE), "biobeads" (mostly PE)	**Environ-mental** (pellets collected from beaches)	**Concentration:** 60 mL microplastics per 240 mL water **Time:** 24 and 72 hours **Temperature:** 18 °C **Lighting:** 24 h darkness **Mixing:** Orbital shaker	**Seawater**	Leachates of PVC, beached nurdles, and biobeads caused developmental abnormalities. Longer leaching times caused more abnormalities. PVC leachates were the most toxic.

Table 9. Summary of existing studies that investigate the difference in toxicity between unaged microplastic (MP) leachates and aged MP leachates, continued.

environmental metals or organic contaminants that could be desorbed into the laboratory leachates to cause further mysid mortality.

My toxicity results also show that leachates of PS, PET, and PC microplastic particles are not acutely toxic, in both their aged and unaged forms. This means that the unaged virgin and consumer particles tested did not contain enough toxic leachable monomers, additives, or other plastic-associated substances to cause acute toxicity under my test conditions. Likewise, the aged versions of the same particles did not accumulate and then desorb enough toxic environmental contaminants into the leachates to cause acute toxicity. Though toxic organic contaminants such as PCBs and PAHs are known to sorb to environmental microplastics, and the maximum concentration of chemicals sorbed varies with microplastic polymer type (Rochman et al. 2012), the effects of these factors were unable to be detected in my toxicity results. One possible explanation for this could be the short leaching time used. If my aged plastics did sorb any hydrophobic contaminants from the environment, it seems unlikely that toxic amounts would desorb into water from the similarly hydrophobic plastic surface in only 2 days. Also, if the aged plastics sorbed any toxic volatile organic compounds during deployment, those compounds could have been lost during my 4-day long drying time, and consequently not have made it into the leachate and toxicity tests.

It is not entirely clear how the LC_{50} values obtained in this study compare to concentrations observed in the environment, though they are likely higher (**Figure 22**). One study investigating microplastics in surface water found that an average of 88 fibers per liter were present across eight sampling sites in the North Sea, with a maximum concentration of 650 fibers per liter (Dubaish and Liebezeit, 2013). However, due to the difference in units between monitoring studies and this toxicity study (particles/L versus grams/L), the two cannot be directly compared. Additionally,

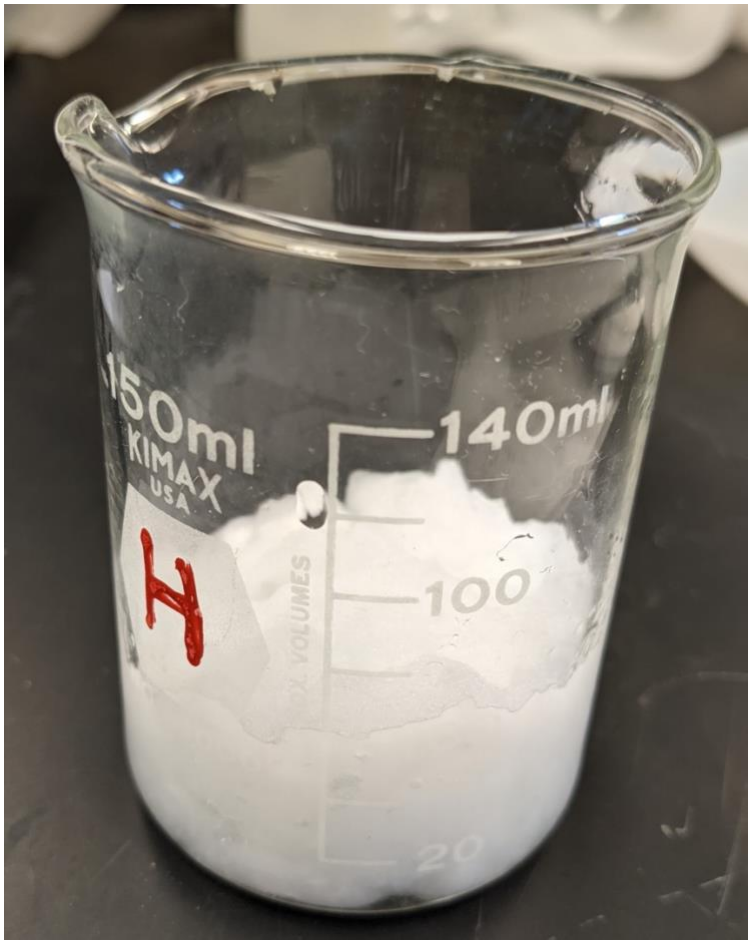


Figure 22. A small sample of unaged white polyester microplastic fibers leaching in seawater at 60 grams per liter. This near the LC_{50} value for this plastic type and the fibers are filling the entire water column, which is not likely to be observed in the environment.

most studies sampling the water column miss the smallest microplastics, which pass through standard filter sizes used while also making up the majority of plastics present (Syberg et al. 2015). Therefore, it is likely that many microplastic monitoring studies, including the one cited here, undercounted their fibers and other microplastic types. This makes determining the ecological relevance of LC_{50} values obtained in toxicity studies even more difficult.

This work also demonstrates that after an environmental deployment of only 76 days (70 days in the case of the GPE), there was a complete mitigation of acute toxicity in the resulting microplastic fiber leachates of all 3 fiber types. This suggests that, regardless of how toxic these fiber leachates are initially, the release of toxic chemicals decreases once they spend enough time in the environment. In addition to releasing additives, microplastics have also been reported to crack, undergo photo and oxidative degradation (Lambert et al. 2017), release unique suites of dissolved organic compounds that depend on sunlight exposure and polymer type (Walsh et al. 2021), and form biofilms (Rummel et al. 2017) when weathering. All these alterations from environmental exposure can create new substances that were not originally associated with the plastic. Therefore, environmental conditions could have also transformed the toxic chemicals in the fibers into less toxic versions, altered the surface chemistry of the fibers in a way that prevented exposure of the mysids to toxic chemicals, or created a biofilm layer changing how the additives were released from the fibers, amongst many other possibilities.

4. Conclusions and Future Directions

4.1 Hypotheses and knowledge gaps addressed

Based on this study's findings, it makes sense to reject all three of my initial null hypotheses and consider the alternative hypotheses.

First, acute toxic effects of microplastic leachates to mysids did significantly differ between microplastic types; the particles were nontoxic, and all three unaged fibers were toxic with significantly different LC_{50} values. At the time of writing, this study is the first to investigate toxicity of microplastic fiber leachates to a marine organism. My results show that microplastic fibers can contain numerous leachable chemicals that are toxic to aquatic life, and within my study, fibers were shown to create more toxic leachates than particles did. These results are consistent with a few other studies comparing the toxic effects of microplastic fibers to particles (Au et al. 2015; Gray & Weinstein 2017; Ziajahromi et al. 2017), though these studies did not distinguish between physical and chemical toxicity.

Second, leachate toxicity to mysids did significantly differ between aged and unaged versions of the same plastic; the microplastic fiber leachates had measurable LC_{50} values in all three unaged versions, while the respective aged versions were nontoxic. Though the finding that microplastic leachate toxicity decreases with aging is not completely consistent with previous studies (Silva et al. 2016; Rummel et al. 2019; Bucci et al. 2021; Rendell-Bhatti et al. 2021), the identity of the plastics in question as consumer plastics helps explain the discrepancy. In another acute toxicity study done with consumer plastics by Bejgarn et al. (2016), the toxicity of 72-hour microplastic leachates made in brackish water to the copepod *Nitocra spinipes* following an artificial aging treatment was found to both increase, decrease, and stay about the same, with

results depending on the type of consumer plastic used to create the leachates. My chemistry, and to some degree toxicity, results suggest that the effects of environmental aging are unique to each type of microplastic.

Finally, the mysid toxic responses were attributable to chemicals released by the microplastics into the laboratory leachates; the effect of metals on the toxicity to mysids was ruled out based on higher concentrations present in other nontoxic leachate samples. 32 organic chemical features were uniquely present in the toxic samples and were prioritized for further identification, results not included here. There are thousands of chemicals likely or possibly associated with plastic packaging alone (Groh et al. 2019), with many of them considered hazardous, and likely many more hazardous chemicals associated with synthetic textiles. Identification of the chemical features unique to the toxic fiber leachates will provide a more complete understanding of environmentally concerning plastic additives, when historically research has been focused on additives associated with hard plastic pre-production nurdles and consumer products.

4.2 Future research priorities

As mentioned previously, my study addresses understudied microplastic fibers. However, it only addresses two fiber polymer types, and all were consumer types, meaning they were dyed or otherwise treated with chemicals to enhance their use. Non-consumer fibers would likely cause different toxic responses in mysids. Also, though my work includes multiple microplastic particle types, there are two common unrepresented types (PE and PVC) and it would be unwise to conclude from my results that microplastic particle leachates pose no acute threat to aquatic organisms, but only that they were not acutely toxic under my test conditions. Though the types

of particle leachates that I tested were nontoxic to mysids, conclusions should be limited to only the specific types of microplastics and test conditions used; more types and test conditions would need to be addressed to make more general conclusions. Also, this study does not include one of the most ubiquitous and metal-laden microplastics, tire particles (Sommer et al. 2018). Another graduate project conducted at WWU recently concluded. This project used the same types of toxicity tests I did, except they were done with various types of unaged and environmentally aged tire wear particles that were deployed a year later in the same location as my microplastics (Roberts et al., 2021).

The leachates in this study were created with specific size ranges of microplastics and at only one leaching time and temperature. Using different sizes of microplastics to create leachates changes the surface area available for leaching, which complicates comparisons between studies and might change toxicity results. Further, if different leachate generation methods (such as altering leaching times, temperatures, lighting, etc.) are used, toxicity and chemistry results could also differ, as was observed in two other leachate studies when temperature and leaching time were varied (Lithner et al. 2012; Rendell-Bhatti et al. 2021). It may be important consider the role of microplastic size, leaching times, and other factors in the chemical composition and toxicity of microplastic leachates in future research.

Finally, it is important to reiterate that this study only considers the acute *chemical* toxicity of microplastics to mysids. It is possible that my microplastic fibers, or even the particles, would elicit different effects if the mysids were directly exposed to them. It is also plausible that the microplastics and their leachable chemicals caused unnoticed sublethal effects, which have been observed in other marine organisms when exposed to microplastics or their leachates (e.g. Li et al. 2015; Silva et al. 2016; Seuront, 2018; Oliviero et al. 2019; Cormier et al. 2021; Rendell-Bhatti et

al. 2021). Another project is being conducted at WWU which investigates changes in respiration and gene expression in mysids exposed to tire particles (Leazer et al., unpublished). Further studies regarding sublethal effects should be conducted for a more complete assessment of microplastic toxicity to marine life.

There is so much left to understand about microplastic toxicity. This work lends strong support to the idea that microplastics should not be considered a single contaminant; rather, they should be a contaminant category, containing all possible polymer types, sizes, shapes, and more, as well as all possible plastic-associated chemicals. Questions about the hazard of microplastics to aquatic organisms will only become more urgent as long as inputs of plastic waste into the environment continue to increase, as they have since the advent of the first synthetic plastic.

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Appendix

Plastic Leachate Type	Aged?	Salinity (‰)	pH	Dissolved Oxygen (mg/L, % saturation)	Toxicity Test Completion Date
Virgin Polystyrene	No	25.5	8.4	7.24, 81.9	3/29/2021
	Yes	26.5	8.1	7.35, 83.4	3/29/2021
Consumer Polystyrene	No	25	8.3	7.24, 82	3/29/2021
	Yes	26	7.9	7.23, 81.2	4/10/2021
Virgin Polyethylene Terephthalate	No	25	8.4	7.31, 82.7	3/29/2021
	Yes	26.5	8.2	6.59, 75.3	3/29/2021
Consumer Polyethylene Terephthalate	No	25	8.4	7.51, 85.1	3/29/2021
	Yes	26.5	8.2	7.32, 82.9	3/29/2021
Virgin Polycarbonate	No	25	8.3	7.38, 83.6	3/29/2021
	Yes	26	8.1	7.45, 84.2	3/29/2021
White Polyester	No	25	8.1	7.42, 82.8	3/29/2021
	Yes	25	7.8	7.28, 82.4	3/29/2021
Green Polyester	No	25	8.0	7.38, 82.6	3/29/2021
	Yes	25	8.3	7.48, 84.2	3/29/2021
Red Polyacrylonitrile	No	25	8.0	7.15, 80.8	3/29/2021
	Yes	24.5	8.1	6.68, 75.3	3/29/2021
Sediment (5 g/L)	/	26.5	7.3	7.64, 88.5	5/4/2021
Sediment (5 g/L)	/	25.5	7.1	Not measured	4/27/2021
Control	/	25	8.0	7.71, 89.7	3/29/2021
Control	/	25	8.7	7.56, 87.5	4/10/2021

Table A1. Water quality parameters of control water and all stock leachates (100 grams per liter for particles, 50 grams per liter for fibers) prior to starting *Americamysis bahia* 96-hour limit tests with various microplastic leachates.

Plastic Leachate Type	Aged?	# Dead Mysids @ 96 hrs	Salinity (‰)	pH	Dissolved Oxygen (mg/L, % saturation)	Toxicity Test Completion Date
Virgin Polystyrene	No	0	26.5	7.7	5.73, 67.5	3/29/2021
		1	26.5	7.7	5.70, 67.1	
	Yes	0	27	7.7	5.82, 67.8	3/29/2021
		0	27	7.8	5.74, 67.0	
Consumer Polystyrene	No	0	26	7.8	5.67, 66.4	3/29/2021
		0	26	7.7	5.44, 63.9	
	Yes	0	26	8.0	5.90, 69.4	4/10/2021
		1	26	8.0	6.09, 71.6	
Virgin Polyethylene Terephthalate	No	0	25.5	7.7	5.66, 66.6	3/29/2021
		0	26	7.8	5.93, 69.7	
	Yes	1	27	7.6	5.74, 67.1	3/29/2021
		0	27.5	7.7	5.78, 67.8	
Consumer Polyethylene Terephthalate	No	0	26	7.7	5.66, 67.3	3/29/2021
		0	26	7.8	5.79, 68.4	
	Yes	0	27	7.8	6.03, 70.9	3/29/2021
		0	27	7.9	6.17, 72.5	
Virgin Polycarbonate	No	0	26	7.7	5.52, 65.3	3/29/2021
		0	26	7.8	5.75, 68.1	
	Yes	0	26.5	7.8	6.07, 70.9	3/29/2021
		1	26.5	7.8	6.19, 72.2	
White Polyester	No	3	26.5	7.7	5.53, 64.9	3/29/2021
		4	26.5	7.7	5.56, 65.4	
	Yes	0	26	7.8	6.14, 71.6	3/29/2021
		0	26.5	7.8	6.22, 72.6	
Green Polyester	No	10	25	7.5	3.67, 43.3	3/29/2021
		10	25	7.5	3.16, 37.5	
	Yes	0	26	7.9	6.34, 74.2	3/29/2021
		1	26	7.9	6.38, 74.6	
Red Polyacrylonitrile	No	10	26	7.7	5.15, 61.1	3/29/2021
		10	25.5	7.7	4.93, 57.4	
	Yes	1	25	7.7	5.50, 64.3	3/29/2021
		0	25	7.8	6.04, 70.6	
Control	/	1	25.5	7.9	6.22, 73.8	3/29/2021
		0	25.5	8.0	6.70, 79.6	
		0	26	8.0	6.24, 73.6	
Control	/	1	25.5	8.1	6.48, 76.5	4/10/2021
		0	25	8.1	6.15, 73.0	
		0	25	8.1	6.03, 71.2	

Table A2. Water quality parameters and dead mysids in every test chamber after 96 hours in *Americamysis bahia* limit tests with various microplastic leachates. Each limit test beaker contained 10 mysids.

Plastic Leachate Type	Aged?	Salinity (‰)	pH	Dissolved Oxygen (mg/L, % saturation)
White Polyester	No	25.5	8.0	7.29, 83.5
Green Polyester	No	25	7.9	7.29, 83.6
Red Polyacrylonitrile	No	25	8.0	7.25, 83.4
Control	/	25	8.2	7.65, 86.4

Table A3. Water quality parameters of control water and all stock leachates (25 grams of fibers per liter) prior to starting *Americamysis bahia* 96-hour range finding tests with various microplastic leachates. All range-finding tests were performed on October 24, 2020 with <24 hour old mysids.

Plastic Leachate Type	Aged?	Test Concentration (g/L)	# Dead Mysids @ 96 hrs	Salinity (‰)	pH	Dissolved Oxygen (mg/L, % saturation)
White Polyester	No	25	0	27	8.2	6.47, 75.6
			0	26	8.2	6.04, 71.4
		2.5	0	25.5	8.2	6.30, 74.5
			0	26	8.2	6.24, 73.6
		0.25	0	27.5	8.2	6.55, 77.5
			1	26.5	8.2	6.22, 73.3
		0.025	0	27	8.2	6.48, 76.6
			0	26	8.2	6.32, 74.5
		0.0025	0	25	8.2	6.37, 75.1
			0	26.5	8.2	5.92, 69.9
Green Polyester	No	25	5	26.5	8.2	6.69, 78.5
			5	25	8.1	6.36, 76.1
		2.5	0	27	8.2	6.39, 75.6
			0	26.5	8.2	6.50, 76.3
		0.25	0	26	8.2	6.35, 74.7
			0	26.5	8.2	6.24, 73.5
		0.025	0	26.5	8.2	6.43, 75.7
			0	26.5	8.2	6.33, 74.8
		0.0025	0	25.5	8.2	6.26, 73.9
			0	26	8.2	6.32, 74.4
Red Polyacrylonitrile	No	25	4	26.5	8.2	6.42, 76.0
			5	25	8.2	6.59, 77.7
		2.5	0	26.5	8.2	6.27, 73.7
			0	27	8.2	6.30, 74.8
		0.25	0	27	8.2	6.51, 76.2
			0	25.5	8.2	6.22, 73.4
		0.025	0	27.5	8.2	6.50, 76.6
			0	26.5	8.2	6.52, 76.6
		0.0025	0	25.5	8.2	6.12, 72.3
			0	25.5	8.2	6.24, 73.7
Control	/	0	0	25.5	8.2	6.39, 75.6
			0	25.5	8.2	6.38, 75.3

Table A4. Water quality parameters and dead mysids in every test chamber after 96 hours in *Americamysis bahia* range-finding tests with various microplastic leachates. All range-finding tests were performed on October 24, 2020 with <24 hour old mysids, and each beaker contained 5 mysids.

Plastic Leachate Type	Aged?	Salinity (‰)	pH	Dissolved Oxygen (mg/L, % saturation)	Toxicity Test Completion Date
White Polyester	No	25	7.7	6.88, 79.6	4/17/2021
Green Polyester	No	25	7.5	6.88, 79.3	4/17/2021
Red Polyacrylonitrile	No	25	7.5	7.00, 78.8	4/10/2021
Control	/	25	8.7	7.56, 87.5	4/10/2021
Control	/	25	8.8	10.41, 121.2	4/17/2021

Table A5. Water quality parameters of control water and all stock leachates (50 grams of fibers per liter) prior to starting *Americamysis bahia* 96-hour definitive tests with various microplastic leachates.

Plastic Leachate Type	Aged?	Test Concentration (g/L)	# Dead Mysids, 96 hrs	Salinity (‰)	pH	Dissolved Oxygen (mg/L, % saturation)	Toxicity Test Completion Date
White Polyester	No	60	7	25	7.8	5.66, 67.7	4/17/2021
			9	25.5	7.9	6.09, 72.8	
			6	25.5	7.8	5.99, 71.7	
		30	0	25	7.5	4.47, 53.5	
			0	25	7.4	4.29, 51.3	
			0	25	7.5	4.55, 54.3	
		15	0	25.5	7.5	4.19, 50.2	
			0	25	7.5	4.37, 51.8	
			0	25	7.6	4.62, 54.9	
		7.5	1	25	7.7	5.23, 62.6	
			0	25	7.6	4.59, 54.8	
			0	25	7.6	4.62, 55.2	
		3.75	0	25	7.7	4.99, 59.5	
			0	25.5	7.8	5.46, 65.3	
			0	25	7.8	5.25, 62.8	
Green Polyester	No	50	10	25	7.6	4.62, 54.6	4/17/2021
			10	25.5	7.5	4.51, 53.2	
			10	25.5	7.6	4.34, 51.4	
		25	9	25.5	7.8	5.55, 66.3	
			10	25	7.7	4.33, 51.1	
			10	25.5	7.7	4.39, 52.0	
		12.5	1	25	7.6	4.73, 56.7	
			2	25	7.6	4.68, 56.0	
			0	25	7.7	5.47, 65.6	
		6.25	0	25	7.8	5.20, 62.5	
			0	25	7.8	5.58, 66.4	
			1	25	7.7	5.37, 64.2	
		3.125	0	25	7.7	5.11, 60.7	
			0	25	7.7	5.14, 61.0	
			0	25	7.7	4.89, 58.4	
Red Polyacrylonitrile	No	50	10	25	7.5	4.69, 54.8	4/10/2021
			10	25	7.7	5.10, 59.7	
			10	25	7.6	4.73, 55.4	
		25	10	25	7.9	5.63, 66.8	
			10	25	7.9	5.79, 68.5	
			10	25	7.9	5.66, 67.0	
		12.5	5	25	8.0	6.15, 72.8	
			7	25	8.0	6.26, 73.9	
			7	25	8.0	6.17, 72.9	
		6.25	0	25.5	8.0	6.31, 74.4	
			0	25	8.1	6.45, 75.9	
			1	25	8.1	6.38, 75.1	
		3.125	0	25.5	8.0	6.48, 76.0	
			1	25	8.1	6.46, 76.1	
			1	25	8.1	6.45, 75.8	
Control	/	0	1	25.5	8.1	6.48, 76.5	4/10/2021
			0	25	8.1	6.15, 73.0	
			0	25	8.1	6.03, 71.2	
Control	/	0	1	25	7.9	5.78, 69.4	4/17/2021
			0	25	8.0	5.99, 71.6	
			0	25	8.0	6.22, 74.2	

Table A6. Water quality parameters and dead mysids in every test chamber after 96 hours in *Americamysis bahia* definitive tests with various microplastic leachates. Each definitive test beaker contained 10 mysids. Percent dissolved oxygen (DO) dipped below 60% (the USEPA recommended minimum DO) in some cases, but never dropped below 50%. It is unlikely that the low DO is what killed mysids in these toxicity tests; some of the lower test concentrations had low DO, and no mysids died.

Reference Test #	Test Concentration (µg $CdCl_2$/L)	# Dead Mysids @ 96 hrs	Salinity (‰)	pH	Dissolved Oxygen (mg/L, % saturation)
One (3/29/2021)	200	10	25.5	7.9	5.52, 64.1
		10	25.5	7.9	5.21, 60.6
	100	10	25.5	7.9	6.43, 76.0
		10	25.5	8.0	6.47, 76.6
	50	7	25.5	7.9	6.30, 74.3
		4	25.5	7.9	6.11, 72.0
	25	0	26	7.9	6.29, 74.4
		0	26	7.9	6.27, 74.1
	12.5	0	25.5	7.9	6.43, 76.0
		0	26.5	7.9	6.32, 74.5
Two (4/10/2021)	200	10	25	8.1	6.19, 73.0
		10	25	8.2	6.13, 72.3
	100	10	25	8.2	6.43, 76.2
		9	25	8.2	6.47, 76.4
	50	2	25	8.1	6.48, 76.7
		3	25	8.1	6.44, 76.1
	25	1	25	8.2	6.49, 76.4
		1	25	8.1	6.53, 77.2
	12.5	1	25	8.1	6.44, 76.5
		0	25	8.1	6.44, 76.4
Three (4/17/2021)	200	10	26	8.2	6.52, 78.7
		10	26	8.2	6.57, 77.2
	100	10	25	7.9	6.07, 78.2
		10	25	8.0	6.01, 71.2
	50	6	25	7.9	5.82, 70.2
		4	26	7.9	5.72, 68.8
	25	0	26	7.9	5.74, 69.0
		1	26	7.9	5.83, 70.0
	12.5	0	25	7.9	5.74, 69.0
		0	26	7.9	5.68, 68.3

Table A7. Water quality parameters and dead mysids in every test chamber after 96 hours in *Americamysis bahia* reference tests with cadmium chloride. Each reference test beaker contained 10 mysids.

Plastic Leachate	Be µg/L	Mg mg/L	Al µg/L	K mg/L	Ca mg/L	V µg/L	Cr µg/L	Mn µg/L	Fe mg/L	Co µg/L	Ni µg/L	Cu µg/L	Zn µg/L	As µg/L	Se µg/L	Mo µg/L	Ag µg/L	Cd µg/L	Sb µg/L	Ba µg/L	Tl µg/L	Pb µg/L	Th µg/L	U µg/L
Unaged Virgin PS	0	845	0	251	263	5.6	6.6	1.0	4.7	0.9	0	**7.4**	0	3.1	12.3	6.9	0.8	0	0.2	6.2	0.4	8.9	0.1	1.8
Unaged Consumer PS	0	851	0	249	263	5.5	6.6	1.7	4.7	0.9	0	4.7	0	3.9	11.1	6.8	0.8	0.6	53.1	31.7	0.3	5.5	0	1.7
Unaged Virgin PET	0	778	0	244	258	4.9	6.4	0.7	4.5	0.8	0	2.8	0	3.7	8.8	9.2	**18.9**	0	11.2	6.0	1.9	6.3	0.1	1.9
Unaged Consumer PET	0	791	0	245	260	5.1	6.4	0.4	4.5	1.4	0	2.3	0	3.5	9.6	8.0	**14.0**	0	2.0	6.2	1.2	6.8	0.1	1.8
Unaged Virgin PC	0	826	0	254	267	5.5	6.8	0	4.7	0.8	0	1.0	0	3.3	10.2	7.3	**4.3**	0	0.2	6.2	0.7	0	0	1.9
Unaged RA	0	887	0	254	267	5.9	6.9	9.1	4.8	1.3	61.4	**7.9**	22.9	2.8	14.8	7.4	0.4	0.4	0.2	7.8	0.1	1.0	0	1.9
Unaged GPE	0	860	0	243	255	5.6	6.2	1.1	4.6	1.0	8.7	3.0	0	2.9	13.1	6.8	0.4	0	7.4	6.2	0.1	0	0	1.8
Unaged WPE	0	867	0	248	259	5.7	6.5	2.3	4.6	1.3	14.2	**5.7**	0	2.8	16.2	6.7	0.4	0	9.0	7.6	0.1	0	0	1.9
Aged Virgin PS	0	905	0	266	276	6.4	7.0	0	4.9	0.9	0	2.8	0	3.9	12.6	7.3	0.7	0	0.2	7.7	0.3	0	0	2.0
Aged Consumer PS	0	900	10.8	264	275	6.2	7.0	0	4.9	0.9	0	**10.8**	0	3.7	15.1	7.4	0.7	0	22.5	8.8	0.2	0	0	1.9
Aged Virgin PET	0	897	0	265	277	6.2	7.1	0	4.9	1.0	0	1.2	0	3.4	14.7	8.2	0.5	0	1.5	8.0	0.2	0	0	2.0
Aged Consumer PET	0	907	0	263	276	5.8	6.9	0	4.9	1.0	0	1.1	0	3.1	13.1	7.5	0.4	0	0.4	7.7	0.2	0	0	2.1
Aged Virgin PC	0	899	0	258	271	6.0	6.8	0	4.8	1.0	0	0.9	0	3.3	14.7	7.1	0.4	0	0.2	8.5	0.2	0	0	2.0
Aged RA	0	1052	36.5	299	313	7.2	8.7	15.6	5.6	1.3	23.3	**6.8**	0	4.2	15.2	8.3	0.3	0	0.2	9.0	0.1	0	0	2.3
Aged GPE	0	1011	0	289	302	7.0	8.1	28.4	5.4	1.9	**123**	**11.9**	56.1	4.1	14.6	8.2	0.4	2.2	0.9	9.2	0.1	1.2	0	2.2
Aged WPE	0	1016	0	287	300	7.3	8.2	33.8	5.4	1.7	**81.1**	**10.6**	23.8	4.3	16.3	8.0	0.3	2.1	1.3	9.2	0.1	0	0	2.1
USEPA CMC (µg/L)	/	/	/	/	/	/	1100	/	/	/	74	4.8	90	69	290	/	1.9	33	/	/	/	210	/	/

Table A8. Concentrations of 24 dissolved metals present in sixteen types of microplastic leachates, analyzed via ICP-MS. Concentrations are highlighted if they exceed established US Environmental Protection Agency Criterion Maximum Concentration (CMC) values for saltwater. Plastic leachate types are highlighted if they were acutely toxic to <48 hour *Americamysis bahia*.