MICROPLASTICS IN BIOTIC AND ENVIRONMENTAL SAMPLES TAKEN NEAR TWO MUNICIPAL WASTEWATER TREATMENTS PLANTS IN THE GRAND RIVER, ONTARIO

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TITLE: Microplastics in biotic and environmental samples taken near two municipal wastewater treatment plants in the Grand River, Ontario

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LAY ABSTRACT

Microplastics (pieces of plastic < 5 mm) are ubiquitous in the environment and pose potential risks to organisms upon ingestion. Municipal wastewater treatment plant (WWTP) effluents are thought to be an important pathway for microplastics release into aquatic ecosystems; however, it is unclear whether organisms living near these discharges are consuming microplastics. This thesis investigated whether microplastic levels in water, sediment, fish, and invertebrates were elevated near the Waterloo and Kitchener WWTP outfalls in the central Grand River, Ontario. Although particle levels did not respond predictably to sample proximity to WWTP outfalls, results do suggest that the Waterloo and Kitchener WWTPs are important sources of microplastics to this system. Results from this thesis also provide baseline microplastics levels for the central Grand River, and contribute to our understanding of the fate of microplastics in freshwaters.

ABSTRACT

Microplastics are present in municipal wastewater treatment plant (WWTP) effluents; however, it is unclear whether these contaminants are ingested by biota living downstream of these outfalls. This study examined whether microplastic levels in caged biota, resident fish, and environmental samples were elevated near the Waterloo and Kitchener WWTP outfalls along the Grand River in the fall of 2019.

Amphipods (*Hyalella azteca*), fluted-shell mussels (*Lasmigona costata*), and rainbow trout (*Oncorhynchus mykiss*) were caged at one upstream reference site and two impacted sites downstream of the Kitchener WWTP for 14 (amphipods and trout) or 28 (mussels) days. Rainbow darter (*Etheostoma caeruleum)* were collected using a backpack electrofisher from 10 sites up and downstream of both the Kitchener and Waterloo WWTPs, along with surface water and sediment samples. Whole body *Hyalella*, fish digestive tracts, and fluted-shell mussel tissues (hemolymph, digestive glands, and gills) were digested in 20% potassium hydroxide. Environmental samples were processed using filtration and density separation, then visual identification of microplastics was done.

Elevated particle counts were found in rainbow trout digestive tracts at the Kitchener outfall site, compared to the upstream reference and downstream farfield sites. Additionally, particle concentrations in sediment were significantly higher at the Waterloo outfall, compared to all other sites (except for one upstream location). However, whole *Hyalella,* fluted-shell mussel tissues (hemolymph, digestive glands, and gills), digestive tracts of rainbow darter, and surface waters did not show elevated counts downstream of these discharges. Across all samples, fibers were the most common morphology, and blue and clear particles were prevalent in samples collected near WWTPs. Overall, these findings suggest that the Kitchener and Waterloo WWTPs

could be important sources of particles to the Grand River, adding to our understanding of the fate of this contaminant in freshwater ecosystems.

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DEFINITIONS & ABBREVIATIONS

Microplastics	Particles less than 5 mm in size, which are comprised of plastic polymers
Microparticles	Particles < 5 mm in size which are of anthropogenic origin, but may not necessarily be made of plastic. All microplastics are considered microparticles.
Microfibers	Fibers < 5 mm in length, which can either be made of plastic or other anthropogenically-modified natural materials (such as cotton)
Plastic debris	Any plastic which is found in the environment
WWTP	Wastewater treatment plant
PP	Polypropylene
LDPE	Low density polyethylene
HDPE	High density polyethylene
PVC	Polyvinylchloride
PU	Polyurethane
PET	Polyethylene terephthalate
PS	Polystyrene
MCE	Mixed cellulose ester
ECCC	Environment and Climate Change Canada
CCIW	Canada Centre for Inland Waters (located in Burlington, Ontario)
ALRF	Aquatic Life Research Facility (located and the Canada Centre for Inland Waters, in Burlington, Ontario)

Microplastics in biotic and environmental samples taken near two municipal wastewater treatment plants in the Grand River, Ontario

1 Introduction

1.1 General introduction to microplastics

The current widespread use and environmental contamination of plastics was propelled by several advancements in plastic synthesis towards the end of the 19th century, when plastic became recognized as a versatile, lightweight, and durable alternative for use in many different sectors. By the 1950's, plastic production began to accelerate, and it is estimated that around 360 million metric tons of plastic are now produced globally each year (PlasticsEurope, 2019). The mass production of plastic products, coupled with poor waste management strategies, has led to the accumulation of plastic debris in ecosystems around the globe. Although the first accounts of plastic debris in the marine environment were in the early 1970's, it was not until nearly 40 years later that this issue garnered considerable scientific attention due to increasing environmental and public health concerns (Alimba & Faggio, 2019; Buchanan, 1971; Carpenter & Smith, 1972). Moreover, the true environmental persistence of this material became apparent as reports of smaller pieces of plastic debris, or microplastics, began to emerge (Ng & Obbard, 2006; Thompson et al., 2004).

Microplastics are ubiquitous environmental pollutants, between 0.1 μm and 5 mm in size, which are broadly split into two categories, primary and secondary. Primary microplastics are manufactured to size and can include pre-production plastic pellets and spheres/beads found personal care products. Secondary microplastics are the result of the breakdown of larger plastic items, either during use or once they enter the environment, and can include textile fibers, tyre dust, or fragments from bottles. Should microplastics continue to fragment, they can become

nanoplastics, which are generally defined as being 1-100 nm in at least one dimension (Rist & Hartmann, 2018).

Both primary and secondary microplastics consist of a variety of plastic polymers. Through the process of polymerization, monomer units are chemically bonded together to form long-chain polymers with high molecular weights. Each of these polymer types allows for plastics to have different physical and chemical properties that suit a multitude of applications (table 1.1). For example, polyethylene (PE) is widely used in the food-packaging sector to make films and flexible packaging (Environment and Climate Change Canada & Health Canada, 2020). To give the plastics additional qualities, chemical additives, such as colourants, functional agents (e.g. stabilizers, flame retardants, plasticizers), fillers, and reinforcers can be added during production and can contribute greatly to the overall weight of the product (Hansen et al., 2013). The term microplastics is therefore used as a catch-all term for a diverse suite of particles that vary in origin, composition, morphology, size, colour, polymer type, and chemical additives (Rochman et al., 2019). Additionally, microplastics sorb various other chemicals, such as persistent organic pollutants and heavy metals, making them both a source and a sink for a complex mix of contaminants (Rochman et al., 2013; Rochman et al., 2014a).

More recently, it has been proposed that heavily-modified natural polymers that are semi-synthetic, such as rayon or viscose, also be included in the definition of plastic debris (Hartmann et al., 2019). Although slightly modified natural polymers, such as dyed natural fibers, are not included in this definition, they are often reported within the microplastics literature and can inflate counts (Suaria et al., 2020). As such, I will be using the term *microparticles* throughout this thesis to refer to any non-chemically confirmed particles which appear to be of anthropogenic origin based on colour or other morphological features.

Table 3.1 Applications of the most commonly used and produced plastic polymers (Environment and Climate Change Canada & Health Canada, 2020; PlasticsEurope, 2017).

Polymer	Acronym	Common Applications
Polypropylene	**PP**	Food containers, packaging, and wrappers Automotive parts Pipes Bank notes
Low Density Polyethylene	**LDPE**	Plastic grocery bags Food packaging (trays, containers, film) Agricultural films
High Density Polyethylene	**HDPE**	Construction (pipes) Milk and shampoo bottles Houseware, toys
Polyvinylchloride	**PVC**	Construction (pipes, flooring, window frames) Electrical cable insulation Plastic sheeting Hoses and inflatable pools
Polyurethane	**PU**	Building insulation, and insulating foams for appliances Pillows, mattresses
Polyethylene Terephthalate	**PET**	Synthetic textile fiber (polyester) Plastic drink bottles, packaging
Polystyrene	**PS**	Packaging, containers (cups, coolers, egg trays) Insulation and foams Eyeglass frames

1.2 Microplastics in freshwaters

In its infancy, microplastics research was largely ocean biased, with relatively little done in freshwaters. While most of the literature still focuses on the marine environment, there is a growing body of work that seeks to understand the presence, fate, and effects of microplastics in freshwaters. Since urban, industrial, and agricultural areas include rivers and lakes, freshwater systems receive numerous inputs of plastic debris from these sources and can serve as vectors for the movement of plastics into larger water bodies (Driedger et al., 2015; Windsor et al., 2019). Additionally, lakes and rivers have large areas of shoreline that can retain particles and further facilitate the accumulation and mechanical degradation of plastics (Eerkes-Medrano et al., 2015). Although the specific transport mechanisms of macro- and microplastics in these systems are not

well understood, the size, shape, density, and surface condition of the plastic likely affect their movement (Windsor et al. 2019; Environment and Climate Change Canada & Health Canada, 2020).

Quantifying and characterizing microplastics in freshwater systems has become increasingly important to better understand their distribution and movement, as well as their uptake by and effects on aquatic organisms. In large urban systems such as the Rhine-Main (Germany) and North Shore Channel (Chicago, Illinois), microplastics concentrations in surface waters and sediments rival those of marine environments (Klein et al., 2015; McCormick et al., 2016). These two rivers, among others, also tend to show spatial differences in microplastics concentrations up- and downstream of point sources, such as wastewater treatment plants (WWTPs), and therefore point towards potential inputs of microplastics to riverine systems (Kay et al., 2018; McCormick et al., 2016). Additionally, spatial differences in microplastics concentrations occur within and between lakes according to currents and proximity to urban centres (Dris et al., 2015a; Eriksen et al., 2013; Grbić et al., 2020; Imhof et al., 2013). In receiving inputs from tributaries, as well as direct inputs from nearby sources, lakes can serve as temporary or long-term sinks for microplastics (Alimi et al., 2018).

Although microplastic pollution has been documented globally (Browne et al., 2011; Li et al., 2020; Li et al., 2018), the Laurentian Great Lakes present a vast setting for studying the distribution of microplastics in freshwaters. Microplastics have been measured in shoreline, beach and benthic sediments (Ballent et al., 2016; Corcoran et al., 2015; Dean et al., 2018; Zbyszewski et al., 2014; Zbyszewski & Corcoran, 2011), as well as in surface waters (Baldwin et al., 2016; Eriksen et al., 2013) in the Great Lakes region. Consistent with other microplastics research, these studies demonstrate heightened microplastics concentrations in samples taken in

regions with more anthropogenic activity. In the Great Lakes, the highest average microplastics levels in surface waters were measured in Lake Erie (0.1055 plastic items/m^2) using a manta trawl net (Eriksen et al., 2013). The Lake Erie basin has a higher population density and more industrial activity than Lakes Superior and Huron, where concentrations were lower (0.0054, 0.0028 plastic items/m^2, respectively). Elevated microplastics concentrations in sediments have also been observed around urban and industrial centres such as the Greater Toronto Area, ON, where microplastics abundances in some samples have exceeded 1000 particles per kilogram of dry sediment (Ballent et al., 2016; Dean et al., 2018).

Microplastics found in and around the Great Lakes vary in their morphology and composition, and therefore likely represent contributions from several different sources (Grbić et al., 2020; Helm, 2017). To better understand these sources, Grbić et al. (2020) quantified and characterized microparticles in surface water samples from Lake Ontario and in nearby source waters (WWTP effluent, and agricultural and stormwater runoff). Both stormwater runoff and WWTP effluents were found to have unique particle signatures, with the former containing rubbery particles associated with tyre wear and the latter being dominated by microfibers. Many studies in urban areas identify fibers as a dominant particle type, indicating that WWTP effluents, along with other potential sources of microfibers such sewage sludge or direct shedding of textiles, are likely important contributors of microparticles to freshwater environments (Carr, 2017; Dean et al., 2018; Grbić et al., 2020; Peller et al., 2021)

1.3 Municipal wastewater as a source of microplastics

WWTPs are seen as both entryways and barriers for microplastics release into the environment. These facilities receive influent wastewater from domestic, commercial, industrial sources, and sometimes surface runoff, which all contain plastics in various forms. For example,

wastewater from domestic sources is known to contain high proportions of microfibers from the laundering of textiles (Vassilenko et al., 2019; Xu et al., 2018). While WWTPs are not designed to remove microparticles (Eriksen et al., 2013; McCormick et al., 2014), they are relatively effective at removing them using a series of up to three levels of treatment before the water is discharged as final effluent to a receiving water body (Gies et al., 2018; Sun et al., 2019; Xu et al., 2018). The first level of treatment, or primary treatment, removes solids using coarse and fine screening followed by primary clarification, which involves holding the effluent in a settling tank where the solids either float to the surface and are skimmed (scum) or they sink to the bottom and are removed with sludge (Gies et al., 2018). It has been observed that larger microplastics are preferentially removed during primary treatment, and that removal efficiencies are typically high, but do vary, likely due to differences in sampling methods among studies, as well as due to inherent differences among WWTPs (Iyare et al., 2020; average 72% removal, range 32-93%; Sun et al., 2019; 50-98% removal). Secondary treatment typically involves using aerobic bacteria in oxygenated environments followed by additional clarification to digest organic materials. This process removes microplastics by trapping them in biological flocs, allowing them to settle in clarification tanks, or potentially through ingestion by microorganisms (Carr et al., 2016; Jeong et al., 2016). It is believed that flocculants such as ferric sulfate might also cause the aggregation of microplastics, although this warrants further investigation (Murphy et al., 2016). After secondary treatment, between 86-98.8% of microplastics are removed (Sun et al., 2019). Finally, some WWTPs employ tertiary treatment, or advanced treatment, that uses different technologies to target the removal of specific dissolved substances such as nitrogen. Microplastics removal by tertiary WWTPs is high, although varies slightly according to particle size and the type of

tertiary treatment used (Iyare et al., 2020; average 94% removal, range 82-99%; Lares et al., 2018; average 98.3% removal).

Although WWTPs are effective at removing microparticles, the high volumes of effluent released by these facilities means that even low concentrations of microplastics can result in large contributions to the environment. This is especially important to consider since municipal wastewater is the largest source of effluent by volume in Canada, and is largely discharged into freshwaters (Environment and Climate Change Canada, 2014). It is estimated that up to 30 billion microparticles are released annually from a WWTP near Vancouver despite it removing between 97-99% of microplastics (Gies et al., 2018). Of the microparticles in the final effluent, microfibers are the most abundant particle type across many studies; however, as mentioned above, not all microfibers are necessarily comprised of plastic polymers (Gies et al., 2018; Grbić et al., 2020; Garneau, et al., 2016; Prata, 2018; Sun et al., 2019). The large proportion of fibers in final effluent can likely be explained by 1) the large quantity of fibers that enter WWTPs, and 2) their ability to pass longitudinally through filters or membranes (Sun et al., 2019; Vassilenko et al., 2019). Further, the microplastics and microfibers captured in sludge during wastewater treatment can be inadvertently applied to agricultural fields with the biosolids used as fertilizer, and therefore this represents an additional source for microplastics in the environment (Grbić et al., 2020).

1.4 Uptake of microplastics by freshwater biota

The large quantities of microplastics and other anthropogenic microparticles being released by WWTPs, as well as those making their way into freshwaters through other routes, such as urban and agricultural runoff, atmospheric deposition, direct losses from industry, or the breakdown of larger pieces of debris, pose a potential risk to aquatic biota. While the physical

threats posed by macroplastics are widely documented and understood, the risks associated with microplastics are less clear. Freshwater species are exposed to microplastics through direct or accidental ingestion (Foley et al., 2018). Direct ingestion can either be active, for example when an organism confuses plastic with prey, or passive, where particles might be ingested accidentally during feeding. Accidental ingestion can be the result of microplastics adhering to natural prey items, such as seaweed, or through absorption through the gills or gut walls (Browne et al., 2008; Foley et al., 2018; Gutow et al., 2016; Watts et al., 2014). Although several studies have evaluated ingestion of microplastics under laboratory settings, where microplastics concentrations are typically very high, few studies have studied microplastics exposures under natural conditions. The remainder of this section will review the feeding ecology of four freshwater species that are used in this study, amphipods (*Hyalella azteca*), mussels (*Lasmigona costata*), and fish (*Oncorhynchus mykiss* and *Etheostoma caeruleum*), and discuss the current literature where microplastics consumption has been reported in these, or similar, species.

1.4.1 Hyalella

Hyalella azteca are benthic macroinvertebrates that are found widely across freshwater habitats and, as such, they are commonly used to assess the toxicity of waterborne contaminants. *Hyalella* selectively feed on bacteria and algae on sediment particles that are less than 65 μm in size (Hargrave, 1970), which is within the size range of microplastic particles. In a laboratory exposure study, Au et al. (2015) observed uptake of polyethylene (PE) microplastic spheres (10-27 μm) and polypropylene (PP) microplastic fibers (length: 20-75 μm, diameter: 20 μm) by juvenile *Hyalella* at concentrations of 10 and 22.5 microplastic particles and fibers/L, respectively. For both particle types, ingestion was significantly and positively related to microplastic exposure concentrations. It was also noted that the egestion time for the PE particles

was approximately 2 hours, which was not significantly different from the amount of time required for normal food items; however, the rounded spheres passed more easily through the gut than the microfibers, indicating that particle morphology can influence retention time in these organisms. For instance, juvenile *Hyalella* indiscriminately consumed irregularly-shaped tyre wear particles, albeit at exposure concentrations exceeding realistic environmental conditions, and retained them for 24-48 hours (Khan et al., 2019). To date, no studies have evaluated whether *Hyalella azteca* consume microplastics under natural conditions, or whether they ingest particles that are not commercially formulated. Microplastics found in the environment vary considerably in their morphology and size, and this will likely affect their uptake and toxicity. Since amphipods are lower-trophic-level organisms, they serve as potential vectors for microplastics to higher trophic levels.

1.4.2 Freshwater Mussels

Freshwater mussels are recognized as one of North America's most imperilled groups, yet they receive little attention in the microplastics literature compared to marine bivalves (Su et al., 2018; Wardlaw & Prosser, 2020). Mussels are filter-feeders, meaning that they filter particles from the water through ciliated gills (Vaughn et al., 2008); their feeding strategy and sessile nature makes them useful bioindicators (Boening, 1999; Su et al., 2018). The fluted-shell mussel, *Lasmigona costata*, is a relatively large (150 mm as adults; Gillis et al., 2017a; Hewitt et al., 2016) unionid mussel which can filter up to 1 L of water every hour. As such, they have the potential to take up microplastics from their environment (Vaughn et al., 2008); however, since mussels can selectively choose which particles enter their digestive gland, or which to expel as pseudo feces, they could be limiting their microplastics ingestion (Ward et al., 2019). Whole body (soft tissues) *L. costata* from the Grand River have relatively low numbers (0-7 particles

per mussel) of microplastics (Wardlaw & Prosser, 2020). Although fluted-shell mussels preferentially consume fine particulate matter (<20 μm in size; Vaughn et al., 2008), Wardlaw and Prosser (2020) observed particles in the range of 21-298 μm (average 63 μm), which is consistent with sizes of microplastics seen in marine bivalves (Kolandhasamy et al., 2018; Qu et al., 2018).

1.4.3 Rainbow Trout

Oncorhynchus mykiss is a species of salmonid native to coldwater tributaries of the Pacific Ocean, however, they have been introduced in numerous regions as a food fish and for sport (Hardy, 2002). They have a varied diet, with juveniles (fry and parr) feeding on zooplankton and aquatic insects, and adults consuming crustaceans, smaller fish, and fish eggs (Hardy, 2002). Because this species is commonly harvested for human consumption, research has focused on their uptake of microplastics spiked with other environmental contaminants, as well as the translocation of plastics from their guts to other tissues. Toxicology studies on rainbow trout tend to use virgin polystyrene (PS) and PE microplastic fragments and spheres between 10-1000 μm added to dietary formulations (Ašmonaite, et al., 2018; Kim et al., 2020; Rummel et al., 2016). Results suggest that rainbow trout are not retaining these particles, nor are they translocated to other tissues (Ašmonaite, et al., 2018; Kim et al., 2020; Rummel et al., 2016). While many of these studies do include different size fractions and some exposures use environmentally relevant concentrations (Kim et al., 2020; Roch et al., 2021), there is little information on the uptake of environmentally-weathered microplastics or particles of varying morphologies. Additionally, since many of these studies administer microplastics in feed, it is unclear whether rainbow trout are consuming microplastics in their natural diets.

1.4.4 Rainbow Darter

Etheostoma caeruleum are small, benthic freshwater fish that feed on benthic macroinvertebrates (Paine et al., 1982). These fish are usually found in stream riffles throughout North America and have a median home range of 5 metres (Hicks et al., 2017; Ray et al., 2006). While no studies have described microplastics consumption by rainbow darter, their association with sediment, where microplastics can settle, along with their diet and limited home range means that these fish will likely encounter and/or consume microplastics. Windsor et al. (2019) sampled streams with inputs from WWTPs and found microplastics in larval Ephemeroptera and Trichoptera, which are common prey items for rainbow darter, including those in the Grand River (Paine et al., 1982; Robinson et al., 2016). Chironomidae larvae were especially abundant in the stomachs of rainbow darter from the Grand River, and this invertebrate has been shown to consume microplastics during laboratory exposures at environmentally relevant concentrations (Ziajahromi et al., 2018).

1.5 Consequences of microplastics ingestion

Similar to macroplastics ingestion by larger organisms, microplastics can cause physical harm to small biota by blocking the digestive tract or causing abrasions (Wright et al., 2013). The presence of microplastics in the gut can also cause reduced feeding and growth, and could result in particle translocation and bioaccumulation (Wright et al., 2013). Several toxicological effects associated with microplastics have also been examined and they generally fall into one of two categories: particle-related (or physical) toxicity and chemical toxicity. The polymer type, size, shape, and surface morphology will primarily drive particle-related toxicity, while chemical toxicity is caused by additives and sorbed compounds (Wagner & Lambert, 2018). It continues to be challenging to tease apart whether particular effects are driven by physical or chemical

toxicity (Foley et al., 2018; Zimmermann et al., 2020), but broadly microplastics have been observed to cause oxidative stress, endocrine and reproductive dysfunction, behavioural changes, and increased mortality in a range of marine and aquatic species (Au et al., 2015; De Felice et al., 2019; Foley et al., 2018; Rochman et al., 2014b; Sussarellu et al., 2016; Xia et al., 2020). Ultimately, the harm posed to organisms will likely vary according to particle concentrations, polymer type, morphology, size, additives, and environmental weathering (Foley et al., 2018; Rochman et al., 2019). As mentioned previously, many lab exposures use virgin plastics which are often of the same polymer type, size, and shape. While these studies are helpful in understanding the effects of that particular particle class, they do not inform us of the effects of microplastics in general because of their immense diversity (Rochman et al., 2019).

The information on microplastics toxicity in *Hyalella azteca* is currently limited to a few studies which evaluate mortality, reproduction, and growth (Au et al., 2015; Halle et al., 2021; Khan et al., 2019; Redondo-Hasselerharm et al., 2018). Juvenile *Hyalella* chronically exposed to PE spheres and PP fibers, had significant reductions in growth (both types) and reproduction (PE only; Au et al., 2015). In 10-day exposures to determine lethal concentrations (LC50), the fibers had greater toxicity and this corresponded to longer residence times in the gut. Additionally, the PP fibers were made from environmentally-weathered marine rope, whereas the PE particles were purchased new; therefore some of the differences in toxicity could be associated with the use of different ages and morphologies of particles (Au et al., 2015). Reductions in growth and reproduction and high mortality were also observed when juvenile *Hyalella* were exposed to tyre wear particles at high concentrations (Halle et al., 2021; Khan et al., 2019).

Although no studies have evaluated whether microplastics exposure affects *Lasmigona costata*, effects have been observed in marine bivalves such as the blue mussel (*Mytilus edulis*).

When exposed to high density polyethylene (HDPE), the digestive cells of *M. edulis* showed a strong inflammatory response and histological changes (Von Moos et al., 2012). Additionally, HDPE particles aggregated in the gills of marine mussels, suggesting an alternate uptake pathway (Kolandhasamy et al., 2018; Von Moos et al., 2012). Cellular alterations were also seen in *Mytilus galloprovincialis* exposed to PE and PS contaminated with pyrene, a polycyclic aromatic hydrocarbon (PAH) commonly sorbed to marine plastic debris (Avio et al., 2015). Along with altered immune responses, neurotoxic effects, and changes in gene expression, the transfer and bioaccumulation of pyrene from plastics into gill tissues has been observed (Avio et al., 2015). The translocation of microplastics has also been reported in *Mytilus edulis*, with PS spheres less than 10 μm in size being present in the hemolymph (Browne et al., 2008). Additional effects of microplastics, such as reductions in attachment strength and filtration rate and alterations in the hemolymph proteome, have also been observed in marine mussels (Green et al., 2019; Woods et al., 2018). Despite these numerous adverse effects, research on freshwater mussels continues to be limited.

Since rainbow trout are a food fish, studies have addressed whether they are affected by microplastics uptake. While some observed no effects when rainbow trout were exposed to pristine microplastics (Ašmonaite, et al., 2018; Rummel et al., 2016), other *in-vivo* and *in-vitro* studies have reported negative effects (Karbalaei et al., 2021; Zwollo et al., 2021). Weathered plastics often sorb environmental contaminants, and therefore their effects on biota might differ from those caused by virgin plastics. Juvenile *O. mykiss* showed different biomarker responses when exposed to pristine and chlorpyrifos (CPF)- loaded PS fragments (Karbalaei et al., 2021). It was observed that PS particles alone caused histopathological changes in the gills, gut, and skin of *O. mykiss*; however, these alterations were more pronounced in mixtures with both PS and

CPF. Another recent study found changes in B-cell development and gene expression when immune cells were exposed to spherical and irregularly shaped PS particles (Zwollo et al., 2021). While these studies are somewhat limited by their near exclusive use of PS, they are beginning to capture some of the complexities associated with microplastics and chemical mixtures and provide some directions for future research.

1.6 Study rationale

There is limited literature investigating microplastics uptake by biota at environmentally relevant concentrations, and it is unclear whether freshwater biota are affected by the presence of microplastics at these levels. WWTPs are an important pathway for microplastics release into the environment, and spatial differences in microplastics concentrations in water and sediment have been found in relation to these point sources (Kay et al., 2018; McCormick et al., 2016); however, it remains unclear whether biota living near these discharges are ingesting elevated quantities of microplastics.

The Grand River is one of the principal tributaries to Lake Erie, which is recognized as a plastic-polluted lake (Hoffman & Hittinger, 2017; Mason et al., 2020). Along its length, the Grand River receives inputs from 30 WWTPs, two of which, the Kitchener and Waterloo WWTPs, serve relatively large populations (>100 000) and have been the focus of previous studies evaluating the effects of wastewater effluents on riverine biota (Fuzzen, 2016; Gillis et al., 2017; Tanna et al., 2013; Tetreault et al., 2013; Tetreault, 2012). Fish downstream of the Kitchener and Waterloo discharges have altered reproductive health and population dynamics, with increased rates of intersex, altered gonadal development, reduced reproductive success, and shifts to more tolerant fish species (Fuzzen, 2016; Tanna et al., 2013; Tetreault et al., 2013; Tetreault, 2012). A 7 km stretch of the river downstream of the Kitchener WWTP outfall is also

a known mussel extirpation zone, indicating that the effluent negatively affects habitat quality and the survival of this imperilled group of organisms (Gillis et al., 2017b). In addition to these WWTP inputs, this system also receives high nutrient loads from surrounding agricultural areas as well as increased chloride concentrations in some locations due to runoff from urban road salt applications (Loomer & Cooke, 2003). It is currently unknown whether microplastics are discharged into the Grand River by the Kitchener and Waterloo WWTPs. This provides a unique opportunity to assess the abundance of microplastics in the biotic and abiotic compartments of this urbanized river.

1.7 Study objectives

This study explored whether proximity to WWTP outfalls influences the concentrations of microplastics in water and sediment, as well as their uptake by biota. Both wild-caught fish and caged organisms were sampled to answer the following questions:

1) Are microplastic levels in the digestive tracts of wild-caught rainbow darter (*Etheostoma caeruleum*), and in water and sediment samples, taken near WWTP outfalls elevated compared to levels at upstream reference sites.

2) Can caged organisms (*Hyaella azteca*, *Lasmigona costata*, and *Oncorhynchus mykiss*) be used as biomonitors to identify areas of microplastics release in riverine environments?

Based on the literature, the following predictions were made. First, it was predicted that environmental samples taken closest to the Waterloo and Kitchener WWTP outfalls would have higher microplastic concentrations than those collected at the reference and farfield sites. Similarly, wild-caught rainbow darter were predicted to have the highest counts of microplastic particles in their digestive tracts near the WWTP discharges. Since *H. azteca* have ingested

microplastics during laboratory exposures (Au et al., 2015), it was predicted that they may consume microplastics under natural conditions; however, their potential use as biomonitors was unclear. In contrast, it was thought that *L. costata* would reflect sites with elevated levels of microplastics as other freshwater bivalves are effective biomonitors for microplastics (Su et al., 2018). Similarly, because other species of fish have been used successfully as biomonitors for microplastics, it was predicted that caged rainbow trout might also be effective biomonitors for identifying areas with elevated microplastic levels (Yan et al., 2021).

This study will serve as a baseline for microplastics concentrations in water and sediments from the Grand River and will contribute to our understanding of the various stressors impacting this watershed. Furthermore, this information will be useful in assessing the effectiveness of future upgrades to the WWTPs at reducing inputs of microplastics and will facilitate future studies seeking to understand the impacts of microplastics on riverine biota at environmentally relevant levels.

2 Methods

2.1 Study area

The Grand River and its tributaries comprise the largest watershed in Southern Ontario, draining an area of approximately 6,800 km^2 into Lake Erie (Anderson & GRWMP Assimilative Capacity Working Group, 2012). This watershed is home to close to 1 million people, who mostly reside in the municipalities of Kitchener, Waterloo, Guelph, Cambridge, and Brantford (Anderson & GRWMP Assimilative Capacity Working Group, 2012).

The region is primarily agricultural, with farms occupying upwards of 70% of the land. Along with agricultural inputs, and urban runoff, the Grand River and its tributaries also receive effluent from 30 WWTPs (GRCA, 2020). Of these, the Waterloo and Kitchener WWTPs service

a population of 139,527 and 242,626 people, respectively (Waterloo Region, 2018), and use secondary-conventional activated sludge treatment. Both the Waterloo and Kitchener WWTPs have been the focus of several studies that have evaluated the effect of WWTP effluent on resident biota in the Grand River, particularly rainbow darter and mussels (Fuzzen et al., 2015; Gillis et al., 2017; Mehdi et al., 2018; Tetreault et al., 2013). For the current study, ten sites were sampled along a 60 km stretch the Grand River in Fall of 2019. Sites were chosen according to their proximity to Waterloo and Kitchener outfalls, with 3 upstream reference sites (REF1-REF3), 3 sites downstream of the Waterloo WWTP (DSW1-DSW3), and 4 sites downstream of the Kitchener WWTP (DSK1-DSK4; figure 2.1). Site coordinates, and proximity to outfalls can be found in table 2.1.

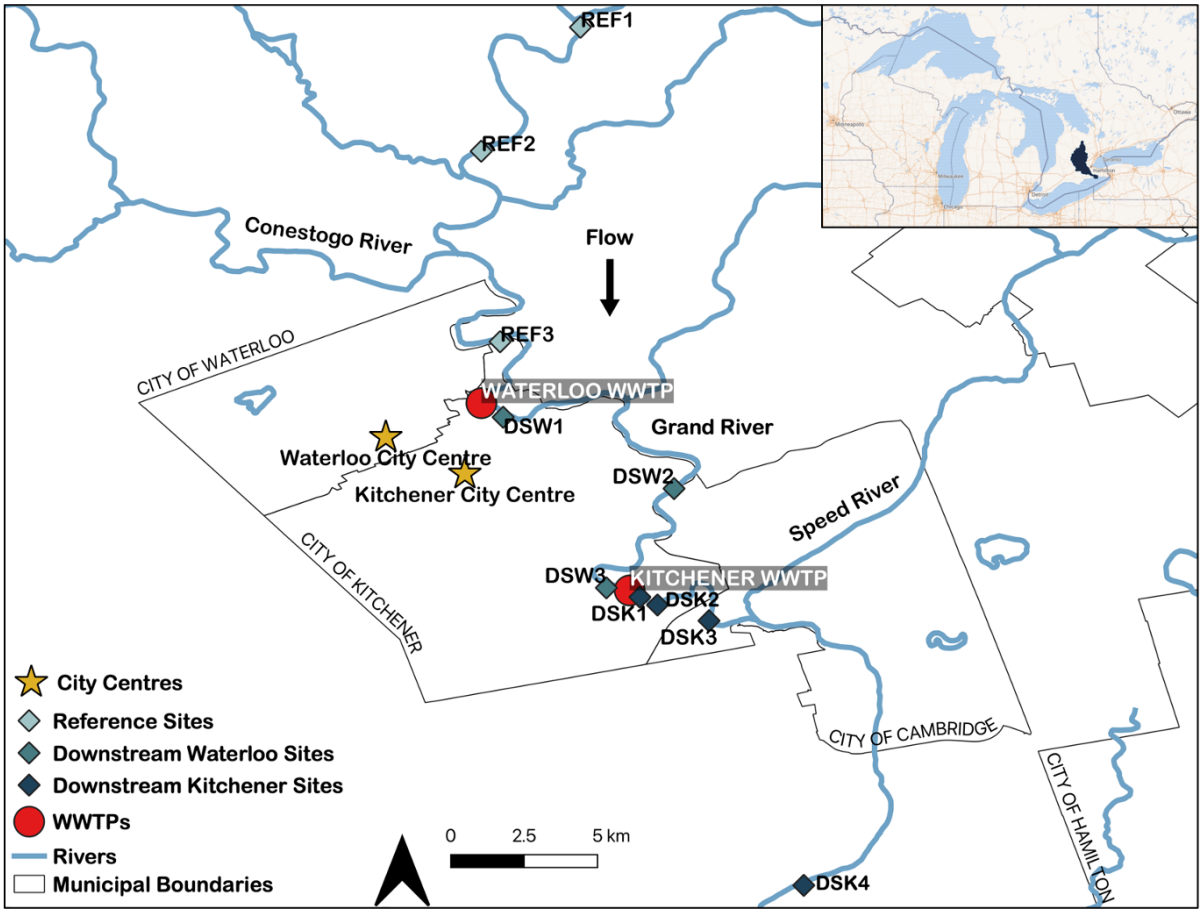


Figure 2.1 Sites sampled in fall 2019 along the Grand River. Site names are described in table 2.1

Table 4.1 Site names, locations, and proximity to WWTP outfalls, and WWTP influence for sites sampled for biotic and environmental samples in October 2019.

Site Name	Location		Proximity to Nearest WWTP (km)	WWTP Influence
	Latitude	Longitude		
REF1 Inverhaugh	43.637147	-80.440662	17.185	Small WWTPs upstream of Waterloo
REF2 West Montrose	43.585147	-80.482303	11.762	Small WWTPs upstream of Waterloo
REF3 Kiwanis	43.505235	-80.474394	2.936	Small WWTPs upstream of Waterloo
DSW1 Economical Insurance Trail	43.47359	-80.4731	0.987	Waterloo
DSW2 Fairway	43.443649	-80.401378	7.713	Waterloo
DSW3 Horseranch	43.402021	-80.429769	9.609	Waterloo
DSK1 Pioneer Tower 1	43.398125	-80.415698	0.605	Kitchener + Waterloo
DSK2 Pioneer Tower 2	43.394828	-80.408326	1.278	Kitchener + Waterloo
DSK3 Blair	43.388209	-80.386709	3.175	Kitchener + Waterloo
DSK4 Glen Morris	43.277221	-80.346926	14.884	Kitchener + Waterloo + Galt

2.2 Environmental sample collection

Water and sediment samples were collected from all 10 sites on the 24th and 25th of October 2019. Three replicates of 20 L of surface water were collected using an orange polypropylene bucket, triple-rinsed with site water, away from the riverbank. The water was pumped through vinyl tubing into a clean empty bucket using a peristaltic pump with 4-in line stainless steel mesh filters. Filters were ordered from largest to smallest fraction (533.4 µm, 228.6 µm, 116.84 µm, 35.56 µm) in the direction of the pump flow. The polypropylene filter casing was rinsed with

deionized (DI) water between replicates, and new filters were inserted. Used filters were stored in PS petri dishes sealed with tape. A blank was done at each site by briefly reversing the flow direction to purge the system, and then re-filtering 20 L of water which had previously passed through the series of filters into the clean bucket.

A metal shovel was used to scoop sediment from near the bank, at an approximate water depth of 30 cm into clean glass jars with metal lids. Four replicates of 500 mL, for a total of 2 L, of sediment was collected at each site. An air blank was collected at each site by opening a clean jar for the approximate duration it would take to collect one sample.

2.3 Environmental sample processing

Stainless steel water filters were transferred from petri dishes into clean glass jars with forceps; petri dishes were rinsed into the glass jar with DI water. The glass jars were topped up with DI water, sealed with a metal lid and sonicated for 1 hour to re-suspend particles that were captured on the filters; filters were removed with clean forceps and rinsed with DI water into the jar. The contents of the glass jar were filtered through a 5.0 μm mixed cellulose ester (MCE) membrane in a vacuum filtration system. Both the glass jar and the sides of the Büchner funnel were rinsed with DI water to ensure all particles were captured. The membrane filters were lifted into glass petri dishes with lids in preparation for visual identification. To limit contamination of samples, glassware associated with the vacuum filtration system was cleaned between samples, and the Büchner funnel was covered with a glass lid as much as possible. The blanks taken from each site were processed the same way as the samples.

In a fumehood, sediment was transferred from sample jars into a clean glass pie dish using a metal spoon. Dishes were covered with tinfoil when not in use to limit airborne contamination. Wet weight was recorded and sediment was placed in a drying oven for ~72 hours at 55°C. Dry

weight was recorded, and sediment was sieved through a sieve stack to separate the sample into two size fractions (>500 μm and >38 μm). When necessary, DI water was used to break up larger clumps of sediment. The two size fractions were transferred into clean glass 1L beakers and covered with tinfoil, and then underwent density separations using $CaCl_2$ (Acros Organics; 96%) at 1.4 g/L which was pre-filtered through a 10 μm filter (Adams et al., 2021). After 24 hours, the floating portion was rinsed on a 38 μm sieve with DI water and transferred to a clean glass petri dish for visual sorting. The jars which were used to collect air blanks at each site were topped up with $CaCl_2$ and were similarly rinsed through the 38 μm sieve and transferred to glass petri dishes.

2.4 Field deployment and sampling of caged organisms

In collaboration with Environment and Climate Change Canada (ECCC), *Hyalella azteca*, *Lasmigona costata*, and *Oncorhynchus mykiss* were caged at one upstream reference site (DSW3) and two impacted sites downstream of the Kitchener WWTP (DSK1 and DSK2) to determine whether they can be used as biomonitors for microplastics. This work was part of a larger study, funded by ECCC's Chemical Management Plan to assess the environmental fate of WWTP-associated chemicals. Planning, fieldwork, and sample collection was led by Patricia Gillis, Gerald Tetreault, and Adrienne Bartlett, and their technicians: Joseph Salerno, Jim Bennett, Jason Miller, and Lisa Brown.

2.4.1 Amphipods (14 days)

Hyalella azteca were obtained from cultures at ECCC's Canada Centre for Inland Waters (CCIW) in Burlington, ON. Amphipods were 10-11 weeks old at the time of deployment and underwent a 14-day exposure between September 24th- October 8th. The cages consisted of clear

acrylic tubing, sealed at each end with 300 μm Nitex mesh (Bartlett et al., 2016). Five cages, each containing 20 individuals, were deployed at each site within a larger metal substrate cage on the riverbed at an approximate water depth of 60 cm. The substrate cage was weighted with a brick and fastened to rebar anchored in the substrate. At the end of the exposure period, individual cages were transported from the stream to the bank in a white polypropylene bucket containing site water. *Hyalella* were rinsed from their cages into a metal tray where they were picked with clean forceps, rinsed with DI water, and placed individually into 1.7 mL polypropylene tubes. Thirty amphipods, 15 each from two cages, were sampled at each site and immediately placed on dry ice; individual amphipods were considered to be replicates. Air blanks were done for every 10 amphipods (3 per site) by exposing the sample tube to air for the duration it would take to pick up an amphipod and transfer it to a tube.

Table 2.2 Whole body *Hyalella* weights (mean ± SD) at reference and impacted sites (n = 10 *Hyalella* per site).

Species	Site	Designation	Whole Body *Hyalella* Weight (mg)
Hyalella azteca	DSW3	Reference	4.94 ± 1.65
	DSK1	Impacted	4.10 ± 1.45
	DKS2	Impacted	3.59 ± 0.61

2.4.2 Mussels (28 days)

Adult fluted-shell mussels were collected from the Grand River (REF3) and held at ECCC's Aquatic Life Research Facility (ALRF; Burlington, ON) in a flow through system with dechlorinated Burlington city tap water and were fed a commercial shellfish diet prior to deployment. Mussels underwent a 4-week exposure from September 24th to October 22nd (DSW3) or 23rd (DSK1 and DSK2). Thirty mussels were deployed at each site in cages

consisting of mussel socks strung across a polyvinylchloride (PVC) frame, which was secured to iron bars in the sediment (Gillis et al., 2014). At the end of 28 days, mussels were stored in aerated coolers filled with site water and were transported for approximately 1 hour to the CCIW in Burlington, ON, for dissections. Hemolymph, gill tissue, and digestive glands were sampled from 10 mussels caged at each site. First, a new 22-gauge syringe was used to draw 500 µL of hemolymph from the anterior abductor muscle (Gillis et al., 2014); the mussel was then rinsed externally with DI water and dissected for portions of the digestive gland and gill tissues. Average masses of tissue collected are shown in table 2.3. A water blank was collected for every 10 hemolymph samples by using a new syringe to transfer DI water, pre-filtered through a 0.45 µm MCE membrane, to a sample tube. Air blanks were collected for both the gill and digestive gland tissues by leaving an empty tube uncapped for the duration of a dissection. All tools and dissection trays were thoroughly cleaned between samples, and materials (mussel socks, blotting papers, syringe tubes) were collected as reference materials for potential sources of contamination.

Table 2.3 Mussel lengths (cm) and weights (g), and masses of tissues (g) sampled for microplastics (mean ± SD) at reference and impacted sites (n = 10 mussels per site).

Species	Site	Designation	Mussel Length (cm)	Whole Mussel Weight (g)	Mass of Tissue Portion (g)	
					Digestive Gland	Gill
Lasmigona costata	DSW3	Reference	9.03 ± 0.84	70.39 ± 17.21	0.31 ± 0.08	0.13 ± 0.06
	DSK1	Impacted	9.27 ± 0.67	73.47 ± 17.20	0.31 ± 0.08	0.14 ± 0.03
	DKS2	Impacted	9.03 ± 0.68	64.05 ± 16.24	0.27 ± 1.10	0.15 ± 0.07

2.4.3 Rainbow trout (14 days)

Juvenile rainbow trout (*Oncorhynchus mykiss*) approximately 10 months in age were obtained from Lyndon Fish Hatcheries and were held at the ALRF for 1 week prior to

deployment. Fish were exposed for two weeks from October 8th-23rd in cages made from PE totes that were secured to a piece of rebar anchored in the sediment. At the end of the exposure period, rainbow trout were transported from the river to a mobile field trailer in aerated 45 L coolers and were processed within 90 minutes of collection. Fish were handled according to ECCC's approved Animal Use Protocol #1958 and were rendered unconscious with a blow to the head, prior to collecting lengths and weights for each individual (table 2.4). Trout were then bled and euthanized by spinal severance before dissections. The digestive tract was removed from 10 fish at each site and placed into triple-rinsed polypropylene tubes. All tools were cleaned with ethanol and DI water between dissections, and an air blank was collected in the trailer for every 10 fish by leaving a clean tube uncapped for the duration of a dissection. Samples were placed on ice and then frozen at -20°C upon return from the field.

Table 2.4 Rainbow trout lengths and weights (mean ± SD) at reference and impacted sites (n = 10 fish per site).

Species	Site	Designation	Length (cm)	Mass (g)
Oncorhynchus mykiss	DSW3	Reference	69.89 ± 14.74	18.65 ± 1.45
	DSK1	Impacted	74.93 ± 17.10	19.03 ± 1.65
	DSK2	Impacted	68.93 ± 12.45	18.69 ± 1.20

2.5 Sampling wild-caught rainbow darter

Ten rainbow darter (*Etheostoma caeruleum*; 5 male, 5 female, >4.5 cm in length) were collected from each of the 10 sites along the Grand River between October 18th-21st 2019 using a backpack electrofisher. The fish were kept in aerated 10 L buckets prior to dissections, which were completed in a mobile lab trailer within two hours of their removal from the river. Rainbow darter were handled according to the University of Waterloo's AUP #40318. Lengths and weights were taken (table 2.5) after rendering the fish unconscious with a blow to the head. Fish

were then euthanized by spinal severance and the whole digestive tract was removed from each of the fish and placed into triple-rinsed 15 mL polypropylene sample tubes. All tools were cleaned between each dissection using ethanol and DI water, and were kept covered with tin foil (fired at 450°C for 6 hours) when not in use. Samples were stored on ice and then frozen at the end of each field day at -20°C. An air blank was collected in the field trailer for every 10 rainbow darter sampled (1 blank for each site) by keeping an empty sampling tube uncapped for the duration of one dissection.

Table 2.5 Rainbow darter lengths and weights (mean ± SD) at reference and impacted sites (n = 10 fish per site).

Species	Site	Designation	Length (cm)	Mass (g)
Etheostoma caeruleum	REF1	Reference	5.61 ± 0.48	2.24 ± 0.63
	REF2	Reference	5.68 ± 0.53	2.36 ± 0.73
	REF3	Reference	5.89 ± 0.81	2.70 ± 1.28
	DSWI	Impacted	5.78 ± 0.32	2.52 ± 0.48
	DSW2	Impacted	5.52 ± 0.40	1.99 ± 0.53
	DSW3	Impacted	5.83 ± 0.43	2.33 ± 0.55
	DSK1	Impacted	5.81 ± 0.59	2.66 ± 1.06
	DSK2	Impacted	5.77 ± 0.36	2.49 ± 0.54
	DSK3	Impacted	5.78 ± 0.41	2.65 ± 0.64
	DSK4	Impacted	5.92 ± 0.54	2.68 ± 0.82

2.6 Biotic sample processing

All biotic samples (whole amphipods, fluted-shell mussel tissues, and rainbow trout and rainbow darter digestive tracts) were digested using 20% KOH (Dehaut et al., 2016; Lusher et al., 2017; Munno et al., 2018; Rochman et al., 2015). Three times the sample volume, or a minimum of 15 mL, of KOH was added to each of the polypropylene sample tubes, which were left at room temperature in the fume hood for 14 days. Digested tissue was rinsed through a 38 µm stainless steel sieve with DI water. In preparation for visual sorting, the contents of the sieve

were then transferred to clean glass petri dishes with lids, which had been blasted with air prior to use (Prata et al., 2020). Field blank tubes were filled with DI water; a laboratory blank was also collected for every 10 samples by filling a triple-rinsed polypropylene sampling tube with KOH. Both the field and laboratory blanks were left for 14 days and underwent the same filtering protocol as the biotic samples.

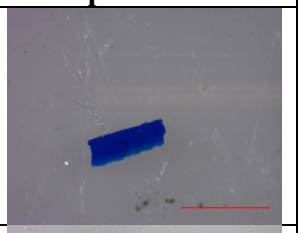
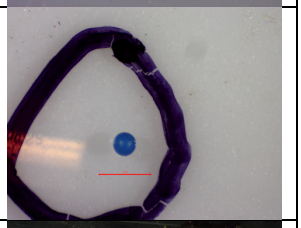
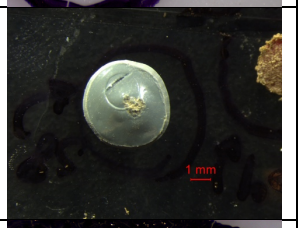
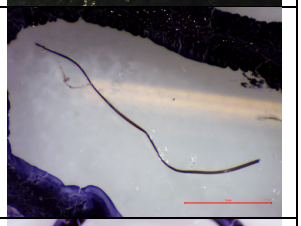
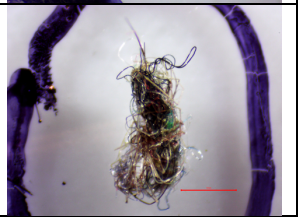
To limit exogenous contamination of the samples in the laboratory, surfaces were wiped thoroughly with 70% EtOH and a Kimwipe; solutions were pre-filtered through 0.45 μm membrane filters; materials were thoroughly cleaned with DI water, Alcojet soap, and a natural sponge; a white cotton lab coat was worn, and clothing composition and colour were recorded. Furthermore, a portable HEPA filtration system was used near microplastics workstations in the McMaster University laboratory to reduce the number of airborne particles. When possible, use of plastic materials was limited, however, since KOH etches glass, polypropylene sampling tubes were used for biotic sample collection and processing. This choice of material is supported by protocols developed by the Rochman lab at the University of Toronto (Munno et al., 2018).

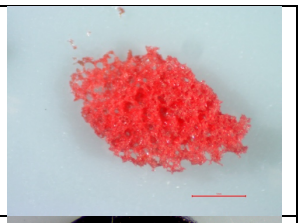
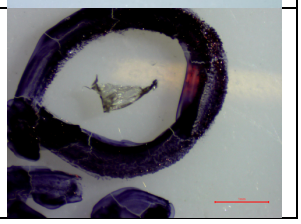
2.7 Visual sorting

Visual sorting was done using an Olympus stereomicroscope (SZX7, magnification range 8x-56x). Particles were removed from the primary glass petri dish, which contained the processed sample, and transferred to a secondary petri dish which was lined with transparency paper and double-sided tape for later chemical analyses. The particle was circled, numbered and recorded according to particle morphology and colour. Seven different morphologies were used to classify particles: fiber, fiber bundle, film, foam, fragment, sphere, pellet (table 2.6; Rochman et al., 2019). Basic colours were used to describe the particle. If a particle was comprised of more than one colour, the predominant colour was used unless indistinguishable, in which case it was

recorded as multi-colour. For samples containing numerous particles of the same colour and morphology combination, only the first 10 were transferred to the secondary petri dish, and afterwards a tally was kept. The data collected during this phase of processing provided a total count of the number, and type, of particles found in each sample. To limit contamination, petri dishes were covered whenever they were not in use, surfaces were cleaned before use, clothing colour and composition were recorded, and all work was done near a HEPA filter.

Table 2.6 Particle sorting categories (as in De Frond and Munno 2019).

Particle Type	Description	Example
Fragment	Rigid edges, hard. Irregular shapes (angular, subangular, rounded, sub-rounded). Do not break easily when compressed.	
Sphere	Perfectly spherical. Can be hemispheres (broken spheres). No irregularities. Smooth, often shiny surface.	
Pellet	Flattened oval shape. Sometimes rectangular or cylindrical with a clear 'machine cut'edge. Larger in size than spheres (typically 3-5 mm).	
Fiber	Strand or string-like. Often equally thick throughout. Can change colour due to bleaching. Can be easily bent and twisted Ends can be flat, pointed, or fraying.	
Fiber Bundle	Tightly wound individual fibers. Cannot be teased apart, separation would cause breakage. Can consist of fibers of different colors.	

Foam	Holes within the particle structure. Will bounce back when compressed.	
Film	Thin, flat, flexible sheets. Can fold or crease Partially or totally transparent.	

2.8 Quality assurance and quality control

To account for contamination during sample collection and processing, blank corrections were done prior to data analysis and chemical identification. Particles were subtracted from samples taken at each site if the procedural blank associated with that site and sample type contained particles of the same colour and morphology combination. Once blank subtractions were complete, remaining particles were photographed and measured for length and width (data not shown). A subsample of 10% of each colour and morphology combination will be sent for chemical confirmation.

2.9 Data analysis

All statistical analyses were completed using R software (version 3.6.1). Model assumptions were first assessed by graphical inspection; to verify homogeneity, residuals versus fitted values were plotted, and normality was assessed by plotting the quantile-quantile plots of the residuals. If it was unclear whether one or both of these assumptions were met, the Shapiro-Wilk test of normality and Levene's test of equal variance were used to decide whether parametric or non-parametric methods should be used. Analysis of variance (ANOVA) were used to compare particle counts across sites for each sample type. If sites were significantly different ($p<0.05$), a

Tukey's honest significance test (HSD) was performed with $\alpha = 0.05$. When assumptions were not met, a Kruskal-Wallis test was conducted to evaluate particles counts across sites; pairwise Wilcoxon tests were done when Kruskal-Wallis tests had significant results. Linear regressions were performed between whole body wet weight, or tissue mass, and the number of particles to determine whether particle counts were affected by the body or sample size of the organism. Finally, Pearson's correlation tests were conducted between different environmental and biotic matrices to evaluate whether levels across different sample types were related.

3 Results

3.1 Microparticles in environmental samples from the Grand River

3.1.1 Abundance and characterization of microparticles in water samples

Particles were found in each of the four size fractions of the water samples; however, counts tended to decrease from the largest to smallest size fraction (figure 3.1). Although each size fraction showed varying spatial trends, statistical differences between sites were only detected for the 533.4 µm and 116.84 µm fractions (ANOVA, $F_{9,20}$=2.85, p = 0.02; $F_{9,20}$=3.08, p = 0.02). For the 533.4 µm fraction, REF1 had significantly elevated counts compared to REF2 (Tukey's, p = 0.03), but there were no other significant pairwise comparisons. In the 116 µm fraction, the Waterloo outfall site (DSW1) had higher counts than REF3 (p=0.03), DSW2 (p = 0.02), and DSK2 (p = 0.04) but the outfall site was not different from the other six sites.

When particle counts from all size fractions within a replicate were summed, another spatial pattern emerged (figure 3.2). While there were site effects ($F_{9,20}$ = 2.61, p = 0.04), no significantly different site pairs were detected (Tukey's, p = 0.09 – 1.0). A trend was observed in the data, where elevated particle counts were found at two of the reference sites (REF1 and

REF3) as well the two WWTP outfall sites (DSW1 and DSK1), when compared to sites further downstream of the outfalls and REF2.

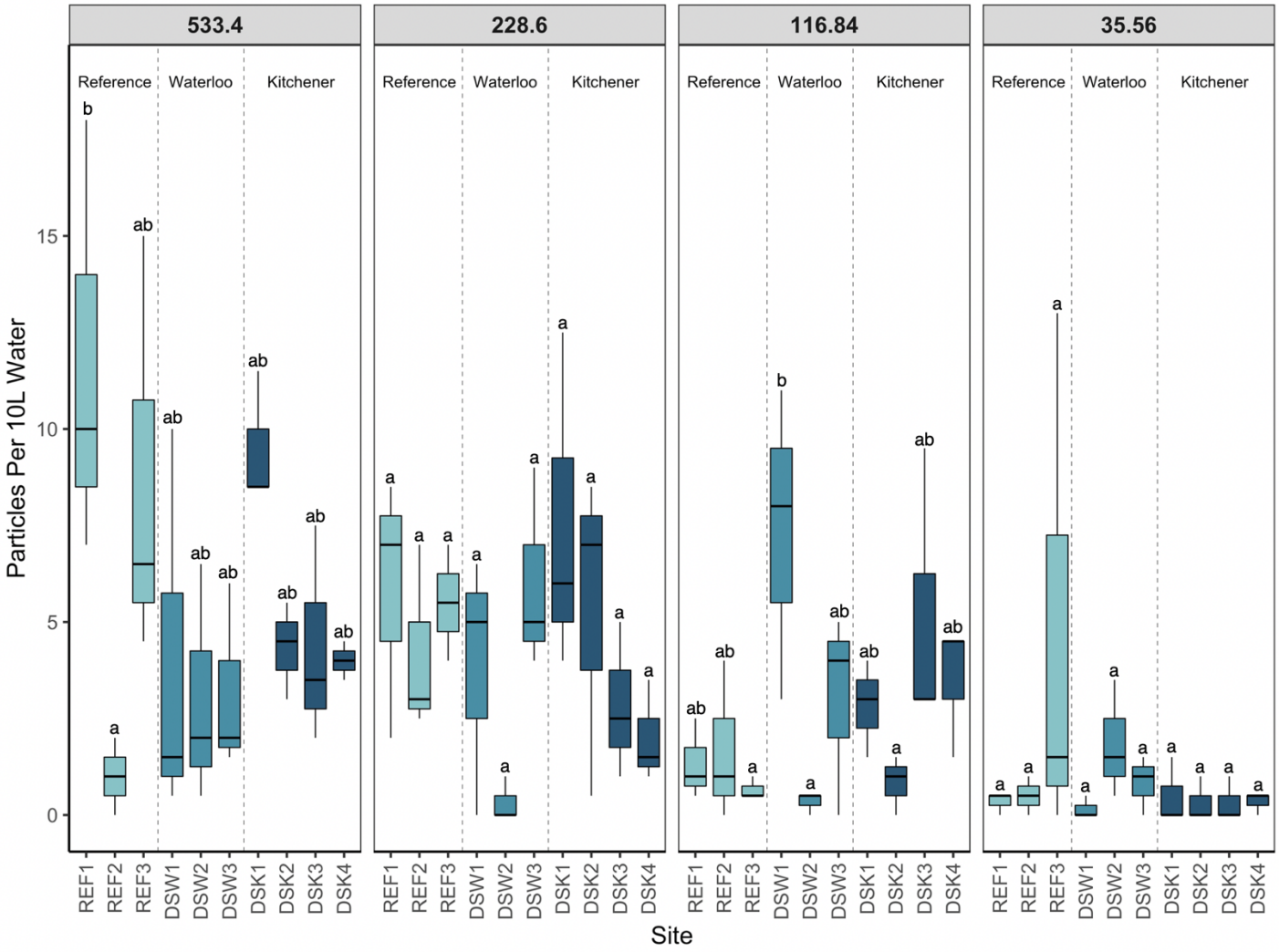


Figure 3.1 Counts of microparticles per 10 L of water from sites along the Grand River, ON (n=3 per site). Plots are organized from largest to smallest filter size fraction (533.4, 228.6, 116.84, 36.56 µm from left to right), which corresponds to the direction of flow through the pump during sampling. Within each size fraction, sites are ordered on the x-axis from upstream (left) to downstream (right). Different letters within each panel indicate significant differences between sites.

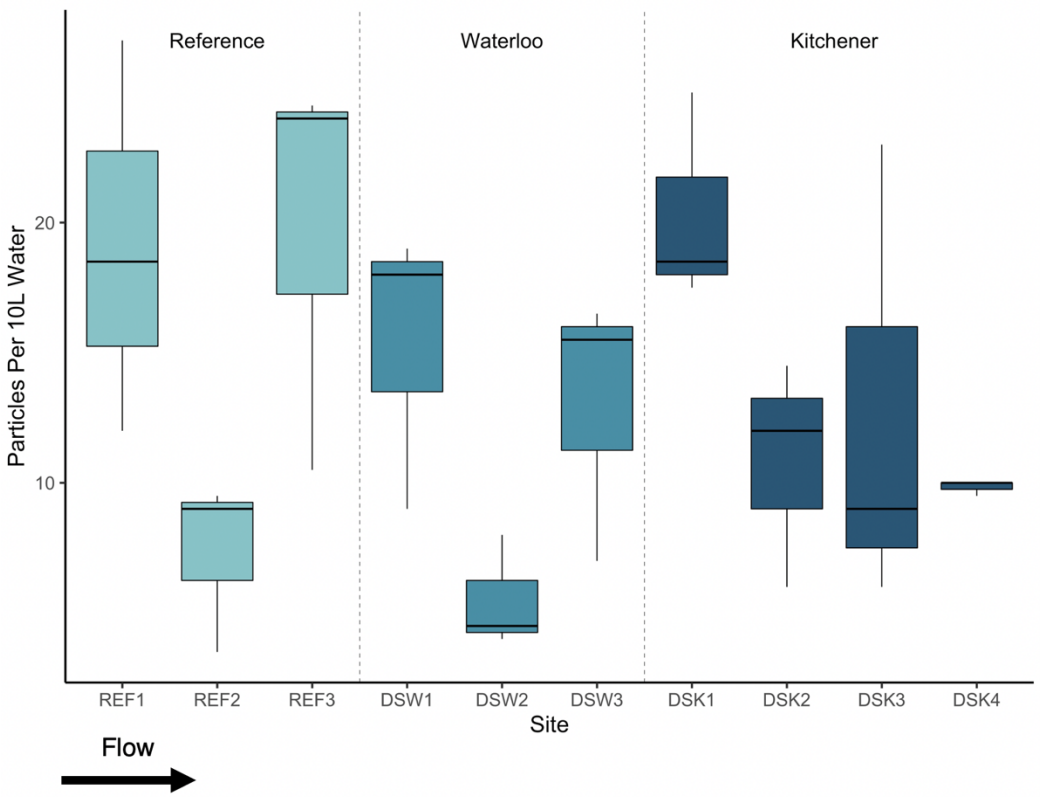


Figure 3.2 Counts of microparticles per 10 L of water from sites along the Grand River, ON (n=3 per site). Counts represent the sum of the particles found all four filters used within a replicate (533.4, 228.6, 116.84, 36.56 µm). Sites are ordered on the x-axis from upstream (left) to downstream (right). There were no significant differences between sites.

Microparticle colours varied across sites as well as between the different size fractions of the water samples (figure 3.3). Fourteen different colours were present in the largest size fraction (533.4 µm), with blue and clear particles appearing commonly across most sites in relatively high proportions; red particles were present in the largest size fraction at 7 of the 10 sites but in low proportions. The 228.6 µm and 116.84 µm fractions both had 10 distinct colours and most sites had high proportions of blue and clear particles (exceptions DSW2 and DSK2 for the 228.6 µm and 116.84 µm fractions, respectively). A total of 8 colours were recognized in the 35.56 µm fraction, and red particles were particularly common, comprising all particles found at two sites downstream of the Kitchener outfall (DSK2 and DSK3) and 50% of particles at REF1 and

DSK4. When all size fractions are combined, blue and clear particles were found at every site, with pink and red particles also being common and appearing at 9 sites each (figure 3.4).

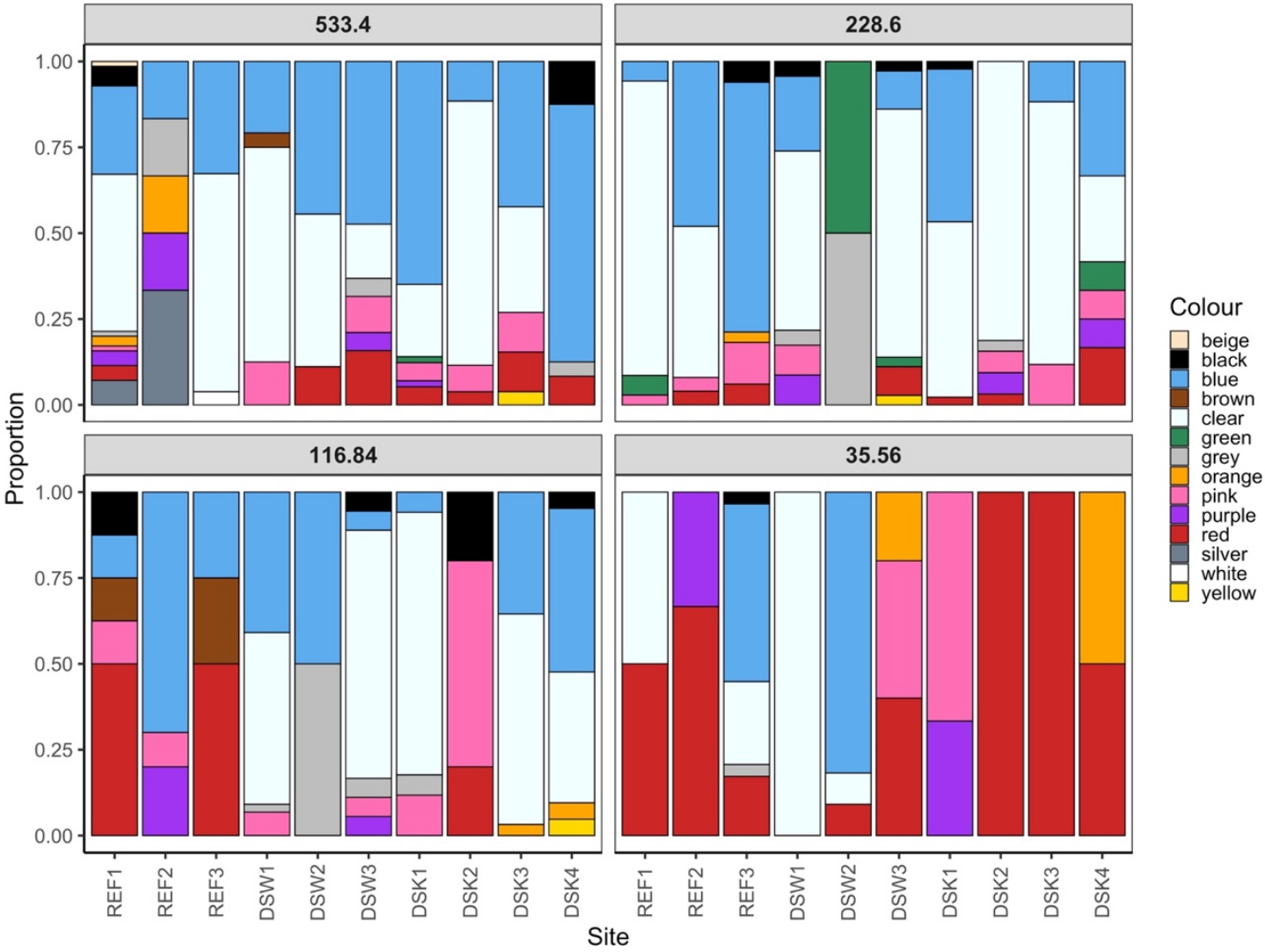


Figure 3.3 Proportions of different particle colours in water samples at sites along the Grand River, ON. Panels are organized according to filter size fraction (533.4, 228.6, 116.84, 36.56 µm), and sites are ordered on the x-axis from upstream (left) to downstream (right).

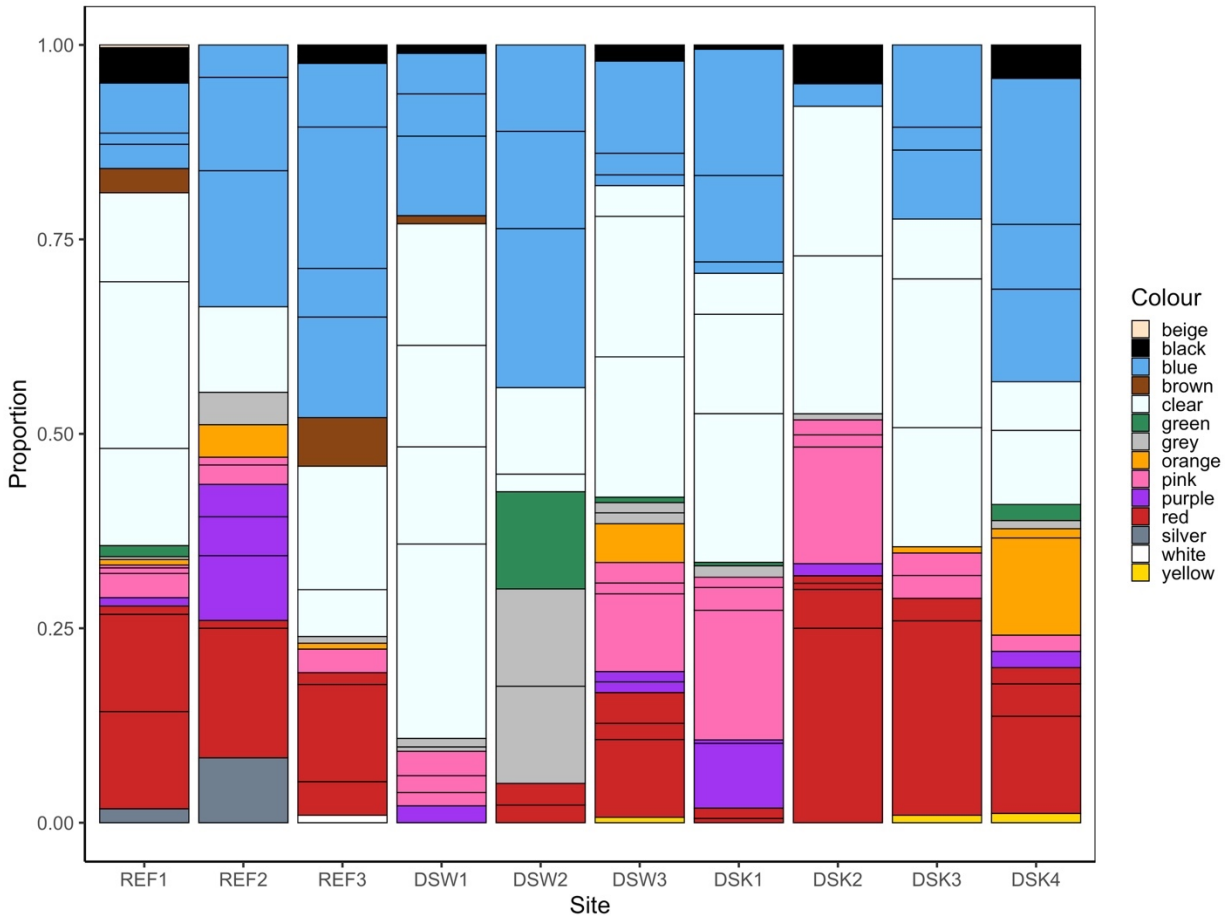


Figure 3.4 Proportions of different particle colours in water samples across all size fractions (533.4, 228.6, 116.84, 36.56 µm) at sites along the Grand River, ON. Black horizontal lines between blocks of the same colour within a site are indicative of contributions from different size fractions. Sites are ordered on the x-axis from upstream (left) to downstream (right).

Across all size fractions, water samples were dominated by fibers, with the smallest size fraction (35.56 µm) consisting entirely of this morphology (figure 3.5). Fragments were the second most frequent category and were found at most sites for the 533.4, 228.6, and 116.84 µm fractions, whereas fiber bundles were rare and were only found at REF1 in the 533.4 µm fraction. Similar to particle colour, the diversity in particle morphology declined in the smaller size fractions. When all size fractions were combined, fibers made up > 75% of particles at each site (figure A1).

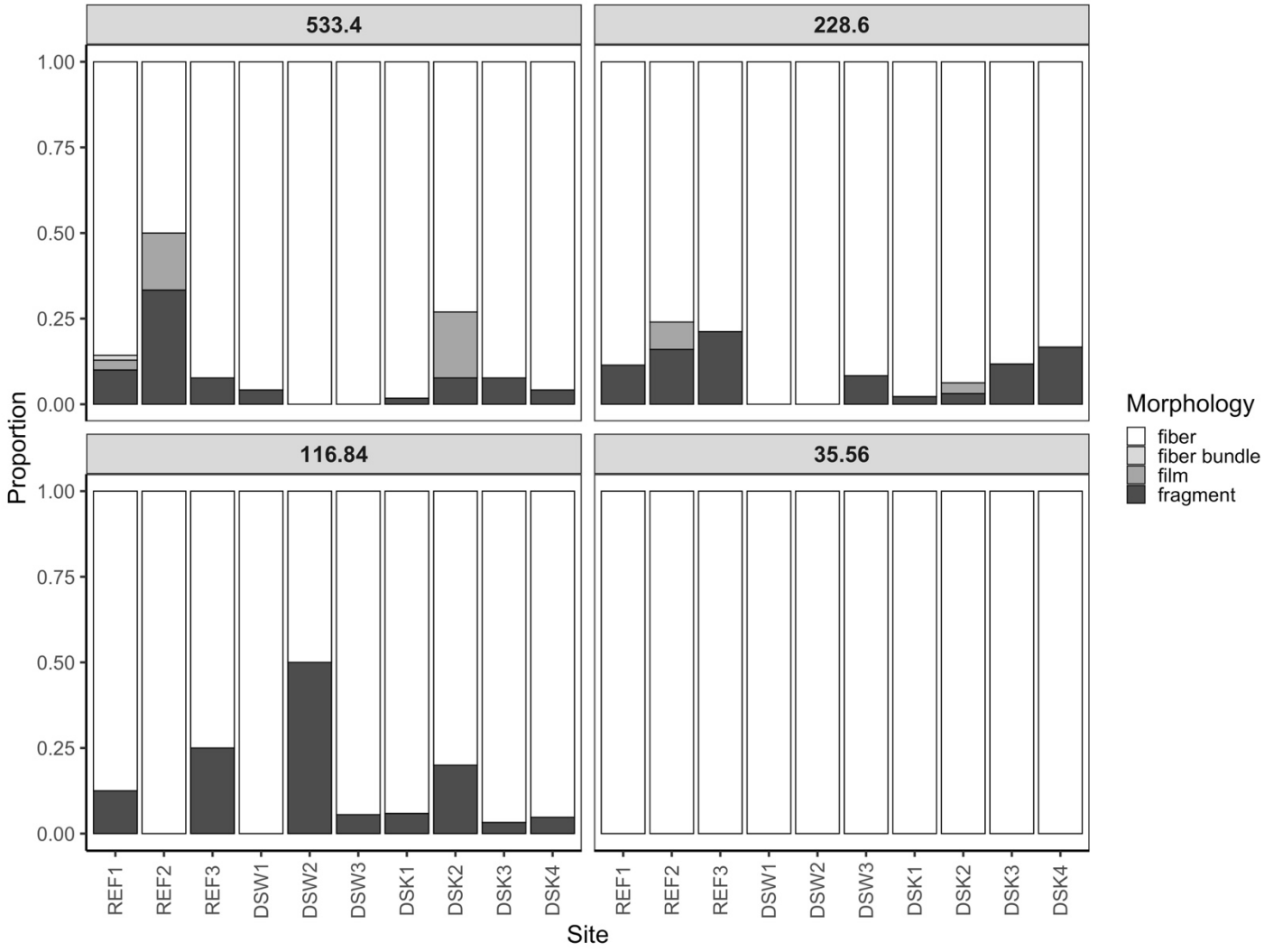


Figure 3.5 Proportions of different particle morphologies in water samples at sites along the Grand River, ON. Panels are organized according to filter size fraction (533.4, 228.6, 116.84, 36.56 µm), and sites are ordered on the x-axis from upstream (left) to downstream (right).

3.1.2 Abundance and characterization of microparticles in sediment samples

Sediments were separated into two size fractions (>500 µm and >38 µm); however, only the >500 µm fraction is included herein since very few particles were detected in the smaller fraction, with counts similar to background levels. There were significantly different microparticle concentrations in sediments (>500 µm) across sites (figure 3.6; ANOVA $F_{9,30}$ = 8.24, p = < 1.0 x 10^{-4}). The Waterloo outfall site (DSW1) had significantly higher counts than

REF2, REF3, and all other downstream sites; however, it was not significantly different from REF1 (table A.1). Particle concentrations at the Kitchener outfall site (DSK1) were relatively low and were not different from surrounding or reference sites, except for REF1 and DSW1 which had significantly higher counts (table A.1).

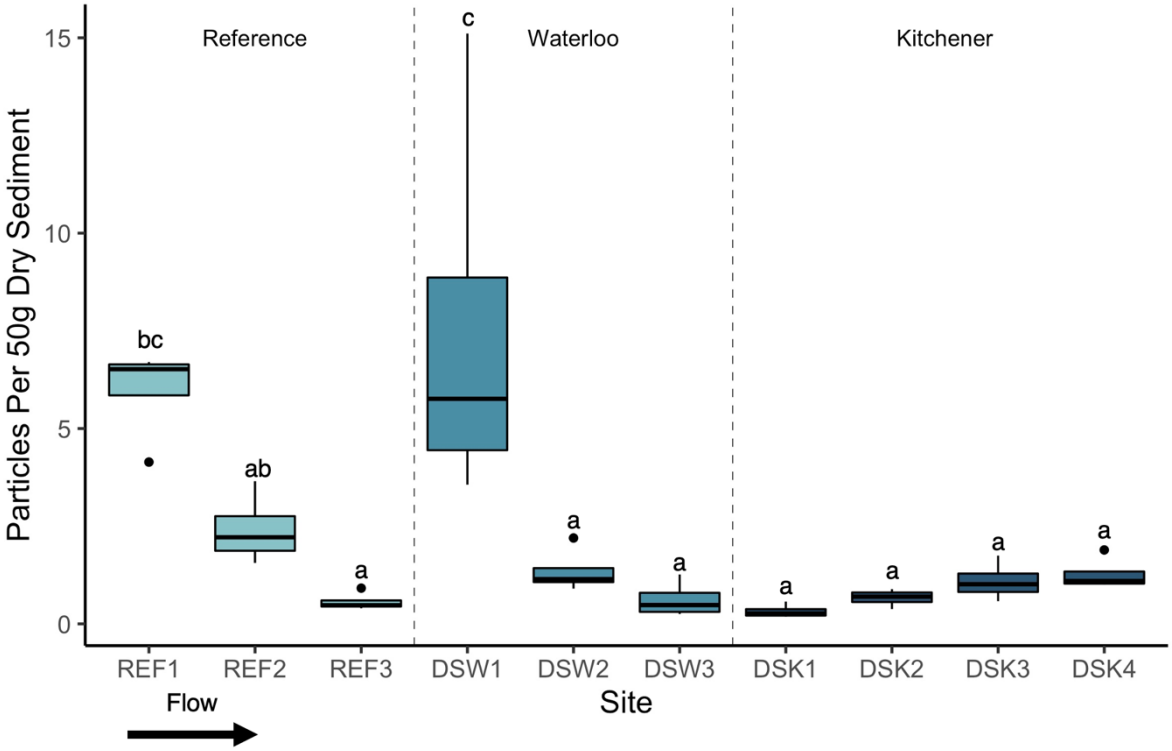


Figure 3.6 Boxplot showing counts of microparticles per 50 g of dry sediment from sites along the Grand River, ON (>500 µm sediment size fraction, n=4 per site). Sites are ordered on the x-axis from upstream (left) to downstream (right). Different letters indicate significant differences between sites.

Twelve different colours, along with some multicolour particles, were found in the sediments from the Grand River (figure 3.7). Black, clear, and red particles were found across all sites, with blue and yellow particles also being present at all but one site each. Clear particles were found in relatively equal proportions across all sites, and black particles declined in relative abundance from upstream to downstream. The proportion of red particles was highest at the two outfall sites, DSW1 and DSK1, comprising 18% and 36% of particles at these sites, respectively.

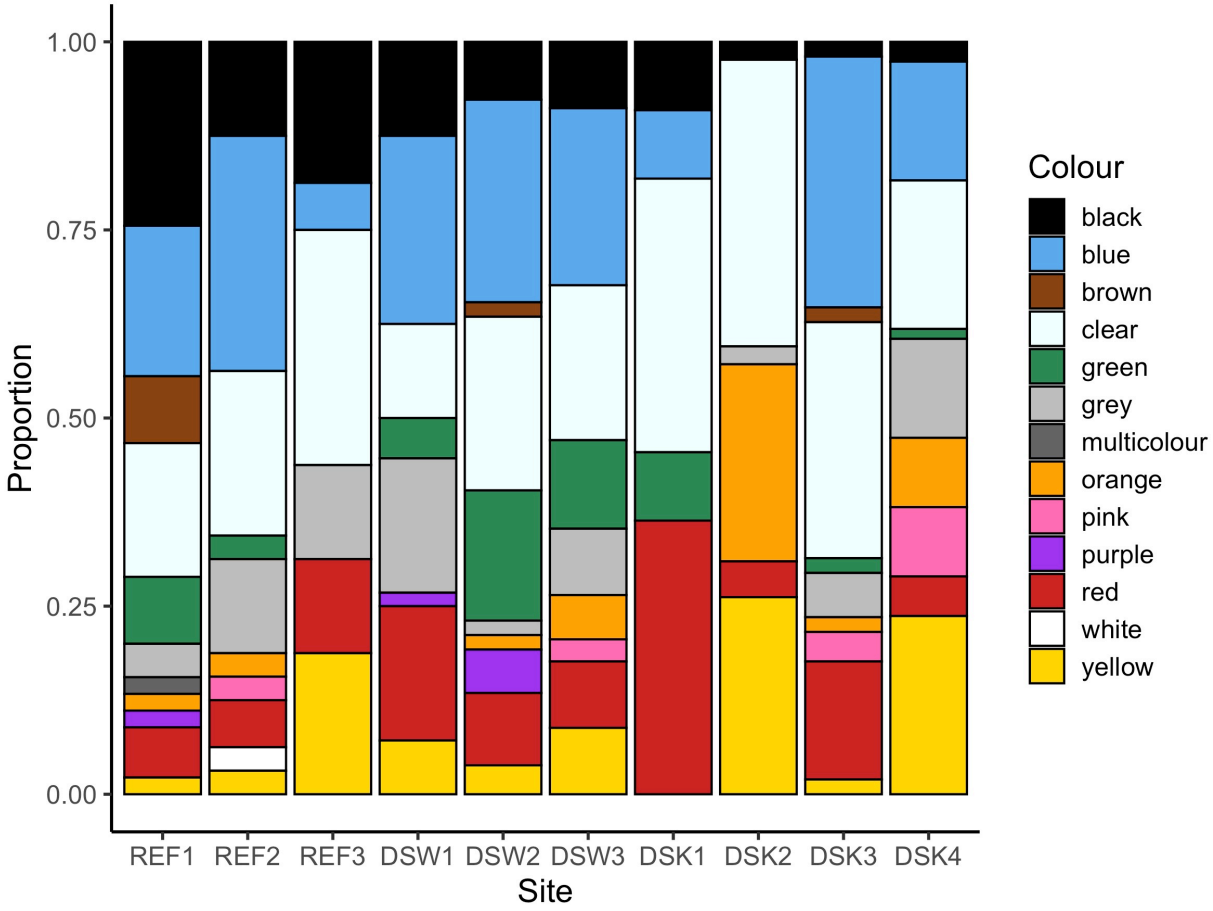


Figure 3.7 Proportions of different particle colours in sediment samples (>500 µm sediment size fraction) at sites along the Grand River, ON. Sites are ordered on the x-axis from upstream (left) to downstream (right).

Five different particle morphologies were identified in sediment samples (figure 3.8)**.** Fibers dominated most sites except for two sites downstream of the Kitchener outfall, DSK2 and DSK4, where films were most common. Films were found at all sites, except for the Kitchener outfall site (DSK1), which only had fibers and fragments. The Waterloo outfall (DSW1) also had low diversity in particle morphology and only had fibers and films. The first reference site (REF1) had the most diversity in particle morphology, with all five different categories represented (fiber, fiber bundle, film, fragment, sphere).

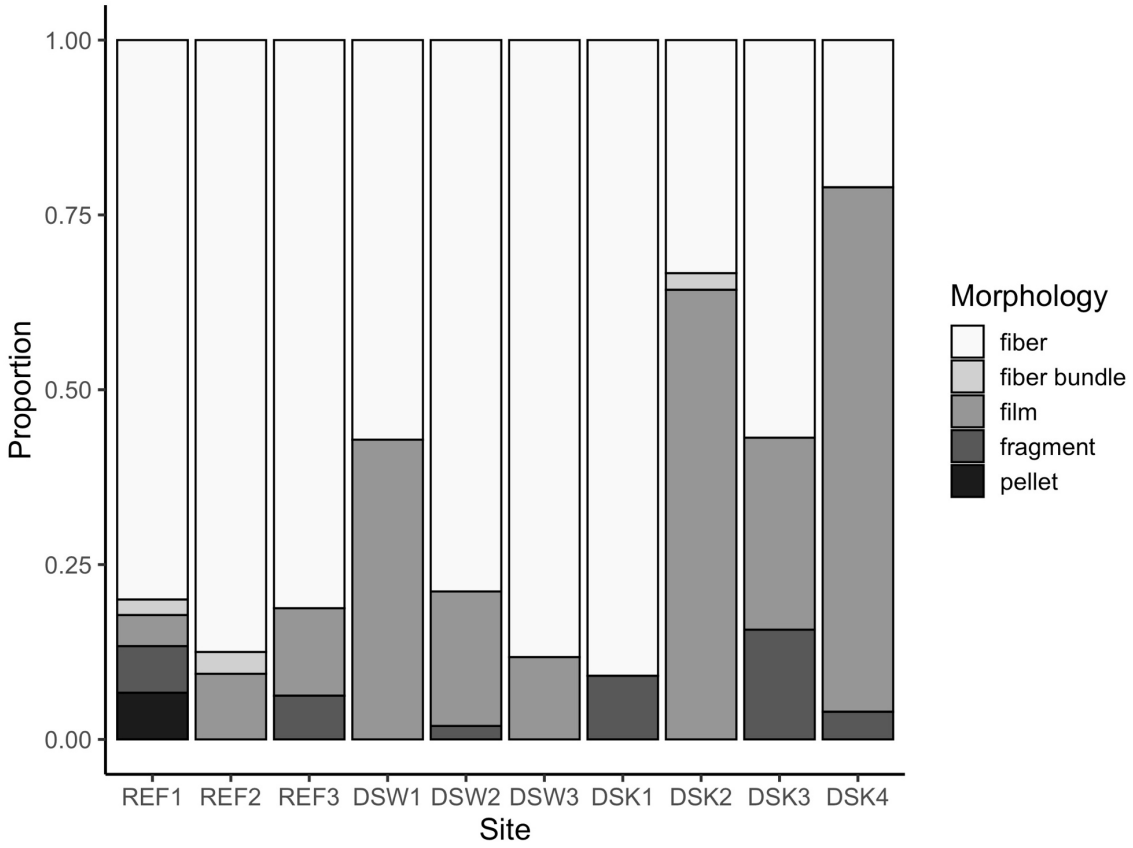


Figure 3.8 Proportions of different particle morphologies in sediment samples (>500 µm sediment size fraction) at sites along the Grand River, ON. Sites are ordered on the x-axis from upstream (left) to downstream (right).

3.1.3 Comparing levels in water and sediment

The average particle counts across replicates for water (n = 3; 533.4 µm water size fraction only) and sediment (n = 4; > 500 µm) were compared among sites. There was no correlation in particle counts between these two compartments ($r_8 = 0.16$, $p = 0.68$).

3.2 Microparticles in organisms caged in the Grand River

3.2.1 Microparticles in caged, whole amphipods

The number of particles per whole *Hyalella* was different across sites (figure 3.9; ANOVA, $F_{2,27} = 5.17$, $p = 0.01$). Particle counts were significantly elevated at the upstream

reference site (DSW3) compared to the Kitchener outfall site (Tukey HSD, p = 0.01). Particle counts further downstream were not significantly different from the reference or outfall sites.

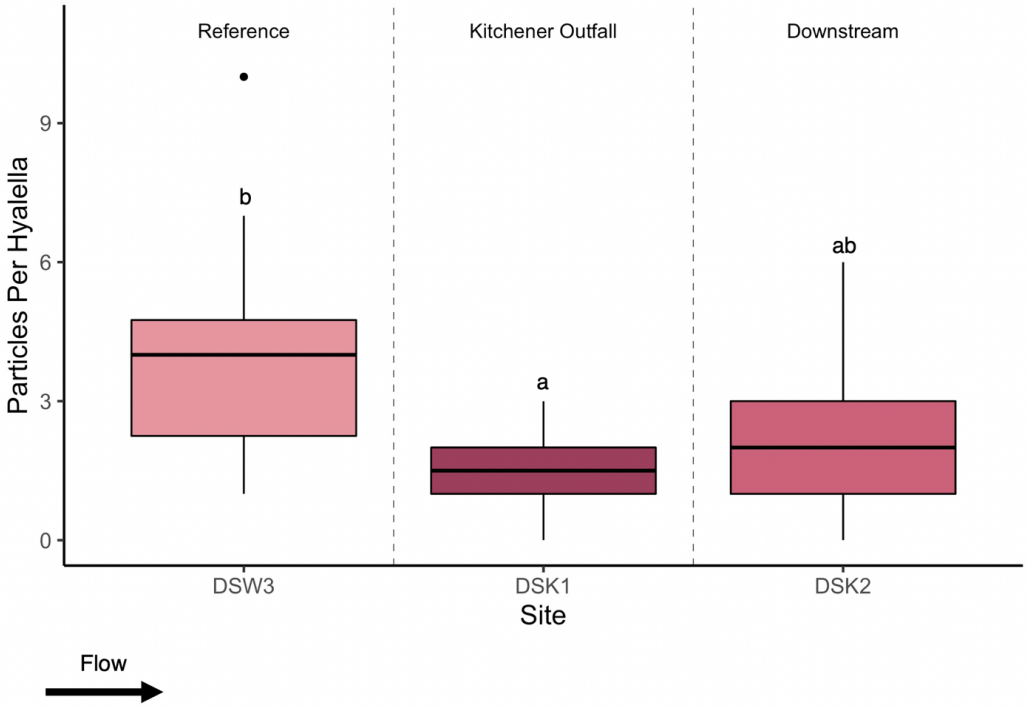


Figure 3.9 Counts of microparticles in whole body amphipods from sites up- and downstream of the Kitchener WWTP outfall, along Grand River, ON (n=10 per site). Sites are ordered on the x-axis from upstream (left) to downstream (right). Different letters indicate significant differences between sites.

Blue, clear, and black particles were found across all sites and together made up more than 75% of the colours found in *Hyalella* (figure 3.10). Clear particles were the most dominant colour across all sites, while green, white, orange, and pink particles were only present at one site each. Four different particle morphologies were observed in *Hyalella*: fibers, films, foams, and fragments (figure 3.11). Particles from the upstream reference (DSW3) and most downstream site (DSK2) primarily consisted of fibers, while the outfall site (DSK1) had fewer fibers but more films. Foams were only found at the reference site (DSW3) and fragments were present in

similar proportions at the reference and outfall sites, but they were less common further downstream.

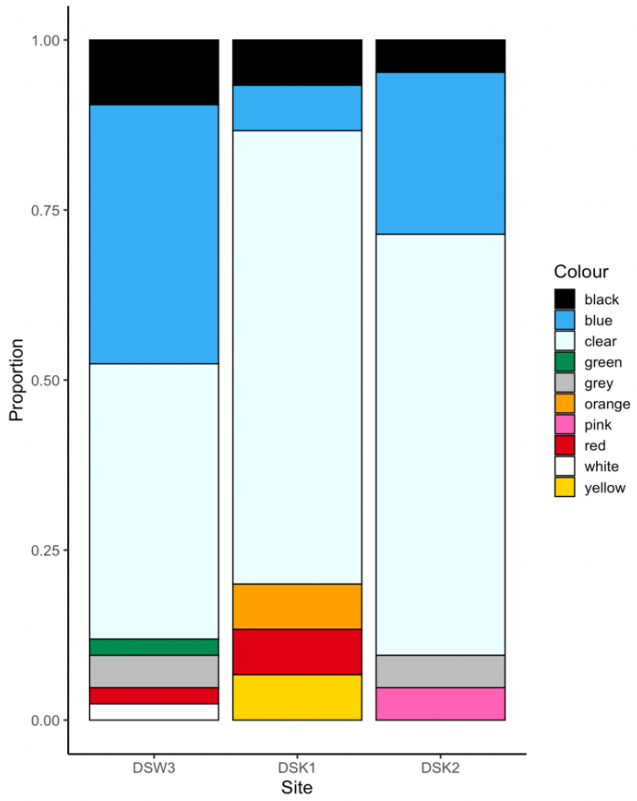


Figure 3.10 Proportions of different particle colours in whole body amphipods from sites along the Grand River, ON. Sites are ordered on the x-axis from upstream (left) to downstream (right).

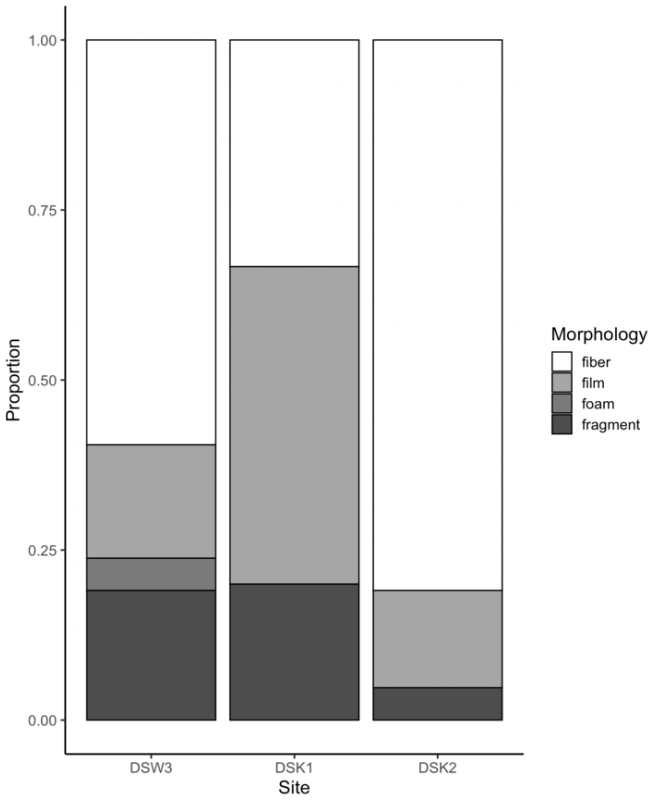


Figure 3.11 Proportions of different particle morphologies in whole body amphipods from sites along the Grand River, ON. Sites are ordered on the x-axis from upstream (left) to downstream (right).

3.2.2 Microparticles in hemolymph, digestive gland, and gill tissues of caged mussels

There were different spatial trends in microparticle concentrations across tissues of fluted-shell mussels. Significant among-site differences in particle concentrations were observed for hemolymph (figure 3.12; Kruskal-Wallis $\chi^2(2) = 11.15$, $p = 4.38 \times 10^{-3}$) and digestive glands (figure 3.14; $\chi^2(2) = 9.16$, $p = 0.01$), but not in gills (figure 3.13; $\chi^2(2) = 3.50$, $p = 0.17$). In mussel hemolymph, particle concentrations were significantly lower at the Kitchener outfall

(DSK1) compared to the reference (DSW3; $p = 0.04$) and downstream sites (DSK2; $p = 5.60 \times 10^{-3}$). The spatial pattern differed for digestive gland tissue, where particle concentrations were significantly elevated at the reference (DSW3; $p = 0.02$) and outfall sites (DSK1; $p = 0.05$) compared to downstream (DSK2). Although particle concentrations for mussel gills appear slightly elevated at the Kitchener outfall (DSK1; figure 3.13), they were not different from the two other sites.

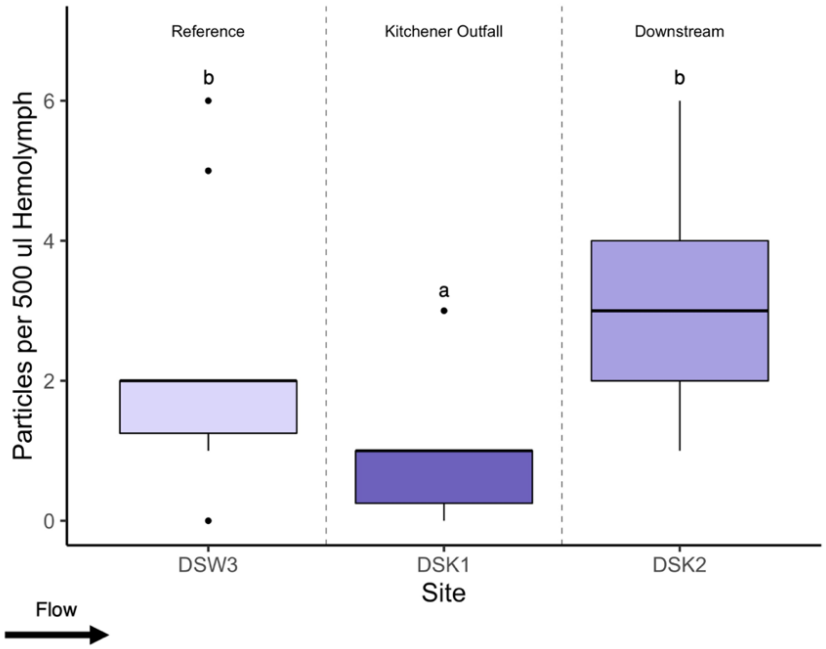


Figure 3.12 Microparticle concentrations in mussel hemolymph (particles per 500 µl) from sites up- and downstream of the Kitchener WWTP outfall, along the Grand River, ON (n=10 per site). Sites are ordered on the x-axis from upstream (left) to downstream (right). Different letters indicate significant differences between sites.

Of the mussel tissues evaluated, digestive glands had the most diversity in particle colour, with 9 colours represented, compared to hemolymph and gills which had 6 colours each (figure 3.14). Across all tissues, clear particles were found at each site. Blue particles were also common but were absent in hemolymph at DSK2 and in the gills of animals caged upstream (DSW3). Beige, brown, and purple particles were unique to digestive gland samples, whereas all tissue

types had some pink and red particles in lower proportions (except for DSW3 where these two colours were more dominant).

There were four different particle morphologies observed in mussel tissues: fibers, fiber bundles, films, and fragments (figure A.2). All tissues and sites were dominated by fibers (>75% at each site). Fiber bundles were only found in low proportions in digestive glands of mussels at two sites. Fragments and films were found in low proportions across most sites for all tissues.

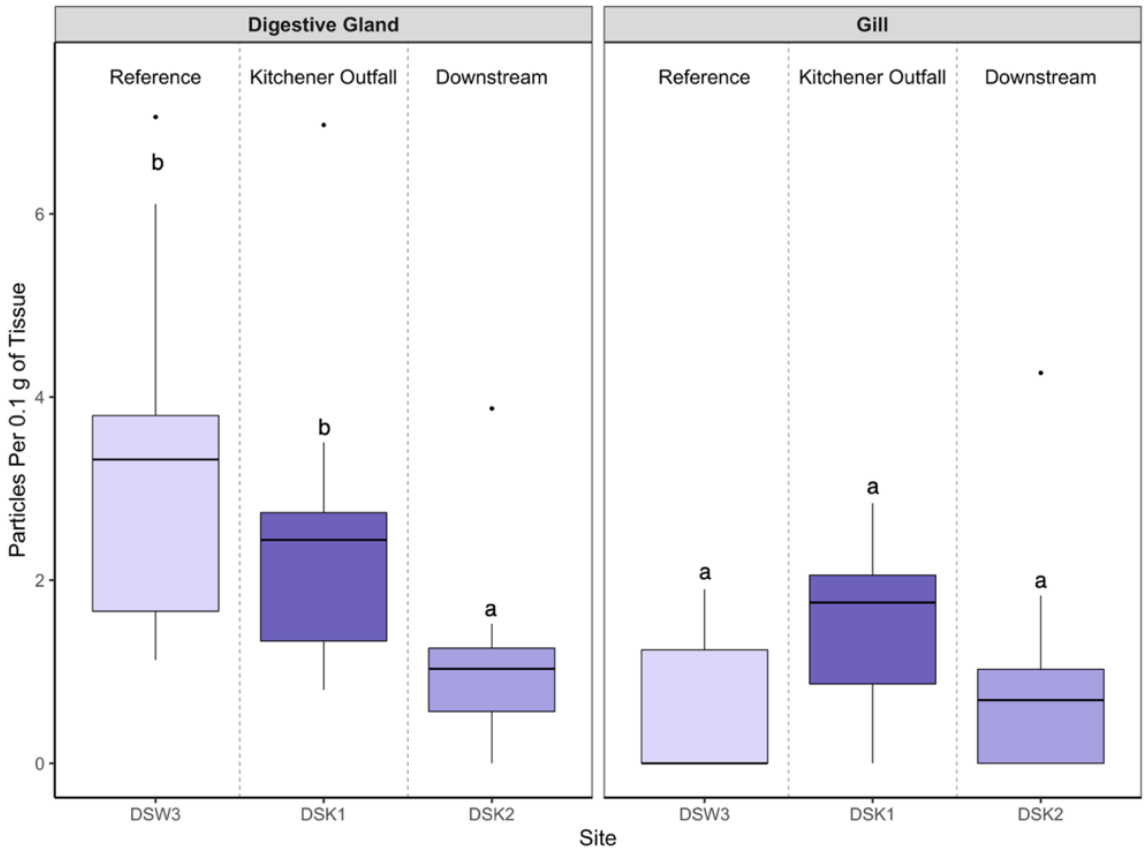


Figure 3.13 Microparticle concentrations (particles per 0.1 g of tissue) in fluted-shell mussel digestive glands and gills from sites up- and downstream of the Kitchener WWTP outfall, along the Grand River, ON (n=10 per site). Sites are ordered on the x-axis from upstream (left) to downstream (right) within each panel. Different letters indicate significant differences between sites for digestive glands; there were no significant differences between sites for mussel gills.

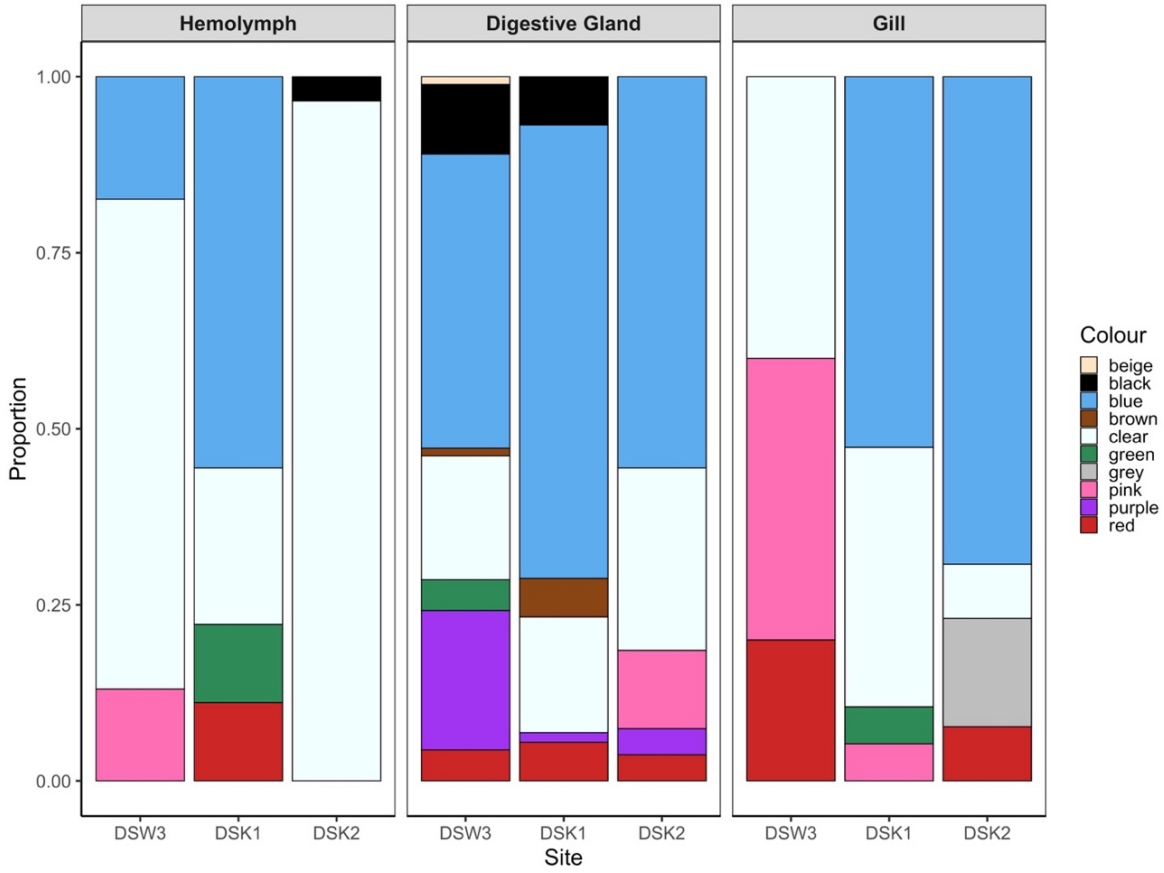


Figure 3.14 Proportions of different particle colours in fluted-shell mussels across different tissue types (hemolymph, digestive gland, and gill) at sites along the Grand River, ON. Sites are ordered on the x-axis from upstream (left) to downstream (right) within each panel.

3.2.3 Microparticles in digestive tracts of caged rainbow trout

Microplastics in the digestive tracts of juvenile rainbow trout were significantly higher at the Kitchener outfall (DSK1) than the upstream reference site (figure 3.15; Tukey HSD, $p = 8.0 \times 10^{-4}$), as well as further downstream of the outfall (Tukey HSD, $p = 5.40 \times 10^{-3}$). However, linear regressions showed no significant relationship between fish mass or tissue mass and the number of particles found in digestive tracts (figure A.3, $R^2 = 0.02$, $p = 0.44$; figure A.4, $R^2 = 0.01$, $p = 0.81$).

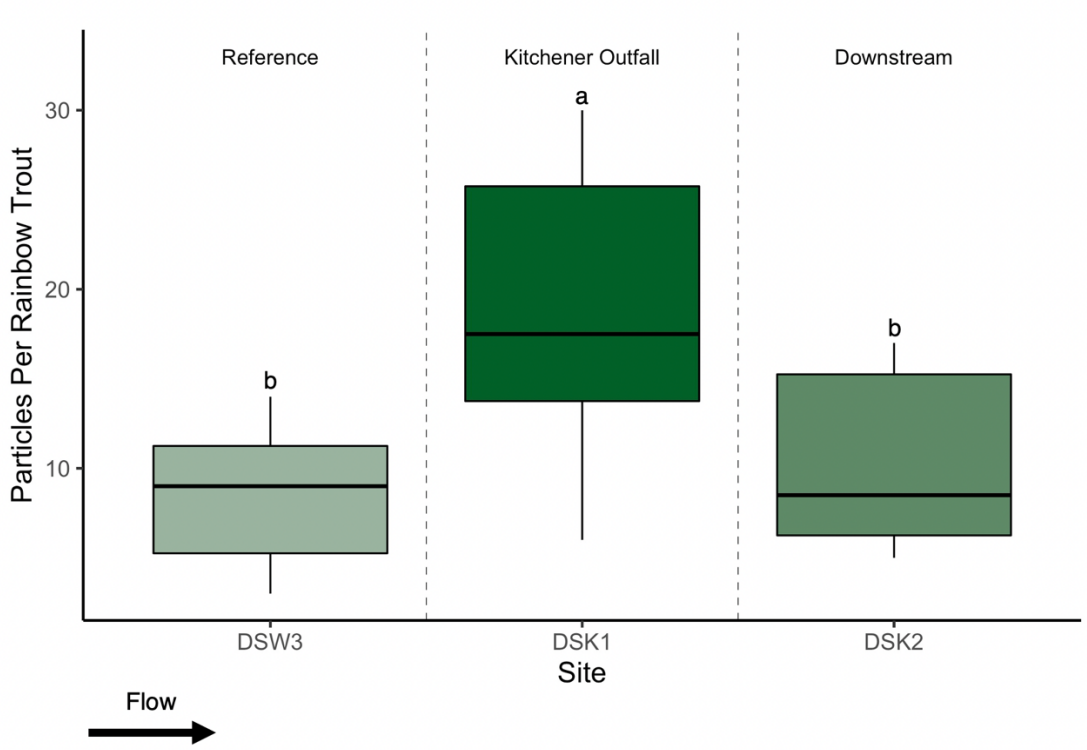


Figure 3.15 Counts of microparticles in rainbow trout digestive tracts from sites up- and downstream of the Kitchener WWTP outfall, along Grand River, ON (n=10 per site). Sites are ordered on the x-axis from upstream (left) to downstream (right). Different letters indicate significant differences between sites.

In the digestive tracts of trout, blue particles were the most dominant colour (figure 3.16), and they were found in relatively equal proportions across all sites (~45%). Compared to the two impacted sites, the upstream reference site (DSW3) had more black and green particles and lacked clear particles. Additionally, the downstream sites had more colours represented, with 12 at the DSK1 and 13 at DSK2 compared to 8 colours at the upstream reference DSW3. Fibers were the most common particle morphology in rainbow trout digestive tracts and made up more than 80% of particles at DSW3 and DSK1, and 70% of particles at DSK2 (figure 3.17). Films and fragments were less dominant morphologies, although they were also present across all sites. A single sphere was found at DSK1; this was the only sphere to be found across all sites in both biotic and abiotic samples.

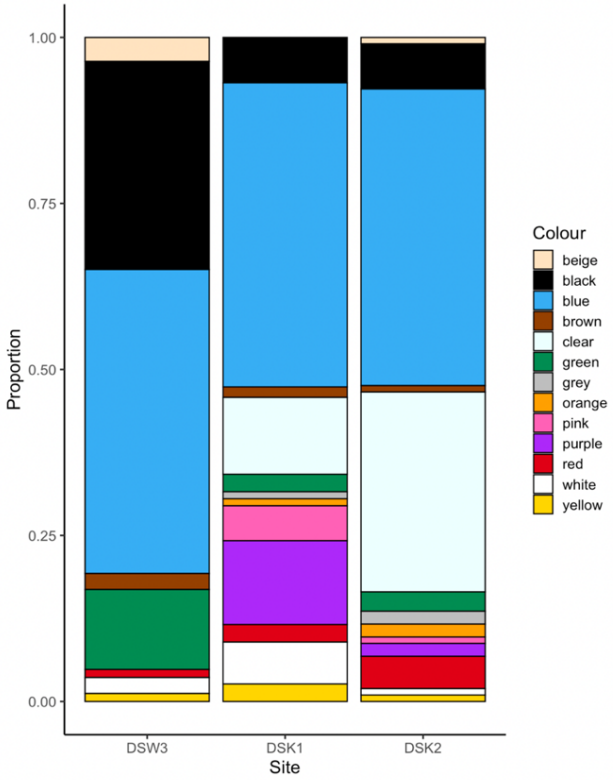


Figure 3.16 Proportions of different particle colours in rainbow trout digestive tract samples at sites along the Grand River, ON. Sites are ordered on the x-axis from upstream (left) to downstream (right).

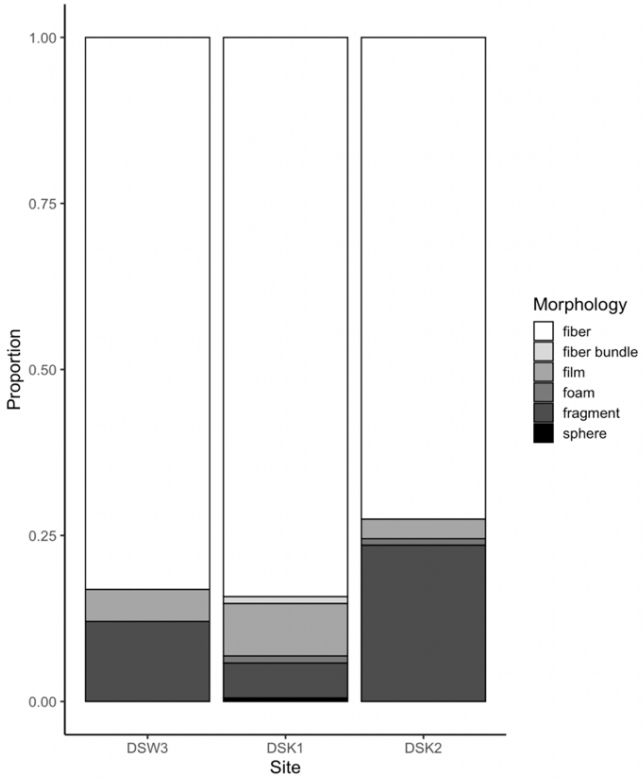


Figure 3.17 Proportions of different particle morphologies in rainbow trout digestive tract samples at sites along the Grand River, ON. Sites are ordered on the x-axis from upstream (left) to downstream (right).

3.2.4 Comparing particle levels in organisms caged in the Grand River to environmental levels

Pearson's correlation tests were conducted to assess whether particle concentrations in the tissues of caged organisms were correlated to environmental levels in water and/or sediment. There were no significant correlations between particle concentrations in biotic and environmental samples (table 3.1).

Table 3.1 Output from Pearson's correlation tests between particle concentrations at each site in the tissues of caged organisms, and levels in water and sediment. Average particle counts across replicates for water (n=3, sum of all size fractions) and sediment (n=4) at each site were included. Correlations used average total particle counts for whole body *H. azteca* (n=10) and digestive tracts of *O. mykiss* (n=10) at each site, while average counts per 500 µl hemolymph and average particle counts per 0.1 g digestive tract and gill tissue at each site were used for *L. costata* (n=10).

Species and Tissue Type	Correlation with Water		Correlation with Sediment	
	p-value	*r*	p-value	*r*
Hyalella azteca Whole Body	0.67	-0.50	0.61	0.58
Lasmigona costata Hemolymph	0.12	-0.98	0.18	0.96
Lasmigona costata Digestive Gland	0.44	0.77	0.38	-0.83
Lasmigona costata Gill	0.76	0.37	0.82	-0.28
Oncorhynchus mykiss Digestive Tract	0.25	0.93	0.18	-0.96

3.3 Microparticles in wild-caught rainbow darter digestive tracts from the Grand River

There were few spatial differences in particle concentrations in the digestive tracts of rainbow darter (figure 3.18). Although there were site effects (ANOVA, $F_{9,90} = 2.91$, $p = 4.18 \times 10^{-3}$), elevated particle counts were not seen at either the Waterloo or Kitchener WWTP outfalls. The second site downstream of the Kitchener outfall (DSK2) had significantly higher microparticle counts than the outfall site (DSK1; Tukey's, $p = 7.30 \times 10^{-3}$) and the furthest downstream site (DSK4; p=0.02), but DSK2 was not significantly different from any of the other sites sampled.

There were 9 different particle colours detected in the rainbow darter samples, with clear and blue particles being the most common (figure 3.19). Blue and clear particles were found at all sites, except for the Kitchener outfall (DSK1) where clear particles were absent. Purple, pink, and black particles were found at several sites; however, orange, red and green particles were only found in digestive tracts at the two sites nearest the Kitchener outfall (DSK1 and DSK2).

Fibers were the dominant morphology in rainbow darter digestive tracts, comprising > 75% of all particles found at each site. Three sites (DSW2, DSK3 and DSK4) only had fibers, while the remainder of the sites had low quantities of films, fragments, and foams (figure A.5).

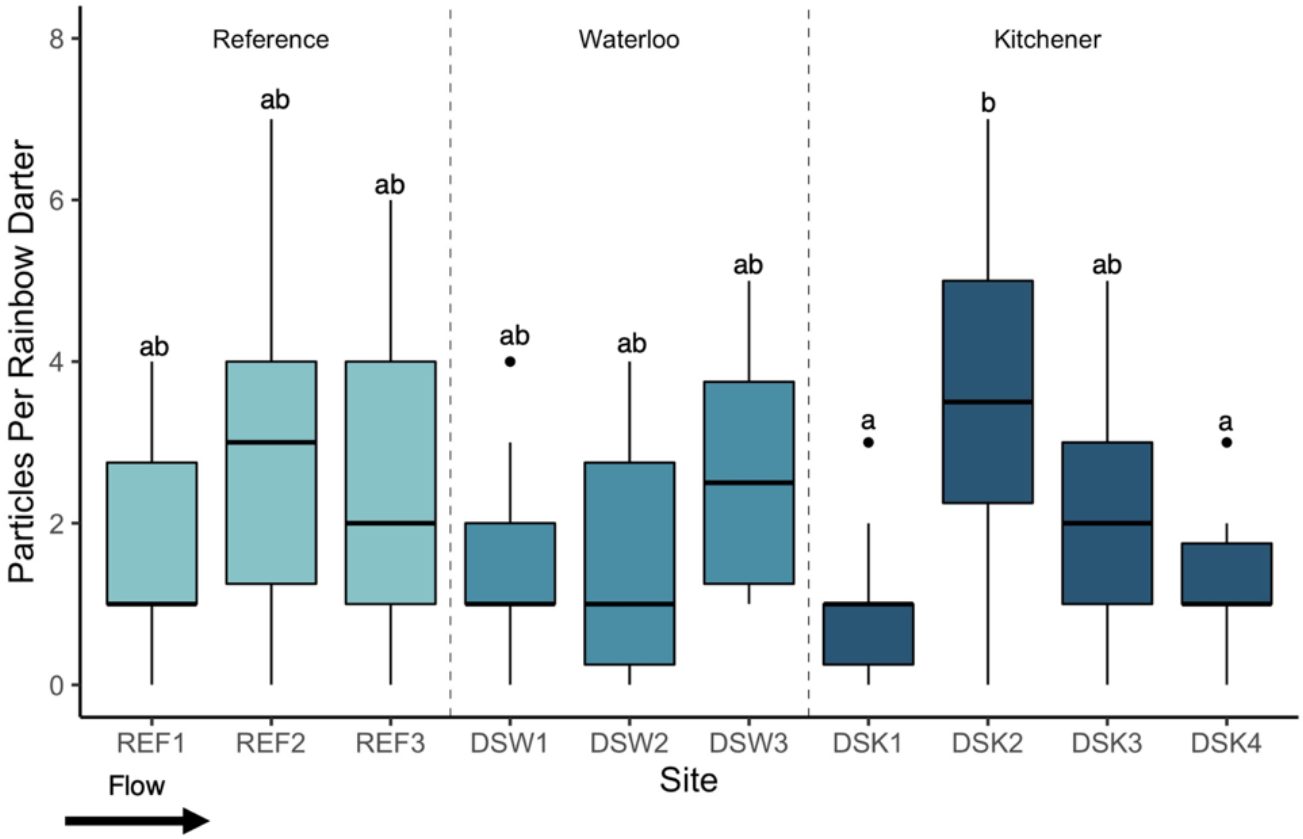


Figure 3.18 Counts of microparticles per rainbow darter digestive tract from sites along the Grand River, ON (n=10 per site). Sites are ordered on the x-axis from upstream (left) to downstream (right). Different letters indicate significant differences between sites.

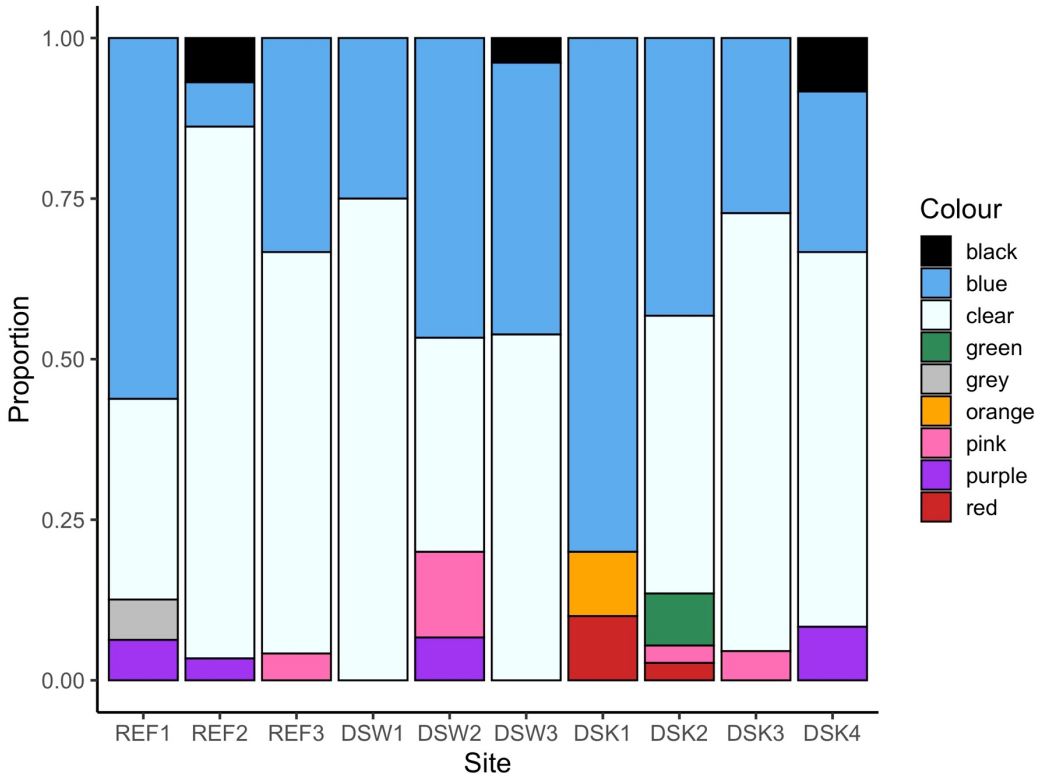


Figure 3.19 Proportions of different particle colours in rainbow darter digestive tract samples at sites along the Grand River, ON. Sites are ordered on the x-axis from upstream (left) to downstream (right).

3.3.1 Comparing particle levels in rainbow darter to fish weight, tissue mass, and environmental levels

There were no significant relationships between the number of particles per fish and their body weight (figure A.6; regression $R^2 = <0.01$, $p = 0.95$) or mass of the digestive tract (figure A.7; $R^2 = <0.01$, $p = 0.40$). Similarly, there were no correlations between average numbers of particles in rainbow darter compared to average particle counts in water (sum of all size fractions per replicate used; $r_8 = -0.28$, $p = 0.44$) or sediment ($r_8 = -0.25$, $p = 0.48$) across sites.

3.3.2 Comparing particle levels in wild-caught rainbow darter and caged rainbow trout

At sites near the Kitchener outfall (DSW3, DSK1 and DSK2), where both trout and darter were sampled, particle counts in the deployed trout were elevated compared to those in wild

darters at all sites (figure 3.20). Rainbow trout also had more particle colours represented in their digestive tracts than rainbow darter; however, both species of fish had high proportions of blue fibers at every site (figure 3.21). Similarly, more particle morphologies were found in rainbow trout compared to rainbow darter, with rainbow trout having noticeably more fragments (figure 3.22). Fibers were the most common morphology, making up more than 75% of particles found at every site for both species (except DSK2 for rainbow trout).

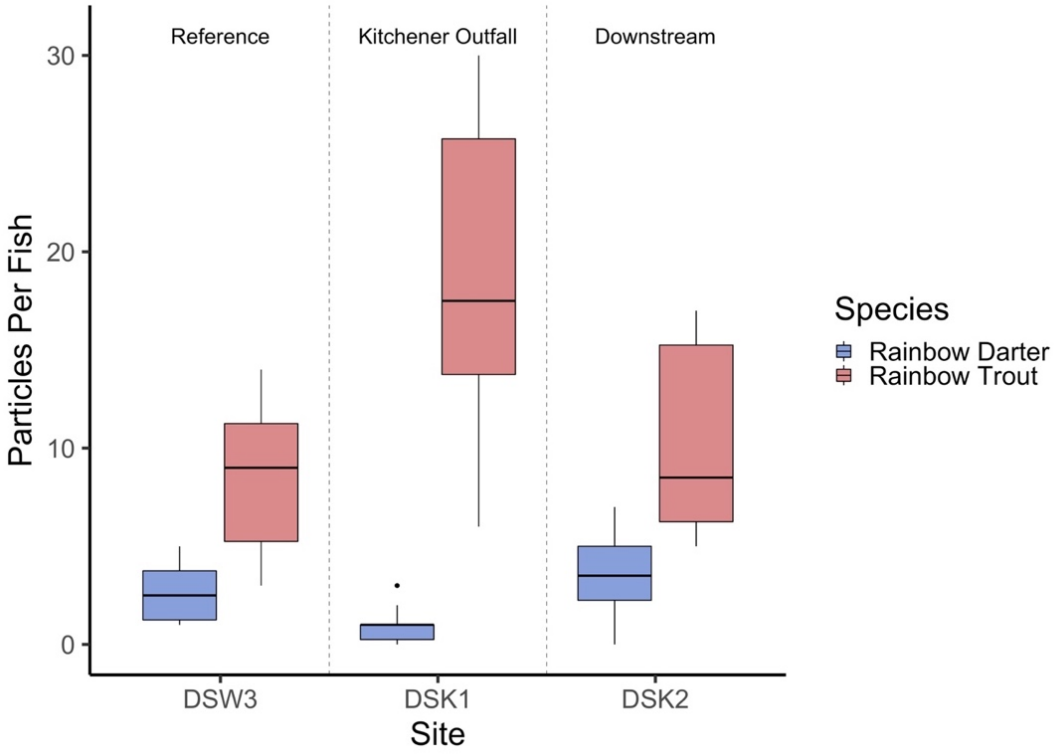


Figure 3.20 Counts of microparticles in wild-caught rainbow darter and field-deployed (i.e. caged for 2 weeks) rainbow trout digestive tracts sampled up- and downstream of the Kitchener WWTP outfall (n=10 per site for each species) Sites are ordered on the x-axis from upstream (left) to downstream (right).

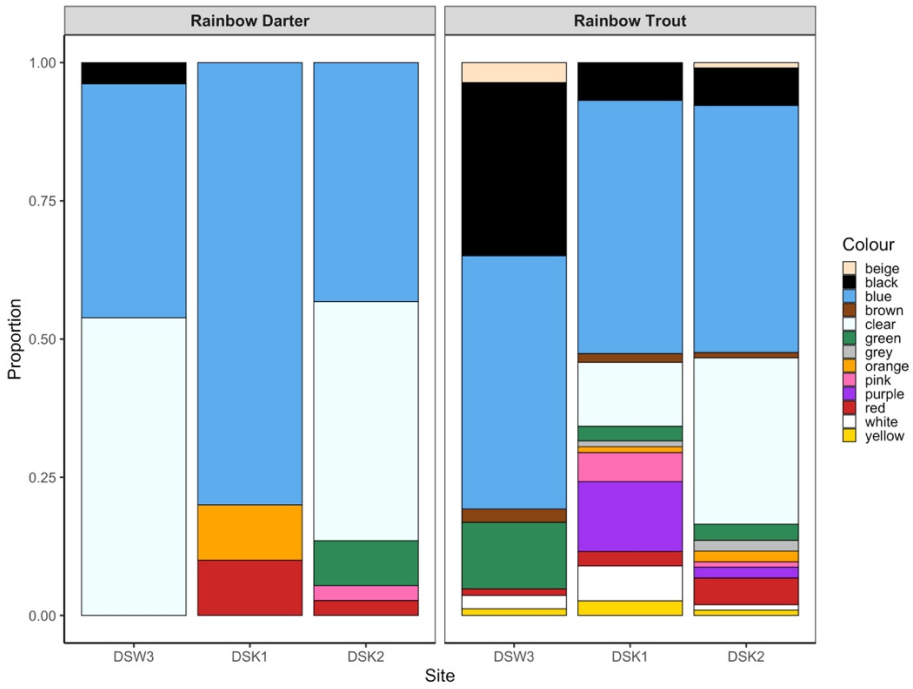


Figure 3.21 Proportions of different particle colours in wild-caught rainbow darter and caged rainbow trout from sites along the Grand River, ON. Sites are ordered on the x-axis from upstream (left) to downstream (right) within each panel.

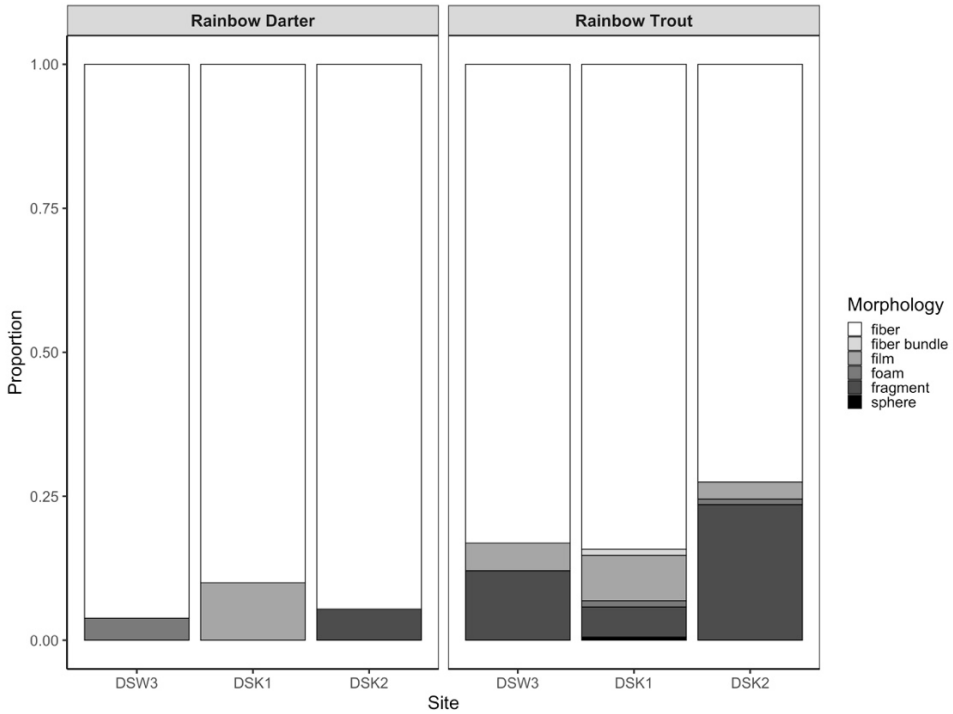


Figure 3.22 Proportions of different particle morphologies in wild-caught rainbow darter and caged rainbow trout from sites along the Grand River, ON. Sites are ordered on the x-axis from upstream (left) to downstream (right) within each panel.

4 Discussion

4.1 Particle abundances

4.1.1 Particle abundance in environmental samples

Particle concentrations in water were between 0.55-2.03 particles L^{-1} (median 1.28 particles L^{-1}, mean ± SD 1.34 ± 0.52 particles L^{-1}) across all sites sampled along the Grand River. This range is consistent with the average (± SD) concentration of anthropogenic microparticles (microplastics, cellulose-based and anthropogenically-dyed particles) measured in Lake Ontario at four sites near Toronto, ON (0.80 ± 0.70 particles L^{-1}; Grbić et al., 2020). Microparticle concentrations in 29 other Great Lakes' tributaries were much lower ($5.0x10^{-5}$ - 3.20×10^{-2} particles L^{-1}; median, 1.90×10^{-3} particles L^{-1}, mean 4.20×10^{-3} particles L^{-1}; Baldwin et al. 2016) than those in the Grand River. More urbanized systems, such as the North Shore Channel in Chicago, Illinois, had higher mean (± SE) microparticle concentrations upstream (1.94 ± 0.81 particles L^{-1}) and downstream (17.93 ± 11.05) of a large activated sludge WWTP than those observed in the Grand River (McCormick et al., 2014). Differences in sampling, processing, and reporting do not allow for other direct comparisons between microparticle abundances in the Grand River and other values from the Great Lakes (Eriksen et al., 2013; Mason et al., 2020; Mason, et al., 2016). Keeping these potential discrepancies in mind, microparticle concentrations in water samples from the Grand River were fairly consistent with samples from nearby areas, but lower than those found in larger urban centres.

Differences in sample collection, processing, and identification may have resulted in some of the discrepancies in particle counts between this study and the above-mentioned ones. Water samples from the Grand River were collected on stainless steel filters (533.4 µm, 228.6 µm, 116.84 µm, 35.56 µm) using a pump system, and did not require chemical digestion for the

removal of organic matter, whereas microparticles in the North Shore Channel were captured in neustron net, were separated using stacked sieves (2 mm and 330 μm), and underwent wet peroxide oxidation (McCormick et al., 2014). Further, numerous studies do not differentiate between microplastics and non-plastic anthropogenic microparticles, and some do not adjust for plastic to non-plastic ratios after chemical identification methods. As such, some results may be overestimates of true microplastic counts and contribute to discrepancies between studies. These differences highlight the need to standardize methods and reporting to facilitate greater reproducibility and comparability between studies (Cowger et al., 2020; Provencher et al., 2020).

Sediments from sites along the Grand River had a larger range in microparticle concentrations than water and were between 6.47-150.98 particles kg^{-1} dry weight (median 23.64 particles kg^{-1}, mean ± SD 43.64 ± 50.21 particles kg^{-1}), with the highest concentration (150.9 particles kg^{-1}) occurring at the Waterloo WWTP outfall. Comparing concentrations of microparticles between different freshwater sediments can be challenging, as studies use different combinations of sampling (grabs, cores, passive traps), processing (sieving, density separation, enumeration), and reporting (particles kg^{-1}, particles m^{-2}). The two studies discussed below mostly collected grab samples, used sodium polytungstate solution with a specific gravity of 1.5 g/mL (similar in density to the 1.4 g/mL $CaCl_2$ used in the current study), and reported particles concentrations per kg of dry sediment. Due to limitations of the chemical identification of particles, including limited numbers of reads and low proportions of confirmed plastic particles, the particles identified in these studies are described as *microparticles* rather than microplastics. Results from my study are in line with microparticle concentrations measured in other tributaries of Lake Erie (10.0 – 462.0 particles kg^{-1}; median 42.0 particles kg^{-1} ; mean ± SD 116.0 ± 194.54; Dean et al., 2018). In particular, concentrations in the central Grand River were

similar to those measured at the mouth of the river (42.0 particles kg^{-1}), where it drains into Lake Erie (Dean et al., 2018). In contrast, concentrations from the Grand River were much lower than those found in urban tributaries around Toronto, ON, where between 40.0-1740.0 particles kg^{-1} (mean 610.0 particles kg^{-1}) were reported (Ballent et al., 2016). While microparticle concentrations in sediments from the Grand River might not be as elevated as more impacted systems, sediments are still a sink for microparticles and might contribute to exposures for benthic species. It is therefore important to continue to monitor freshwater sediments in the Grand River to identify whether microparticle concentrations and assemblages are changing over time, and to better assess exposure risks to sediment-dwelling biota.

4.1.2 Particle abundances in biotic samples

Between 0-10 microparticles were found in caged whole body *Hyalella* (median 2, mean ± SD 2.60 ± 2.24 per individual). Other information about microplastics ingestion by *Hyalella* is currently limited to laboratory exposures. Currently, there is no evidence to indicate that microplastics are taken up into tissues; however, whole body counts in juvenile *Hyalella* are greater with increasing microplastics concentrations during acute exposures (Au et al., 2015). Gut passage for microplastic spheres in juvenile *Hyalella* is comparable to egestion time for regular food items (~2 hours), but fibers take much longer to pass (~4-10 hours; Au et al., 2015). This raises the possibility that wild amphipods might reflect elevated particle concentrations in their environment, particularly when high numbers of fibers are present. Microplastics have been found in other freshwater invertebrates upstream and downstream of WWTP effluent outfalls in South Wales, UK (Windsor et al., 2019). Microparticle concentrations in *Hyalella* caged in the Grand River (0.66 ± 0.60 particles mg^{-1}; whole body *Hyalella* 4.21 ± 1.39 mg) were much higher than those observed in Baetidae, Heptageniidae and Hydropsychidae (average 0.01 – 0.04

particles mg^{-1}, and up to 0.14 particles mg^{-1}; Windsor et al., 2019). It could be that differences in feeding strategies and habitat preferences could explain some of these differences in particle concentrations. Deposit feeders or grazers, for example, might inadvertently ingest microparticles, whereas, filter feeders could be more selective in what they choose to ingest; nevertheless, evidence of this occurring under natural conditions is limited and conflicting (Windsor et al., 2019; Xu et al., 2020). Generally, more work is needed to better quantify microplastics uptake by freshwater invertebrates, including amphipods, at environmentally realistic levels.

Mussels from the Grand River had relatively high counts of microparticles per individual when compared to the other taxa examined herein, but their presence varied among tissue type. On average (± SD), 6.37 (± 4.28) particles g^{-1} of tissue were found in the digestive gland, whereas gill tissue and hemolymph had fewer particles (1.25 ± 1.29 g^{-1} of tissue; 4.21 ± 3.48 per mL of hemolymph, respectively). The highest counts in digestive glands aligns with previous work assessing tissue distribution of microplastics in marine mussels (Avio et al., 2015; Kolandhasamy et al., 2018).

While larger particles have been found within mussel digestive glands, and they adhere to other tissues (Kolandhasamy et al., 2018), there is also evidence that microplastics are taken up into mussel tissues. In marine mussels, microplastics (0-80 μm) were found in digestive cells (Von Moos et al., 2012), and even smaller particles < 10 μm have been found in hemolymph and could translocate between tissues (Browne et al., 2008). Gut residence times for microplastics in mussels vary considerably according to species, gut emptiness, as well the size and morphology of the particles. In *Mytilus galloprovincialis* the gut residence times of irregularly-shaped microplastics can exceed times for regular food items, and some particles were retained in

digestive tracts after 6 days of depuration (Fernández & Albentosa, 2019). Since mussels are selective feeders they can also eliminate unwanted items prior to ingestion, thus it is possible that the types of particles found in the digestive tract do not accurately reflect a mussel's true exposure to microplastics (Ward et al., 2019). Therefore, monitoring microparticles in whole body mussels, or including additional tissues where alternative modes of uptake are known to occur (adherence in gills, and translocation of small particles <10 μm to hemolymph) provides a more complete understanding of microplastics exposure in mussels (Browne et al., 2008; Kolandhasamy et al., 2018).

Microparticle counts in caged mussel tissues herein were elevated compared to whole body fluted-shell mussels from other sites in the central Grand River, where the highest number of microplastics found in an individual was 7 particles (Wardlaw & Prosser, 2020). However, in the current study, mussels caged at DSK1 were very close (<0.5 km downstream) to the outfall of a large WWTP, and those in Wardlaw & Prosser (2020) were either collected further downstream (> 7 km) of the same WWTP, or from downstream of smaller WWTPs. Since the microparticles from my study have yet to be chemically confirmed as plastic, it is possible that some of these particles, particularly the fibers, could be other anthropogenically-modified materials such as cellulose. In another unionid mussel, *Margaritifera margaritifera,* collected from rural tributaries of the Saint John River, NB, 14 ± 21 microfibers were found on average in whole body mussels; however, up to 163 microfibers were observed in one individual (Doucet et al., 2021). While the number of microparticles per gram of tissue were still higher in mussels caged in the Grand River, it is difficult to discern whether this is because environmental levels were elevated in the Grand River compared to the Saint John, as no environmental samples were collected there, or because microparticles are more likely to concentrate in the digestive gland compared to other

tissues (Kolandhasamy et al., 2018). Additionally, differences in reporting (microplastics in Wardlaw & Presser, 2020; microfibers in Doucet et al., 2021) make it challenging to compare particle counts between studies.

There was high variability in the number of particles found in caged rainbow trout (3-30 particles per digestive tract; median 12, mean ± SD 12.5 ± 7.29). While there is limited evidence of rainbow trout consuming microplastics under natural conditions, several fish species in Lake Ontario and Lake Superior, such as lake trout, ingest anthropogenic microparticles and microplastics, with particle counting methods similar to those employed here for Grand River samples (Munno et al., 2021). Counts in the digestive tracts of field-deployed rainbow trout from the Grand River were lower than those in wild lake trout from Lake Superior (median 27, mean ± SD 37.0 ± 29.0), but generally within the same range as those found across all fish species from Lake Ontario (median 26, mean ± SD 59.0 ± 104.0), and Lake Superior (median 9, mean ± SD 26.0 ± 74.0; Munno et al., 2021). Since rainbow trout in my study were caged for a short period, rather than being wild-caught, this could have resulted in differences in particle counts between studies. Namely, there was little evidence of feeding in the caged rainbow-trout in the current study, whereas wild-caught fish could have had greater access to prey items that may have facilitated higher uptake of microparticles, either through direct consumption or trophic transfer (Hasegawa & Nakaoka, 2021; McNeish et al., 2018).

Information on microplastics uptake by small-bodied benthic fish, like rainbow darter, is limited in the literature but this study found that rainbow darter from the Grand River ingested between 0-7 microparticles each (median 2, mean ± SD 2.07 ± 1.69). Fathead minnow (*Pimephales promelas*) are similar in size to rainbow darter and are also benthic feeders, permitting some comparisons to be made between systems. Fathead minnow sampled from the

Humber Bay and River in Toronto, ON, had much higher microparticle counts per fish (median 17, mean ± SD 19.0 ± 11) than rainbow darter from the Grand River (Munno et al., 2021); however, particle numbers in *P. promelas* from Milwaukee River, WI (mean 4.60 particles per fish), a major tributary to Lake Michigan, were more comparable to levels in rainbow darter from the Grand River (McNeish et al., 2018). It is possible for benthic feeders to have higher abundances of microparticles compared to fish that feed in the upper region of the water column, since microparticle concentrations in water tend to be higher in the benthic zone compared to those in surface waters (Hoellein et al., 2017). The digestive tracts of benthic and benthopelagic fish from Lake Ziway (Ethiopia) had significantly higher microparticle abundances than pelagic planktivorous species, indicating that feeding behaviour and habitat use are important factors in microparticle accumulation (Merga et al., 2020). In Lake Ontario, no significant differences in microparticle concentrations were found between benthopelagic and demersal fish species due to the high variation between individuals within each habitat; however, the mean number of particles in the digestive tracts of demersal species (median 25, mean ± SD 62.0 ± 110.0) was nearly double that of benthopelagic species (median 27, mean ± SD 35.0 ± 22.0; Munno et al., 2021). Evidence that habitat influences microparticle abundances in freshwater fish is currently limited to studies in lakes, and thus, it is unclear whether fish occupying different riverine habitats will differ in their microparticle levels.

Most of the biota sampled from the Grand River contained microparticles in their tissues (100% of rainbow trout and mussels, 87% of amphipods, and 84% of rainbow darter), and could suffer adverse health effects as a result. Additionally, fibers were a common particle morphology found in the Grand River and they have longer residence times and higher toxicity in *Hyalella* compared to spherical particles (Au et al., 2015). More research is therefore needed to determine

if microfibers and other microparticles are harmful to freshwater organisms at environmentally relevant levels.

4.1.3 Using caged organisms for microplastics biomonitoring

Rainbow trout, mussels, and amphipods caged in the Grand River each showed different spatial trends in particle abundances and their values were not correlated with environmental levels, making it difficult to discern which organism might be the most suitable biomonitor in this system. However, the design of the cages might, in part, account for some of the spatial differences seen among biota. For example, the 300 μm Nitex mesh used on the exterior could have prevented the passage of larger particles into *Hyalella* cages, whereas mussels were strung on a PVC frame in mesh netting (> 1 cm openings) and therefore would have been exposed to microparticles in water > 300 μm. Rainbow trout were enclosed within a PE totes with holes to allow for the movement of water and prey; however, it is possible that particles settled within this container, or did not move as they would normally in the river, resulting in higher concentrations within the cage. Additionally, differences in feeding strategy, diet, and positioning of the cages within the water column could have further contributed to the discrepancies between species.

The lack of correlations between environmental levels of microparticles and those found in caged biota might be due to the one-time sampling of water and sediment, because they are likely more variable over time than biotic samples, although this is speculative. Caged organisms are therefore a valuable tool for microplastics biomonitoring since they may provide more information about microparticle accumulation in resident riverine biota that may not be captured in environmental samples alone. Further, deployed biota can sometimes be advantageous to use in place of wild-caught organisms. Caged organisms can be continuously monitored or extracted

after different exposure periods and their conditions and placement are controlled. Deployed biota may also act as proxies for at-risk species whose wild populations might suffer from sampling. In addition, using organisms that occupy different trophic positions in microplastics caging studies might facilitate a more complete understanding of exposures in riverine biota since different species characteristics can influence microparticle ingestion. For example, microparticle abundance in the digestive tracts of riverine fish is positively related to fish trophic level, and predatory fish tended to have greater numbers of particles compared to omnivores and detrivores (McNeish et al., 2018). The inclusion of various species (invertebrates and vertebrates) in caging efforts therefore has the potential to inform questions surrounding trophic transfer of plastics (depending on how they feed within the cage), as well as differences in microplastics uptake according to species traits.

4.1.4 Biotic and environmental particle concentrations not correlated

There were no correlations between particle concentrations for water and sediment. Although microparticle concentrations in both sediment and water appeared elevated at the Waterloo outfall (DSW1), and at the most upstream reference site (REF1), they did not have other spatial similarities. The movement of microplastics through urban river systems is complex, with particle density and flow velocity thought to be important factors (Ballent et al., 2016; He et al., 2021). Riverine sediments are considered a sink for microplastics, especially dense plastics which tend to settle near their source; however, high bottom velocities can facilitate the movement of these particles over longer distances (He et al., 2021). In general, sites with elevated concentrations in sediment might reflect areas with high local contributions, whereas concentrations in surface waters might be more variable with changing river flows, even with similar inputs from sources over time.

There also were no correlations between microparticle levels in biotic and environmental samples from the same sites; the lack of correlations could be due to the inherent differences between these sample types. Water and sediment samples were collected once from each site and provide a snapshot of particle concentrations in those compartments on the day of sampling. In contrast, biotic samples can integrate information from longer timescales (days to weeks, depending on particle size, morphology, and egestion times) and may respond more slowly to fluctuations in environmental microplastic concentrations. Regular environmental sampling would be helpful to better understand how environmental microplastics concentrations fluctuate within this system, in response to seasonal variations in WWTP effluent loads and river flows, and how biota might respond to these changes.

4.1.5 Rainbow darter and rainbow trout show different spatial patterns

Despite being sampled from the same sites near the Kitchener WWTP outfall, rainbow trout and rainbow darter showed dissimilar spatial trends in particle counts within their digestive tracts. In caged rainbow trout, particle counts were highest at the nearfield site closest to the Kitchener outfall (DSK1) and lowest at the upstream reference (DSW3) and far field (DSK2) sites. The opposite was seen in wild-caught rainbow darter, where counts were lowest at the outfall (DSK1), elevated at the farfield (DSK2) location, and significantly elevated at the reference (DSW3) site, when compared to the outfall. In addition, particle consumption was also generally much lower in rainbow darter compared to rainbow trout (figure 3.21). Several factors could be influencing differences in spatial trends between these two species, as well as the number of particles they consumed. Firstly, the juvenile rainbow trout were held in cages, and therefore their interactions with their surrounding environment would have differed from wild rainbow darter. For example, the cages did not allow for rainbow trout to have direct contact

with sediments, limiting their feeding to prey items suspended within the water column. These two fish species were also at different life stages and varied in body size, with juvenile rainbow trout being much larger (~18-20 cm) than rainbow darter (~5-6 cm); as such the trout had a larger gape size and higher nutritional demands. While there is limited information on gape size and microplastics ingestion in fish, larval fathead minnow, which are smaller in size than rainbow darter, were able to consume microparticles up to 500 µm in size (Bucci et al., 2021). Therefore the direct ingestion of microparticles and prey items > 500 µm may be gape-limited in rainbow darter compared to rainbow trout and this is supported by the difference in average particle lengths between rainbow trout (1797 µm) and rainbow darter (1109 µm; data not shown). Finally, rainbow darter had some mobility near the discharge, and they may have not have been continuously exposed to the WWTPs plume at the outfall site, unlike the caged trout that were held in place. To better understand how these two species are interacting with their surrounding environment, stable isotope analyses and sampling of tissues for pharmaceuticals could be insightful to identify individuals with the greatest exposure to the effluents.

4.2 Particle colour

There were several consistencies in particle colour between abiotic and biotic samples from the Grand River, with blue and clear particles being commonly found across most sites for all matrices examined herein and these results are similar to other studies. Of all the particles identified, ~70% were blue or clear (34.9% and 34.8%, respectively), while black (5.8%) and red (5.5%) were the next most common. Clear, blue, red and black microparticles are consistently found in urban rivers with inputs from WWTPs. In tributaries to the Saint John River, NB, the four most abundant microfiber colours in freshwater mussels were blue (44%), clear (25%), red (15%), and black (13% ; Doucet et al., 2021). Similarly, clear, blue, and red particles were

common in fish and water samples taken from three major tributaries to Lake Michigan (McNeish et al., 2018). Wastewater effluent sampled at three WWTPs in Toronto, ON also consisted of 32% blue, 24% clear/white, and 13% clear-blue particles (Grbić et al., 2020). The shedding of fibers from blue denim jeans during laundering is a common source of blue fibers to urban rivers (Athey et al., 2020; McQueen et al., 2017), and clear or white particles could originate from the flushable wipes or sanitary products or result from the loss of dyes from microparticles during treatment or environmental weathering (Grbić et al., 2020; Munoz et al., 2018). Although microparticle abundances in biotic and environmental samples from the Grand River were not correlated, the colours of microparticles found in biota were similar to those in water and sediment, likely reflecting the interactions between biotic and abiotic compartments. Effluent samples from the Waterloo and Kitchener WWTPs would therefore be helpful to investigate whether common particle colours and morphologies found in biotic and environmental samples are also reflected in the effluents. Further, Raman or FTIR spectroscopy could be used to chemically match dyed particles in the aquatic environment to those found in WWTP effluent (Athey et al., 2020; Xu et al., 2018).

4.3 Particle morphology

Fibers were the dominant morphology observed in Grand River biotic and abiotic samples and made up 82.1% of all particles found, and these results are consistent with other studies which have primarily found fibers in WWTP effluents (Mason et al., 2016; Grbić et al., 2020; Prata et al., 2020), as well as in biotic and abiotic samples from urban rivers (Baldwin et al., 2016; Doucet et al., 2021; Frond, 2019; McNeish et al., 2018) and nearshore Lake Ontario (Athey et al., 2020; Munno et al., 2021). The high numbers of fibers being discharged from WWTPs can be linked to domestic sources, and are largely shed during the laundering of textiles

(Vassilenko et al., 2019; Xu et al., 2018). Additionally, atmospheric deposition, as well as runoff from agricultural areas using biosolids as fertilizer, can also contribute microfibers to freshwaters (Carr, 2017; Grbić et al., 2020; Peller et al., 2021).

Films and fragments were the next most common particle categories, and made up 10.4% and 6.6% of all particles, respectively. Primary microplastics, such as spheres and pellets, were uncommon in this system. Across all samples, only one sphere, or microbead, was found in a rainbow trout digestive tract. The relative absence of spheres within Grand River samples could be related to the microbead ban in 2018, which prohibits the use of plastic microbeads in personal care products and thus prevents their release into the surrounding environment by WWTPs (Government of Canada, 2018). Overall, results from the current work suggest that the number of microfibers being found in environmental and biotic samples could be reduced by limiting inputs from WWTPs to the Grand River.

4.4 Study limitations

As with other studies quantifying and characterizing microplastics, this study had some limitations. While several steps were taken to limit contamination in the field and during laboratory processing, such as reducing sample exposure time to air and thoroughly cleaning all surfaces and equipment, exogenous particles were still present in procedural blanks. To address issues of contamination, blank subtractions of samples were done and material from potential sources were kept as reference material for future chemical analyses. While plastic materials were kept to a minimum, they were sometimes necessary to use in sample collection and processing. For example, since potassium hydroxide etches glass, polypropylene tubes were used in sample collections and processing to avoid damage to glassware, and to keep the transfer of samples between containers to a minimum. Similarly, the filters used in the collection of water

samples were held within polypropylene casings. While procedural blanks would have accounted for the use of these materials, further efforts could be made to streamline sample handling and reduce contamination of samples. Finally, field sampling, baseline samples of caged organisms were not taken prior to exposures and would be valuable to include in future studies.

The generalization of this work is also restricted by the one-time sampling. Since samples for this study were all collected during the month of October in 2019, they do not capture how microplastics concentrations might vary seasonally with changing inputs from the WWTPs and other diffuse sources, as well as river flows. Increased laundering of textiles in the winter could, for example, result in WWTPs releasing more microfibers to the Grand River (Ben-David et al., 2021; Browne et al., 2011; Vignola, 2020). Additionally, since the Waterloo and Kitchener WWTPs both use forms of secondary treatment, WWTPs with primary or tertiary treatment may differ in their effectiveness at removing microparticles from final effluent. The volume of effluent discharged, flow of the receiving body, as well as surrounding land use should be also considered when comparing microparticle levels found in this study to other riverine systems.

Despite the Waterloo and Kitchener WWTPs being the focus of this study, there are numerous other potential sources of this plastics to this system that should not be overlooked, including several other WWTPs within the watershed. Biosolids produced by WWTPs are also becoming recognized as an important source of fibers to the environment (Carr, 2017). While biosolids produced in the region of Waterloo are transported out of the region for end use or disposal, it is unclear whether other fertilizers applied to agricultural fields contain plastics, or whether microplastic films are being released from crop wraps in this area (Qi et al., 2020; Region of Waterloo, 2018; Rochman et al., 2019). In addition, atmospheric deposition of microplastics, industrial inputs, tyre wear particles, and urban runoff could also be sources to the

Grand River (Dris et al., 2015b; Grbić et al., 2020; Kole et al., 2017). Finally, as microparticles from this study have yet to be chemically analyzed to determine their composition, it is not possible to match materials to possible sources or compare material types between studies.

4.5 Conclusions and future directions

With much of the microplastics literature to date focusing on the marine environment, there is a need for an improved understanding of the sources and fate of microplastics in freshwaters. While the presence of microplastics in wastewater influent and effluent has been reported (Ben-David et al., 2021; Carr et al., 2016; Gies et al., 2018; Iyare et al., 2020; Sun et al., 2019), and microplastics have been observed in biotic and environmental samples near WWTP discharges (Kay et al., 2018; McCormick et al., 2014; Windsor et al., 2019), these studies typically do not integrate information from multiple sample matrices across a large spatial gradient. This current study quantified and characterized microparticles across a suite of biotic and environmental samples taken along the Grand River, ON, to determine whether microplastics concentrations were affected by proximity to two WWTP outfalls.

I predicted that abiotic and biotic samples (caged and wild) collected near WWTP outfalls would have elevated microparticles but this prediction was only partly satisfied. Sediments in the central Grand River had significantly elevated particle counts at the Waterloo WWTP outfall site, and surface waters had highest, albeit non-significant, counts at both the Waterloo and Kitchener outfalls. In biotic samples, only caged rainbow trout had significantly elevated particle counts near the Kitchener outfall, but with caged amphipods and mussels, as well as wild-caught rainbow darter, having other spatial trends. While high particle counts were observed at the outfall sites for some sample types, particle concentrations did not respond predictably to sample proximity to WWTP outfalls and elevated levels of microparticles were

also observed at some upstream reference sites. These different spatial trends across biotic and abiotic compartments provide insights into the fate of microplastics within this riverine system, and potentially point to additional sources beyond WWTPs, such as agricultural inputs, to explore in future studies.

This study contributes baseline data on microparticle concentrations for sediments and water in the central Grand River, which may be helpful in assessing the effectiveness of future upgrades to the Waterloo and Kitchener WWTPs or of changes in policies aimed at reducing inputs of microparticles to this system. However, as previously mentioned, regular sampling of WWTP effluents from facilities that release to the Grand River would give a better understanding of seasonal variation in their microplastics inputs to the watershed. It would also be valuable to sample biotic and environmental samples near WWTPs employing different types of treatment to better understand how it affects discharges, as well as to assess whether WWTPs have unique microplastic profiles according to their primary sources of influent (e.g. industrial, commercial, municipal; Franco et al., 2020; Grbić et al., 2020; Mason et al., 2016).

Particle counts in wild-caught and caged biota as well as water and sediments from the Grand River will also guide future lab studies seeking to understand the effects of microplastics on biota at environmentally relevant levels. Further, current studies typically use pristine microplastic spheres or commercial powders for exposures (Browne et al., 2008; Rochman et al., 2019; Rummel et al., 2016; Von Moos et al., 2012); however, the high numbers of fibers found in this riverine system, as well as in other studies, point towards a need for more widespread inclusion of microfibers in toxicity studies to better understand their impacts on aquatic biota. Finally, it remains unclear how microplastics are transported through riverine food webs, and it is therefore difficult to account for all possible sources of plastics to these organisms. Sampling

of additional resident organisms, such as sediment-dwelling invertebrates, and other fish might help inform how different species within this environment are interacting with anthropogenic microparticles at the same sites.

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APPENDIX A

Appendix A contains supplementary information in support of the results section of this thesis.

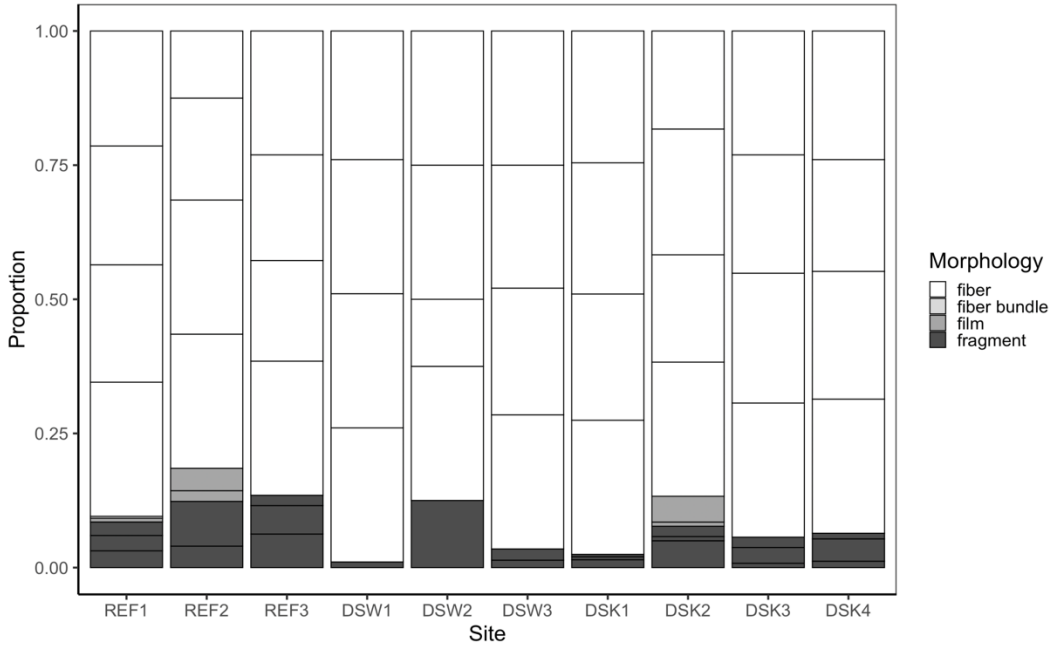

Figure A.1 Proportions of different particle morphologies in all water size fractions at sites along the Grand River, ON. Sites are ordered on the x-axis from upstream (left) to downstream (right). Horizontal black lines separate contributions from the different size fractions (533.4 µm, 228.6 µm, 116.84 µm, 35.56 µm).

Table A.1 Tukey HSD pairwise comparisons calculated between sites for sediment. Statistically significant values are shown in red.

ANOVA F9,30 = 8.239, p = <0.0001			
		Tukey HSD Values	
Site	**Site**	**Diff**	**P adjusted**
REF1	REF2	3.5590	0.1566
REF1	REF3	5.4029	0.0046
REF1	DSW1	-1.5784	0.9515
REF1	DSW2	4.6220	0.0231
REF1	DSW3	5.3498	0.0052
REF1	DSK1	5.6470	0.0027
REF1	DSK2	5.3046	0.0057
REF1	DSK3	4.8808	0.0137
REF1	DSK4	4.6966	0.0199

REF2	REF3	1.8439	0.8854
REF2	DSW1	-5.1374	0.0081
REF2	DSW2	1.0630	0.9967
REF2	DSW3	1.7908	0.9015
REF2	DSK1	2.0880	0.7933
REF2	DSK2	1.7456	0.9141
REF2	DSK3	1.3218	0.9843
REF2	DSK4	1.1376	0.9945
REF3	DSW1	-6.9813	0.0001
REF3	DSW2	-0.7809	0.9997
REF3	DSW3	-0.0531	1.0000
REF3	DSK1	0.2441	1.0000
REF3	DSK2	-0.0983	1.0000
REF3	DSK3	-0.5221	1.0000
REF3	DSK4	-0.7063	0.9999
DSW1	DSW2	6.2004	0.0008
DSW1	DSW3	6.9281	0.0002
DSW1	DSK1	7.2254	0.0001
DSW1	DSK2	6.8829	0.0002
DSW1	DSK3	6.4592	0.0005
DSW1	DSK4	6.2749	0.0007
DSW2	DSW3	0.7278	0.9998
DSW2	DSK1	1.0250	0.9975
DSW2	DSK2	0.6826	0.9999
DSW2	DSK3	0.2588	1.0000
DSW2	DSK4	0.0746	1.0000
DSW3	DSK1	0.2973	1.0000
DSW3	DSK2	-0.0452	1.0000
DSW3	DSK3	-0.4689	1.0000
DSW3	DSK4	-0.6532	0.9999
DSK1	DSK2	-0.3424	1.0000
DSK1	DSK3	-0.7662	0.9997
DSK1	DSK4	-0.9504	0.9986
DSK2	DSK3	-0.4237	1.0000
DSK2	DSK4	-0.6080	1.0000
DSK3	DSK4	-0.1842	1.0000

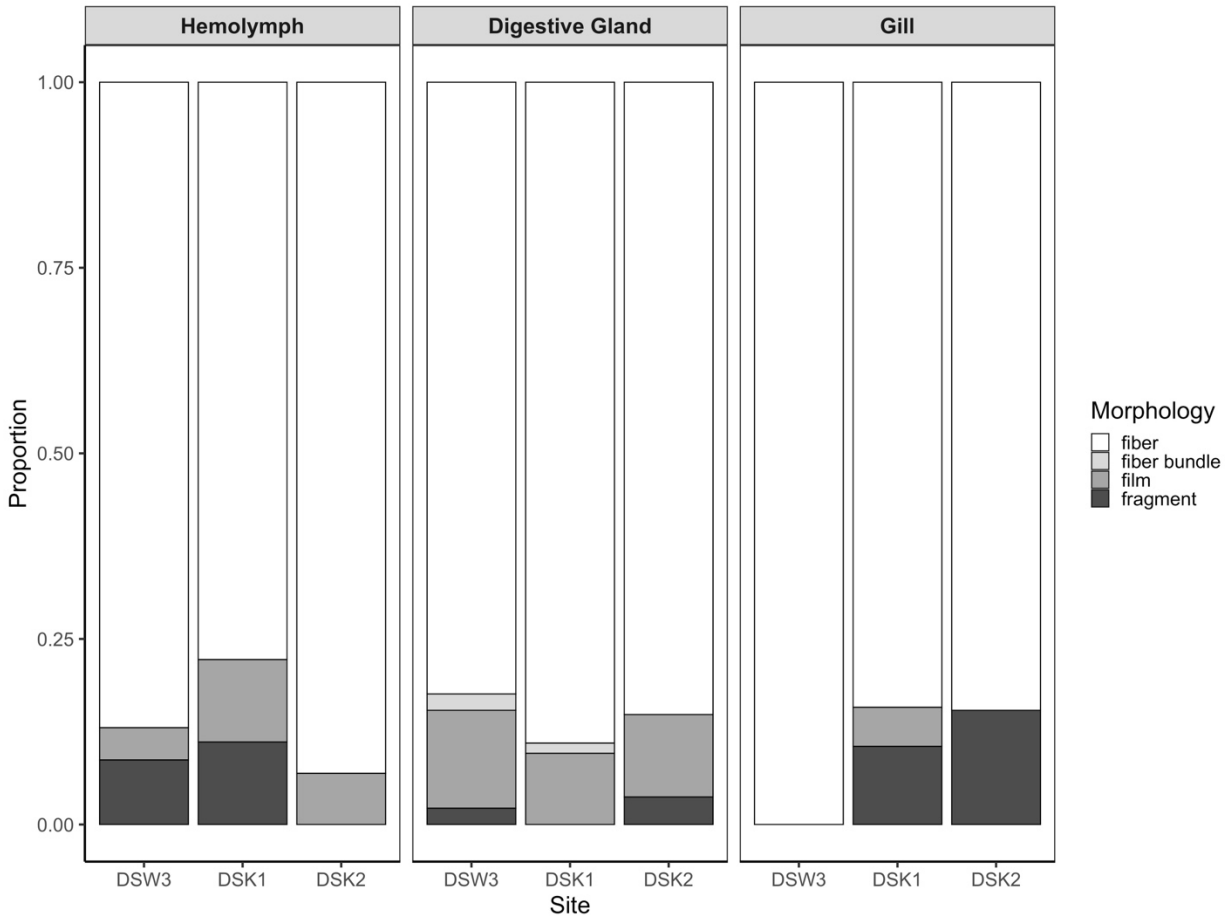

Figure A.2 Proportions of different particle morphologies in mussel tissues at sites along the Grand River, ON. Sites are ordered on the x-axis from upstream (left) to downstream (right).

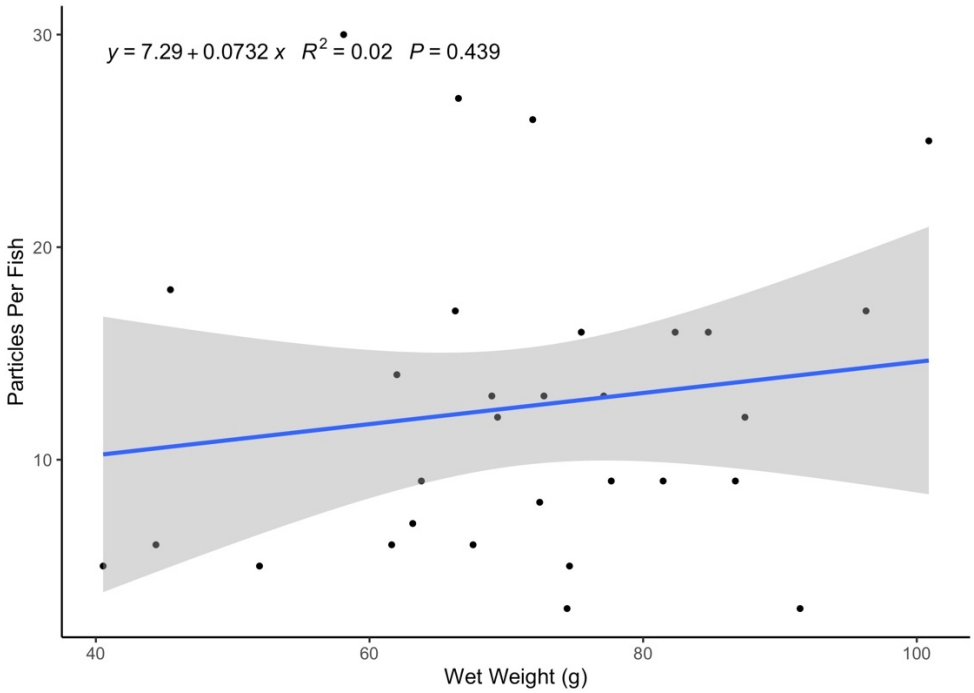

Figure A.3 Linear regression between the number of particles per digestive tract and whole body wet weight (g) for rainbow trout caged in the Grand River, ON.

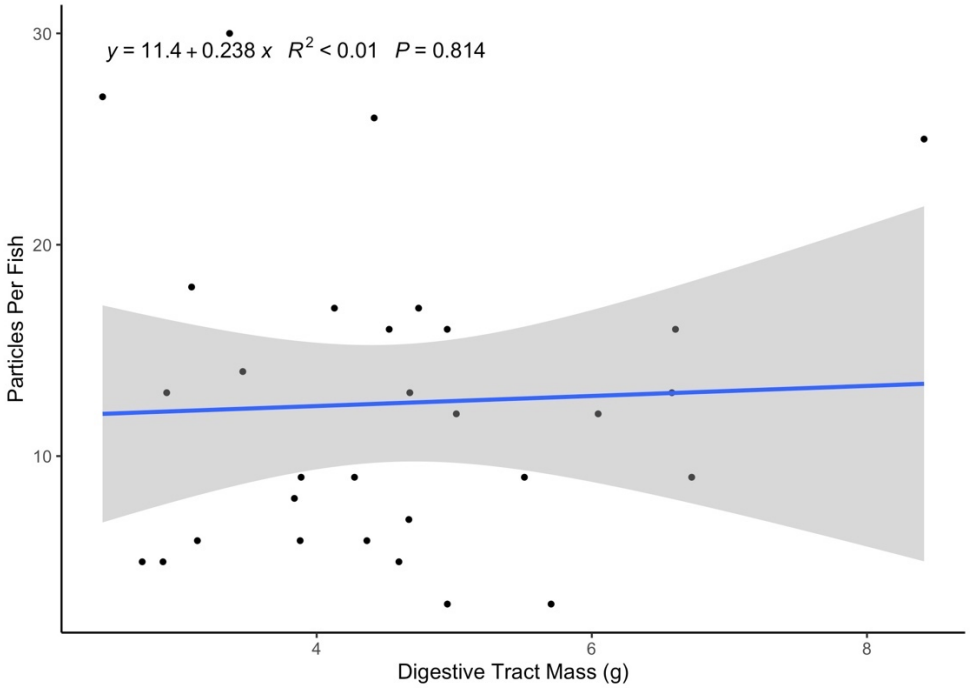

Figure A.4 Linear regression between the number of particles per digestive tract and digestive tract tissue mass (g) for rainbow trout caged in the Grand River, ON.

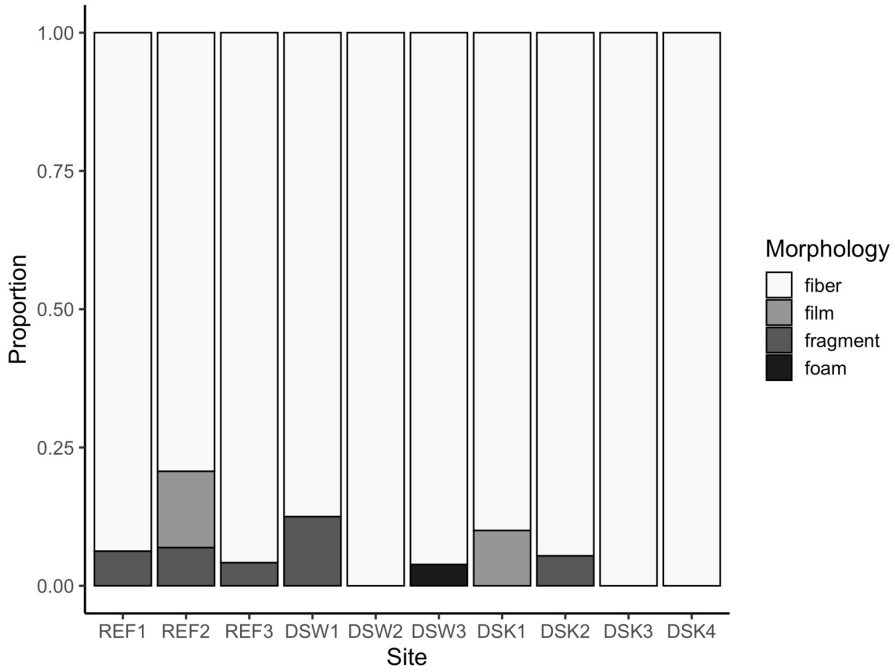

Figure A.5. Proportions of different particle morphologies in rainbow darter digestive tracts at sites along the Grand River, ON. Sites are ordered on the x-axis from upstream (left) to downstream (right).

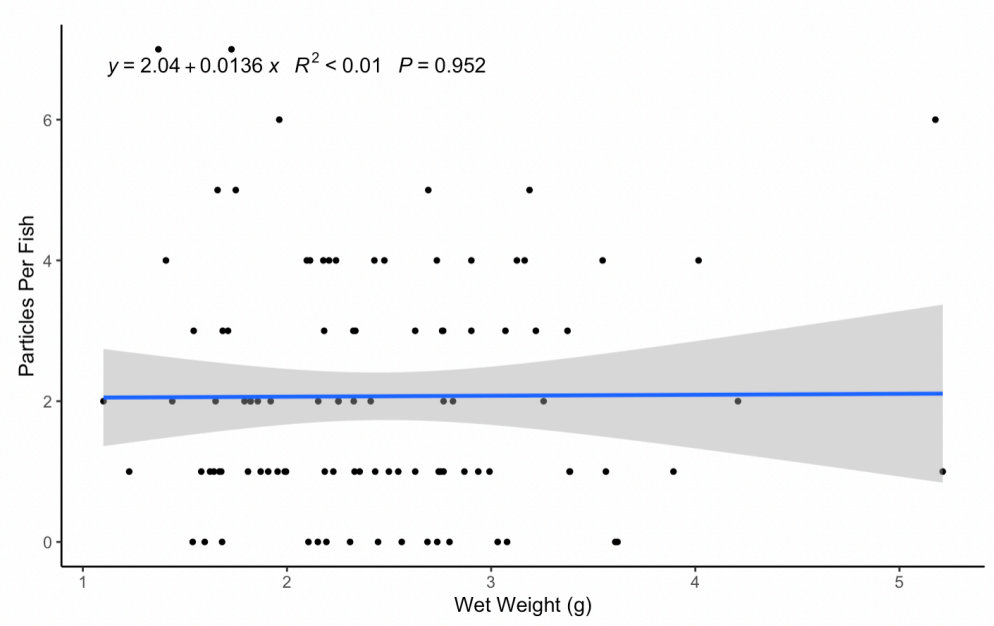

Figure A.6 Linear regression between the number of particles per digestive tract and whole body wet weight (g) for wild-caught rainbow darter from the Grand River, ON.

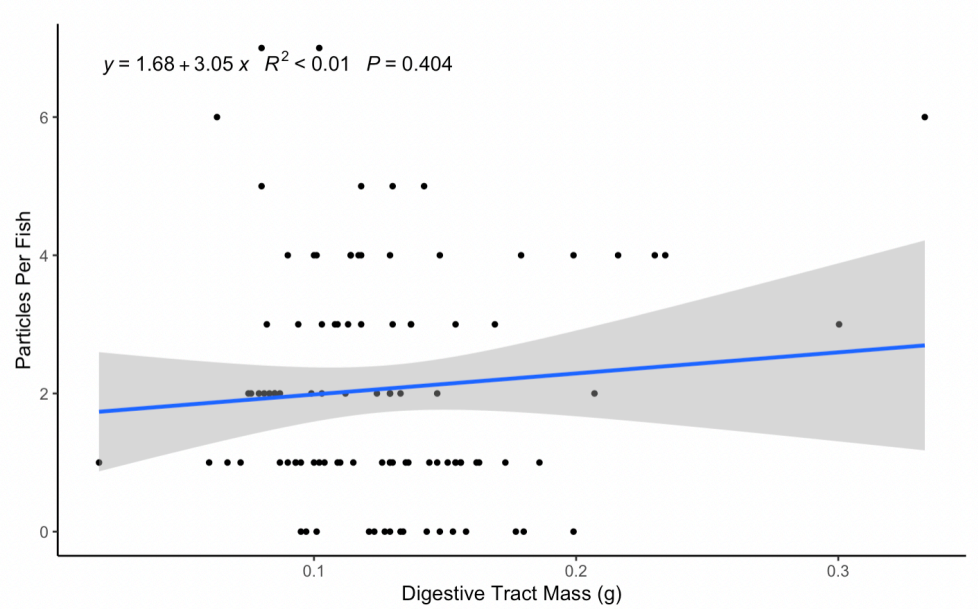

Figure A.7 Linear regression between the number of particles per digestive tract and digestive tract tissue mass (g) for wild-caught rainbow darter from the Grand River, ON.