MICROPARTICLES IN FRESHWATER BIVALVES EXPOSED TO WASTEWATER EFFLUENT

MICROPARTICLES IN FRESHWATER BIVALVES CHRONICALLY EXPOSED TO WASTEWATER EFFLUENT IN THE GRAND RIVER, ONTARIO, CANADA

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LAY ABSTRACT

Microplastics are found in nearly every environment, especially freshwater ecosystems. These plastics come from a variety of sources, and this study focuses on assessing the characteristics of microparticles in freshwater clams and mussels (bivalves) that have been exposed to municipal wastewaters. Bivalves and water samples were collected from 5 locations along the Grand River (Ontario, Canada) in 2021-2022, and microparticles were extracted and analyzed from each sample. Fibers were the most abundant type of microparticle, with colours consisting mostly of clear, blue, and black. Clams had the highest number of microparticles per mass of tissue collected and the lowest counts were found in water samples. Higher microparticle counts were only seen in one (mussel gill) of the four tissues from bivalves collected downstream of wastewater outfalls. This study provides baseline data on microparticle characteristics in freshwater bivalves and will guide future studies on the toxicity of microparticles to these animals.

ABSTRACT

Microparticles enter aquatic environments through many sources, including wastewater treatment plants (WWTPs), but their uptake by aquatic organisms is poorly understood. Freshwater bivalves accumulate multiple contaminants, making them potential bioindicators for MP pollution. This study aims to understand the abundance and characteristics of microparticles that accumulate in wild bivalves. Samples were collected from 5 locations along the Grand River (Ontario, Canada) in 2021-2022, including 3 municipal WWTPs where both an upstream and downstream site were sampled. At each site, fingernail clams (*Sphaeriidae*, n=5 composite samples), flutedshell mussels (*Lasmigona costata*, n=10), and surface water (n=3) were sampled. Within the mussels, the gill, digestive gland, and hemolymph tissues were targeted and compared. Microparticles were isolated and quantified via stereomicroscopy but have not yet been confirmed as plastic; as such, they will be referred to herein as microparticles. Fibers were the dominant morphology and clear, blue, and black were the most common colours, but there were some differences among sites and sample types. Most microparticles were between 80 μm and 1 mm in length. Fingernail clams contained the highest microparticle counts per mass of tissue at 35.5 ± 29.4 microparticles/g, mussel tissues ranged from 4.3 ± 4.2 microparticles/mL to 6.5 ± 8.1 microparticles/g, and water samples contained the lowest counts at 0.0055 ± 0.0028 microparticles/mL. Elevated microparticle counts at downstream sites were only seen in mussel gills and not other bivalve tissues. Surface water samples did not show elevated counts downstream of the WWTPs and microparticle exposures were similar across sites. This study provides baseline data for

future monitoring and informs toxicity studies to fully assess the risk of microparticles to vulnerable freshwater bivalves and other aquatic organisms. It also suggests microparticles in freshwater bivalves are coming from sources in addition to WWTPs and are ubiquitous in the Grand River.

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DEFINITIONS & ABBREVIATIONS

Bioindicator – biological organisms used to indicate environmental conditions (Grabarkiewicz and Davis, 2008). This is a qualitative measure.

Biomarker – measurable indicator of biological conditions, often change in response to environmental conditions. This is a quantitative measure.

Biosolids – solid sewage sludge that settles during wastewater treatment processes, is often used in agriculture as fertilizer as high in nutrients.

Bivalves – mollusc organisms with within the class Bivalvia and include a shell made up of two valves.

Digestive Gland – tissue within freshwater bivalves which is made up of digestive diverticula and encompasses the stomach of freshwater mussels and where absorption of nutrients occurs (Cummings and Graf, 2009).

Fingernail Clams – freshwater bivalves in the family Sphaeriidae. Fingernail clams are also referred to as pea or pill clams.

Freshwater mussels – freshwater bivalves in the family Unionidae. In the current study all organisms were flutedshell mussels, *Lasmigona costata.* Freshwater mussels are also referred to as naiads or pearly mussels.

Gills – organ within freshwater bivalves which is made up of cilia and ctenidia and can produce a current that facilitates filter feeding and where preliminary particle sorting occurs (Cummings and Graf, 2009).

Glochidia – freshwater mussel larvae.

Gravid – female mussels that contain brooding glochidia.

Hemolymph – circulatory fluid in freshwater bivalves which transports oxygen throughout the animal. It is clear and does not contain any hemoglobin or hemocyanin (Cummings and Graf, 2009).

Microparticles – anthropogenically-derived particles less than 5 mm, not spectroscopically confirmed to be made of plastic materials.

Microplastics – plastic particles less than 5 mm in size, ubiquitous in the environment.

AR – Ayr study site (WWTP).

CD – Caledonia study site (WWTP).

DDT – dichlorodiphenyltrichloroethane, an insecticide.

GDP – gross domestic product, representing the monetary values of goods and services produced per country per year.

HE – Hespeler study site (WWTP).

HEPA – high **e**fficiency purified air, a type of filter commonly used to remove particulate matter from the air.

IUCN – International Union for Conservation of Nature.

KOH – potassium hydroxide solution, used to digest organic matter and isolate plastic particles in biological samples.

MPs – microplastics

PA – polyamide, a plastic polymer.

PAH – polycyclic aromatic hydrocarbons, a group of natural chemicals that occur in many petroleum products and are a byproduct of incomplete combustion.

PCB – polychlorinated biphenyls, a group of synthetic chemicals that occur in many industrial and consumer products.

PE – polyethylene, a plastic polymer.

PET – polyethylene terephthalate, a plastic polymer.

PP – polypropylene, a plastic polymer.

PS – polystyrene, a plastic polymer.

PVC – polyvinyl chloride, a plastic polymer.

WMT – West Montrose study site.

WWTP – wastewater treatment plant.

YK – York study site.

DECLARATION OF ACADEMIC ACHIEVEMENT

I, Emily Robson, declare this thesis to be my own work and am the sole author of this work. The collection, processing, and analysis of most fingernail clam samples was done by Evlyn Sun as part of her Honours thesis at McMaster, and I co-supervised her thesis project. No part of this work has been published or submitted for publication or for a degree at another institution. My supervisor, Dr. Karen Kidd, and members of my supervisory committee Dr. Pat Chow-Fraser, Dr. Patricia Gillis, and Dr. Ryan Prosser, have provided guidance and support throughout the project and on this document, but the analysis is my own.

1 INTRODUCTION

Plastic synthesis began in the late 1800s, with a notable increase in production starting in the 1950s (Geyer, 2020; Geyer et al., 2017). Their properties, including durability, versatility, and low cost, make plastics a desirable material used in a wide variety of products, leading to an increase in plastic production over time (Cowan et al., 2021). Commonly used plastics are not biodegradable and persist in the environment (Geyer et al., 2017). Despite public awareness leading to bans of single-use plastics in some countries (Schnurr et al., 2018), plastic pollution continues to be an environmental concern. Of specific interest are microplastics (MPs), anthropogenic plastic particles <5 mm in size (Arthur et al., 2009), as their small size means that they are easily transported throughout the environment and can impact a wide variety of organisms, from very small to very large.

1.1 Microplastic Pollution

Microplastics have become ubiquitous in the environment, from densely populated areas to the remote poles (Hamid et al., 2018). Due to the wide variety of MPs in the environment, they must not be considered as a single contaminant but instead as a diverse group of contaminants (Rochman et al., 2019). Microplastics can be categorized by source, composition, and physical characteristics. Two main sources of MPs exist; primary microplastics that are manufactured in a small size while secondary microplastics have degraded from larger plastics (Wagner et al., 2014). Plastics degrade in the environment through a variety of processes including biodegradation, photodegradation, thermo-oxidative degradation, thermal degradation, and hydrolysis (Andrady, 2011). A

wide array of MP compositions exists, including but not limited to polyethylene (PE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyethylene terephthalate (PET), and polyamide (PA), each of which differs in attributes, such as density, which dictate their fate in the environment (Geyer, 2020). Microplastics can further be categorized by their size, colour, and morphology (fiber, fiber bundle, fragment, film, foam, sphere, and pellet), which can help determine the source of the plastic (Rochman et al., 2019). The physical and chemical properties of MPs impacts their fate in freshwater ecosystems (Atugoda et al., 2020).

Their small size also means microplastics have a large surface-area to volume ratio, and therefore a large surface area for chemicals to adsorb to and desorb from (Weber et al., 2021). During production, a wide variety of additives are mixed with plastics to give them additional properties (Wagner et al., 2014). Some additives include thermal stabilizers, plasticizers, flame retardants, UV stabilizers, colour, and lustre additives (Andrady and Neal, 2009), and these can leach from the plastic into organisms after ingestion (Franzellitti et al., 2019). Plastics also have the potential to sorb additional persistent organic pollutants from the environment, including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and dichlorodiphenyltrichloroethane (DDT; Rios et al., 2007; Velzeboer et al., 2014). These additives and pollutants can also impact organisms exposed to microplastics.

1.2 Sources of Microplastics to Freshwater Ecosystems

Microplastics enter freshwater environments from a variety of point and non-point sources. They can enter freshwater environments through atmospheric deposition,

wastewater and industrial effluents, as well as urban and agricultural runoff (Eerkes-Medrano et al., 2015). The abundance and characteristics of microplastics vary greatly depending on the source, however, the relative contribution from each source to fresh waters is poorly understood (Li et al., 2020). Higher microplastic concentrations are usually observed in urban areas and positively related to factors associated with anthropogenic activity, such as gross domestic product (GDP), urban land area, population size, and agricultural land use (Cera et al., 2020; Li et al., 2023). Since this is a relatively new field of study, investigation is needed to better understand the sources and fate of microplastics in freshwater systems.

Wastewater treatment plants have been identified as a major source of microplastics to freshwater environments (Kay et al., 2018; Li et al., 2018). High removal rates of MPs during wastewater treatment have been reported, ranging from 95 – 99 % (Horton, Walton, et al., 2017). Preliminary and primary treatment of wastewater involves screening, surface skimming, and flocculation during gravity separation, and MP removals range from 35 – 59% and 50 – 90% respectively, removing mostly fibers (Sun et al., 2019). Secondary treatment typically involves biological treatment and clarification and may lead to biofilm formation on MPs, with removal efficiency of an additional 0.2 – 14%, removing mostly fragments (Sun et al., 2019). Tertiary treatment involves a wide variety of technologies to remove nutrients and pathogens, with additional MP removal of 0.2 – 2%, as most have been removed from earlier stages (Sun et al., 2019). The majority of microplastics in wastewater effluents are fibers, as they are released from clothing (both synthetic and natural textiles) when washed and are not as effectively removed as

other morphologies during conventional wastewater treatment (Browne et al., 2011; Sun et al., 2019). The abundance and characteristics of microplastics in effluent is affected by a variety of factors including population served, wastewater sources (residential, commercial, or industrial), and types of treatment (Sun et al., 2019); this suggests that WWTPs contribute different microplastic loads to freshwater environments depending on their characteristics. Even though WWTPs have high removal efficiencies, the large volumes of effluent mean high numbers of MPs are released into freshwater environments (Sun et al., 2019; Ziajahromi et al., 2017) and potentially impacting downstream organisms.

The field of freshwater microplastics research is relatively new, compared with decades of research in marine environments, but it is revealing widespread contamination in this ecosystem (Wagner et al., 2014). Microplastics assessments have been conducted in the Laurentian Great Lakes and high occurrences were observed; one spatial survey of surface waters contained, on average, a staggering 43,000 particles/km^2 (Eriksen et al., 2013). Higher occurrences of MPs in surface waters and on beaches are associated with large industrial regions and densely populated urban areas in the Great Lakes region (Dean et al., 2018; Eriksen et al., 2013; Zbyszewski et al., 2014; Zbyszewski and Corcoran, 2011). Despite a growing number of studies in the Great Lakes basin, there is less focus on the surrounding tributaries than the lakes themselves (Dean et al., 2018; Lenaker et al., 2021; Munno et al., 2022). One of the only studies on tributaries to the Great Lakes found that all samples contained MPs and morphologies differed from those reported in the lakes (Baldwin et al., 2016). Given that rivers are a source of MPs to lakes

and contain organisms potentially at risk from these exposures, the occurrence and sources of MPs and their uptake into biota in these systems needs further investigation.

1.3 Microplastics Uptake in Freshwater Organisms

Given their ubiquitous nature in freshwater environments, MP uptake in aquatic organisms is occurring but is not well understood. Most microplastic uptake occurs during feeding and the functional feeding groups of organisms influences the number of MPs ingested; with filter feeding organisms typically ingesting more MPs than those with other strategies (Scherer et al., 2017). Microplastics may bioaccumulate and biomagnify in aquatic ecosystems, therefore adversely affecting older organisms and those at higher trophic levels (Wagner et al., 2014). MPs have been found in organisms from nearly all trophic levels, however the process of biomagnification has not yet been characterized in aquatic ecosystems (Krause et al., 2021). In freshwater environments, most field studies have focused on fish, with a very small percentage of studies (~23%) investigating freshwater invertebrates (de Sá et al., 2018). As MPs are ingested, they can impact organisms through physical, chemical, and biological means (Li et al., 2018). Due to their small size, some microplastics can pass through cellular membranes and enter tissues (Franzellitti et al., 2019). Overall, microplastics can reduce feeding and reproduction, impact oxidative stress, delay growth and be genotoxic and neurotoxic (de Sá et al., 2018). Lab toxicity studies have shown that microplastics can have adverse effects on freshwater organisms, but to fully assess their risk we must also understand the exposures occurring in the environment.

1.4 Freshwater Bivalves as Bioindicators

Freshwater bivalves are an abundant group of freshwater molluscs that are found worldwide and perform many key functions in ecosystems (Haag, 2012). They filter a high volume of water which improves water quality, facilitate nutrient and oxygen cycling, provide habitat and stabilize substrates, and are an important food source for larger organisms (Haag, 2012; Ostroumov, 2005). Freshwater bivalve populations are declining, with over 200 species on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species due to habitat destruction, introduction of non-native species, overfishing, and pollution (Lydeard et al., 2004; Metcalfe-Smith et al., 2000). The present study focuses on two of the dominant families of these freshwater bivalves; Sphaeriidae (fingernail clams) and Unionidae (freshwater mussels) (Cummings and Graf, 2009; Hornbach et al., 1984).

Both groups of bivalves are primarily filter feeders but are also known to deposit feed (Haag, 2012; Nichols et al., 2005; Way, 1989). Fingernail clams can filter up to 30 mL/hour and also use their foot and siphons to deposit feed in the sediment (Way, 1989). Freshwater mussels use cilia in their gills to produce a current of water through the body at rates up to 1 L/hour and these currents can be strong enough to pull particles from both the water column and sediment into the animal (Haag, 2012; Nichols et al., 2005). Juvenile mussels are known to actively pedal feed, using their foot to bring sediment into the body for ingestion (Yeager et al., 1994). Both groups of bivalves feed on small particulate matter (typically <20 μm), including phytoplankton, zooplankton, bacteria,

and detritus (Strayer, 2008; Vaughn et al., 2008), and therefore have the potential to ingest small microplastics.

The risk of microplastics to freshwater bivalves is largely unknown. Recent literature on marine bivalves suggests they accumulate more microplastics than other invertebrates (Setälä et al., 2016) and strong positive correlations between MP counts in bivalves and in the environment have been reported (Bom and Sá, 2021). The filter and deposit feeding of freshwater bivalves means they are exposed to contaminants in the water column as well as the sediment (Haag, 2012; Way, 1989), and organisms with these feeding strategies are known to ingest microplastics (Bour et al., 2018; Scherer et al., 2017). In some freshwater bivalves, specifically zebra mussels, microplastics impacted energy reserves and caused oxidative stress after long exposure periods (Weber et al., 2021). Since bivalves are known to impact the cycling of some contaminants (Way, 1989), they may also affect the fate of microplastics in the environment. Because much of the literature is focused on marine bivalves, there is a need to better understand the presence and impact of microparticles to freshwater bivalves.

Marine bivalves have been recommended as a bioindicator for microplastic pollution in marine ecosystems, and freshwater bivalves have many of the same traits potentially making them good bioindicators for this group of contaminants in freshwater ecosystems (Li et al., 2019). Fingernail clams are a relatively short-lived (1-4 years) and are abundant in freshwater environments (Carr and Hiltunen, 1965). Their cosmopolitan behaviour means they are commonly found in large assemblages, typically on the sediment but they have also been found burrowed and attached to aquatic plants (Martin,

1998). Fingernail clams have been used to monitor metals, including cadmium, copper, zinc, and lead (Anderson, 1977), and in toxicity testing (Kullman et al., 2007; Verrengia Guerrero et al., 2007). Freshwater mussels are much longer-lived organisms (some 70+ years), and although widespread, populations are not as abundant as fingernail clams (Grabarkiewicz and Davis, 2008; Strayer, 2008). Freshwater mussels are sedentary, with many species remaining within a few meters during their lifetime (Haag, 2012). They have long been used as bioindicators, including for metals, ammonia, chlorine, herbicides, insecticides, and pharmaceuticals and personal care product (De Solla et al., 2016; Grabarkiewicz and Davis, 2008). Many biomarkers are well characterized in freshwater mussels, and they have been used to understand the effects of urban runoff, wastewater effluent, organic compounds, and pharmaceuticals (Gagné et al., 2004; Gillis, 2012; Gillis, Gagné, et al., 2014; Jasinska et al., 2015). Both groups of freshwater bivalves are potential indicators of microplastic pollution in aquatic environments, but it must be determined if there is a correlation between MPs found in bivalves and the environment.

1.5 Study Rationale

The Grand River watershed in Southern Ontario is home to approximately 1,000,000 people, and discharges into Lake Erie, which has higher microplastic counts than other Great Lakes (Eriksen et al., 2013; GRCA, 2020). Within the watershed, approximately 60% of the land use is agricultural, ~14% is urban, ~16% is forest, and ~10% is wetland (GRCA, 2020). To support a growing population, there are 30 WWTPs releasing effluent to the mainstem of the river and its tributaries (GRCA, 2020). Urbanization of the watershed and WWTP effluents have led to many regions along the

Grand River being classified with low water quality scores (Anderson, 2012; GRCA, 2020). Although improvements to wastewater treatment have been made in recent decades (Anderson, 2012; Metcalfe-Smith et al., 2000), there are still releases of excess nutrients (Cooke, 2006), metals (Gillis, Higgins, et al., 2014), as well as pharmaceuticals and personal care products (De Solla et al., 2016; Gillis, Gagné, et al., 2014); all of which have the potential to negatively impact bivalves.

The presence of microplastics has previously been confirmed in freshwater bivalves in the Grand River. First, 71% of freshwater mussels collected from six sites contained microplastics, although potential sources were not assessed (Wardlaw and Prosser, 2020). Second, the potential input of microparticles from wastewater effluent was investigated by caging freshwater mussels upstream and downstream of a large wastewater treatment plant on the Grand River for 28 days (Weir et al., 2023). Microparticle counts differed across sites in each of the three tissues analyzed, with high particle counts in hemolymph and gill tissues at downstream sites but no differences in digestive gland counts (Weir et al., 2023). The current study characterizes microplastics in two groups of freshwater bivalves from the Grand River, compares the microplastics found in surface waters to those found in bivalves to assess their potential for biomonitoring, and further investigates the impact of wastewater effluents on microplastic uptake.

1.6 Study Objectives

The main objective of this study was to assess microparticles (herein called microparticles as they have not been chemically confirmed as plastic) in freshwater

bivalves chronically exposed to wastewater effluents. This was done by examining the abundance and characteristics of microparticles in bivalve and surface water samples collected upstream and downstream of 3 WWTPs along the Grand River, as well as from sites upstream and downstream of the study area. This research aimed to answer the following questions:

1) Do bivalves collected downstream of wastewater outfalls have higher microparticle counts than bivalves collected upstream?
2) In which group(s) and tissue(s) are microparticles accumulating in freshwater bivalves?
3) Are the microparticles found in bivalves representative of the microplastics found in the environment?

It is predicted that bivalves collected downstream of WWTPs will have higher microparticle abundance than those collected upstream due to chronic exposure. Freshwater mussels are expected to have higher microparticle counts (particles/g) than fingernail clams as they are longer-lived and filter higher volumes of water. Within freshwater mussels, it is predicted that the digestive gland will have the highest microparticle counts, as it is the tissue most directly associated with digestion of particles. Finally, it is also predicted that the abundance of microparticles in bivalves will reflect the abundance of microparticles in surface water samples. This work will help to advance our understanding of the prevalence and types of microparticles in freshwater ecosystems and WWTPs as a source of microparticles to freshwaters and provide baseline data for monitoring in vulnerable freshwater bivalves.

2 METHODS

2.1 Study Location

Sampling was conducted along the Grand River watershed in Southern Ontario, Canada. It is important to acknowledge that the land 10 km on each side of the Grand River was deeded to the people of Six Nations in 1784 for their assistance during the American revolutionary war, but today the Six Nations occupy less than 5% of this area (Bruechert, 2018; Lewis, 2015; Wilson and Anderson, 2021). Five locations were sampled; 3 at WWTPs, as well as 1 site upstream and 1 site downstream of the study area (Figure 2.1). West Montrose (WMT) and York (YK) are the most upstream and downstream locations, respectively, and are not directly associated with WWTP discharge. The three WWTPs sampled vary in the type of treatment and size of population served (Table 2.1), and 2 of the 3 are located on tributaries to the Grand River. At each WWTP location, a site upstream and downstream of the outfall were sampled, for a total of 8 sites sampled throughout the watershed (2 at each WWTP and 1 at each WMT and YK).

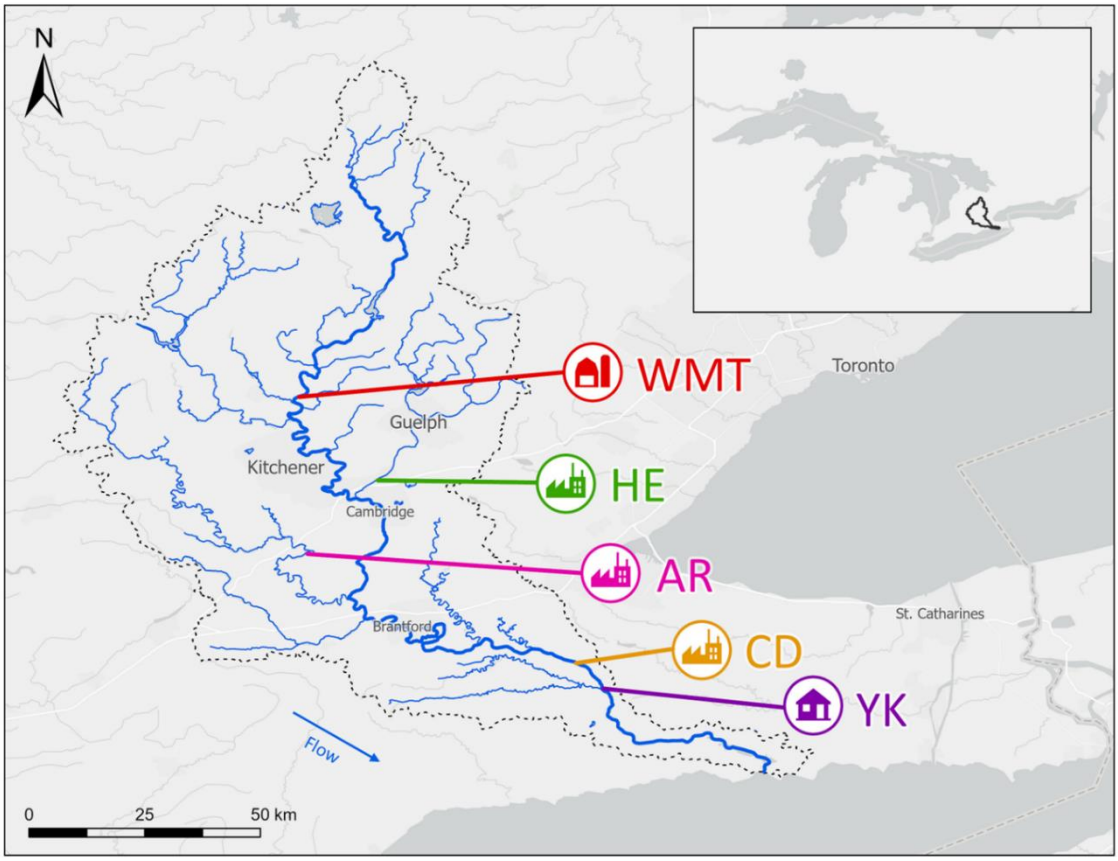


Figure 2.1 Map of study locations sampled in the Grand River watershed, ON, in summer 2021. Locations are ordered from most upstream (top) to downstream (bottom). The West Montrose (WMT) and York (YK) locations are at the upstream and downstream limits of the study area and do not receive inputs from a nearby WWTP. The Hespeler (HE) and Ayr (AR) locations are on tributaries, the Speed and Nith Rivers, respectively, while all other sites are on the main branch of the river. HE, AR, and Caledonia (CD) are locations where a WWTP was sampled.

Table 2.1 Characteristics of each wastewater treatment plant (WWTP) sampled in the Grand River watershed, ON, in summer 2021. Data for Hespeler and Ayr are current as of December 2020 (Region of Waterloo, 2018, 2021), while data for Caledonia are current as of February 2019 (Haldimand County, 2019). Treatment plants are ordered generally from most upstream (top) to downstream (bottom).

Treatment Plant	Discharge Water Body	Population Served	Average Flow (m^3/day)	Level of Treatment	Treatment Used
Hespeler	Speed River	25,970	6,396	Secondary	Aerated grit removal, extended aeration, secondary clarification, sodium hypochlorite disinfection, de-chlorination. Addition of alum
Ayr	Nith River	5,784	1,428	Tertiary	Screening, aerated grit removal, extended aeration, secondary clarification, tertiary filtration, UV disinfection. Addition of ferric chloride
Caledonia	Grand River	9,674	3,208	Tertiary	Stacked activated sludge, tertiary filtration, aerobic digestion, chlorine disinfection, de-chlorination

2.2 Field Collection

Most samples were collected between Aug. 17 and Sept. 29, 2021; with the exception of water samples collected from Ayr downstream on Nov. 4, 2021, and fingernail clams collected from Ayr upstream and downstream on Sept. 8, 2022. The sites upstream and downstream of each WWTP were similar distances from the outfall and always within 100 m (Figure 2.2). The plume of the WWTP effluent was determined visually and confirmed by high conductivity readings using a multi-probe YSI. For fingernail clams, a far-field site was also sampled (except for Ayr) approximately 60 m further downstream of the outfall, where conductivity levels were similar to readings at the upstream site.

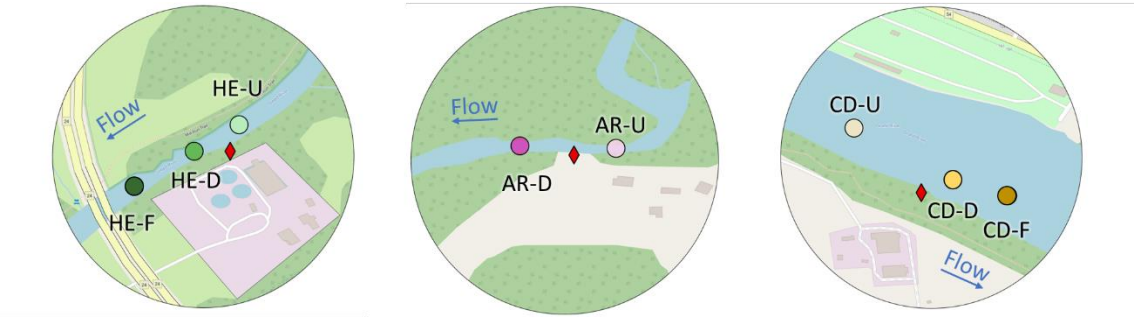


Figure 2.2 Map of each wastewater treatment plant sampled in the Grand River watershed, ON, in summer 2021. At each WWTP location, an upstream and downstream sample similar distances from the outfall were collected. A far-field sample was also collected for fingernail clam samples two locations. Maps are ordered from left to right are Hespeler (HE), Ayr (AR), and Caledonia (CD). Light coloured circles represent the upstream (U) sampling sites, darker coloured circles represent the downstream (D) sites, and the darkest circles represent the far-field (F) sites. Red diamonds represent the location of the WWTP outfall. Blue arrow shows direction of flow at each site.

2.2.1 Fingernail Clam Collection

At each site, n=5 composite samples of 10 Sphaeriidae clams each were collected, for a total of 50 clams/site. For each replicate, a 0.5 m quadrat was used to search for clams. Sediment was collected from within the quadrat and sieved using a 1 mm sieve to locate clams. Preference was given to larger clams when possible. If not enough clams were found within one quadrat, a second quadrat was also searched. Immediately after collection clams were rinsed with filtered DI water (see section 2.6 Quality Assurance) to remove any external contamination, stored in 20 mL glass vials on ice in the field, and then put in the freezer at -20ºC until dissection. At each site, a blank was collected by mimicking transferring clams from the sieve into a glass vial using forceps, and the forceps were rinsed with DI water into the vial.

In the laboratory, clams were dissected in a clean glass Petri dish using a stereomicroscope. The total wet weight (including shells, ±0.0001 mg) of the composite sample was determined using a microbalance. Each clam was individually photographed before the soft tissue was dissected from the shell and transferred to an aluminum foil pouch. Once all clams in the replicate were dissected, the wet weight of the composite tissue sample was measured (excluding shells, ±0.0001 mg). The Petri dish was subsequently rinsed after each replicate, and the rinsate was stored in the same glass vial in the fridge at 4ºC until sample extraction. The blank collected in the field was left open during one of the dissections and the cleaned Petri dish was also rinsed into the vial to represent any aerial deposition of microplastics that may have occurred during the dissection process.

Following dissections, the images of each clam were measured using ImageJ software to determine the mean length, height, and width (±0.01 mm) of each replicate. The total wet weight (shell included) and tissue wet weight (after dissection, shell excluded) per clam were calculated for each replicate by dividing the total mass by the number of clams. The mean wet tissue mass per clam was determined to be 60 mg, and this value was used when analyzing microparticle counts to calculate the approximate number of microparticles found per clam.

2.2.2 Freshwater Mussel Collection

At each site, n=10 flutedshell mussels (*Lasmigona costata*) were collected. Mussels were collected using underwater viewers and identified to species. Dissections were conducted in the field due to Covid-19 restrictions. Microplastics enter through the

gills, accumulate in the gut, and transfer into the circulatory system in marine bivalves (Browne et al., 2008; Kolandhasamy et al., 2018). For this reason, the gills, digestive gland, and hemolymph were targeted in this study.

Before dissections, the total length (longest part of mussel, parallel to hinge, ±0.1 mm) and wet weight (shell included, ±0.1 g) of the mussel were recorded. Approximately 1.5 mL of hemolymph was collected from the posterior adductor muscle using a polypropylene syringe (21 gauge with 0.514 mm inner diameter) and stored in a 2 mL polypropylene vial. The mussel was then opened using a shucking knife and both adductor muscles cut using a stainless-steel scalpel. It was then determined if the mussel was gravid (contained glochidia) or not based on the thickness of the gills. Flutedshell mussels are not sexually dimorphic, so sex could not be determined if individuals were not gravid; gravid mussels were classified as female while non-gravid mussels could be male or female. The entire gills and digestive gland were then dissected from the mussel using stainless-steel forceps and scissors, each placed in an aluminum foil pouch, immediately weighed, and stored on ice until frozen at -20ºC later the same day.

Between each dissection, the glass dissecting surface was cleaned with bleach, ethanol, and ultrapure water and wiped with a Kimwipe. The dissector also rinsed their dissection tools in the same three solutions and changed their gloves. At each sampling location, a blank for the hemolymph was collected by transferring clean filtered DI water using a new syringe into a 2 mL vial. A blank for the tissues was also collected by leaving a foil pouch open to air contamination for at least 10 minutes and moving tools above it to mimic tissue dissections. The tissue blank was used for both the digestive gland and gill

tissues. Mussel shells were saved, and in the lab the approximate age of the mussel was determined by counting the external annuli rings on the left valve.

2.2.3 Surface Water Collection

At each site, n=3 surface water samples were collected within 2 weeks of sampling bivalves (except at Ayr) to determine environmental microparticle concentrations. A 20 L grab sample of surface water was collected in an orange polypropylene bucket, away from the bank and typically near the center of the river facing upstream (similar to Nan et al., 2020). The bucket was immediately covered with a lid of the same material to limit airborne contamination. The water was then passed through a GeoPump peristaltic pump using Tygon tubing through 4 in-line filters. Each contained a stainless-steel filter decreasing in mesh size (533.4 μm, 228.6 μm, 116.84 μm, and 35.56 μm), allowing for *in-situ* partitioning of particles by size (Ziajahromi et al., 2017). After filtration was complete, each of the four filters were transferred to polystyrene Petri dishes using clean forceps. At each sampling site, a blank was collected by pumping 20 L of already filtered river water through the pump onto new blank filters. Each polypropylene filter holder and forceps were rinsed with filtered DI water between each replicate.

2.3 Microparticle Extraction

2.3.1 Extraction in Bivalve Samples

Biological tissues were digested in a potassium hydroxide (KOH) solution to dissolve organic material but retain any plastic particles (Tsangaris et al., 2021). The digestions ran for at least 14 days at room temperature in polypropylene tubes (Falcon),

as KOH is known to damage glass (Munno et al., 2018; Wakkaf et al., 2020). Fingernail clam tissues were digested in a solution of 10% KOH while mussel samples were digested in a 20% KOH solution since mussel tissues masses were much larger than composite clam tissues and consequently more difficult to digest. Tissues were transferred from the foil pouch to the Falcon tubes using clean stainless-steel forceps. The foil pouch was then briefly rinsed with filtered DI water to collect any remaining tissues, as aluminum reacts with the KOH. To account for any dilution due to this step, the KOH solution was slightly super-saturated. After transferring the tissues, the tube was topped up with KOH ensuring there was at least 3x the volume of the tissues. Tubes were inverted every few days to ensure complete mixing and digestion.

Fingernail clam tissues were mixed with the rinsate from the dissection dish in a 50 mL Falcon tube and topped up with KOH. Mussel digestive gland tissue was digested in a 15 mL tube while the gills were digested in a 50 mL tube due to their larger size. Mussel hemolymph was poured into a 15 mL tube and the cryovial was triple rinsed with KOH. After the digestion period, the solutions were filtered through a 38 μm sieve, the same lower limit as the water samples; as particles smaller than 45 μm are difficult to handle using forceps (Miller et al., 2021). Any particulate matter caught in the sieve was rinsed into a glass Petri dish or glass jar for storage until visual identification.

2.3.2 Extraction in Water Samples

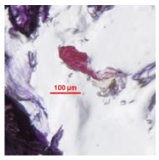
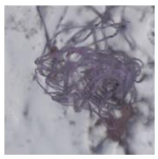
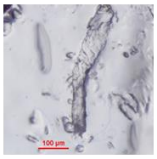
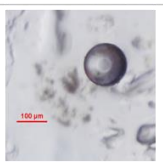
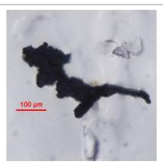
Each stainless-steel filter was transferred to a glass jar and covered in ~300 mL of filtered DI water. These jars were then sonicated for 1 hour to re-suspend particulates. Each filter was thoroughly rinsed with filtered DI water at least three times, and all the

water and rinsate were then filtered through a 0.2 μm gridded membrane filter (Whatman). These filter papers were transferred back into the plastic Petri dish, which had been air-blasted to remove any remaining particulate matter, and stored until visual identification.

2.4 Visual Identification of Microparticles

Once microparticles were extracted, the samples were analyzed under a stereomicroscope (Olympus SZX7 and Motic SMZ-171) and every particle identified (except for water samples, see below). Suspected microplastics were picked from the sample and mounted onto double-sided tape, numbered, and identified by morphology and colour (Rochman et al., 2019; Table 2.2). For some, the colour was faded but any hint of colour was used to classify it. Any interesting characteristics or suspected sources of the particle (e.g., suspected tire wear particles) were also noted. Cross-validation between observers was conducted; I reviewed all particles picked for a subset of samples completed by each observer to ensure uniform classification of morphology and colour. All particles remaining after blank subtraction (detailed below) were photographed and the longest length and widest width (±0.1 μm) measured using the Olympus Lumenera program (Teledyne Lumenera INFINITY ANALYZE v6 and v7). A small number of particles were lost during transfer to the double-sided tape and consequently were not measured.

Table 2.2 Microplastic morphology classification. Adapted from (Rochman et al., 2019). Note that pellets were not found in the current study. Red scale line indicates 100 µm.

Morphology	Description	Example Image	Morphology	Description	Example Image
Fiber	Long and thin, even thickness throughout. Flexible but resistant to breakage.		Fragment	Rigid pieces of plastic, irregular in shape. Film and Foams can also be considered fragments.	
Fiber Bundle	3+ fibers tangled together. Shape is not long and thin like a fiber, fibers could not be untangled.		Film	Thin, flat and malleable. Tend to be translucent due to thin plastic.	
Sphere	Perfectly round in shape, typically very smooth.		Foam	Soft and compressible, often porous.	
Pellet	Rounded or cylindrical shape, typically larger than spheres.	*did not find any pellets in current study			

Due to time constraints, water filters were subsampled and only some of the mussel gill replicates were completed. For the water, 20% of the total area (30 grids out of ~150) of each filter was analyzed. Grids were chosen at random, although grids on the edge of the filter were not analyzed. Once a grid had been sampled, it was marked to ensure it was not re-analyzed. The number of particles found was then multiplied by 5 to represent particles on the entire filter, assuming particle distribution was homogeneous. A subset of 10 filters were used to examine the subsampling efficiencies and a comparison of the counts for 20% and 100% of the filter found relative percent differences (RPDs) of 22.2 to 102.0%, indicating sub-sampling led to an overestimation of the number of particles per filter (Appendix Table A1). Gill samples from West Montrose (WMT), Ayr

(AR-U and AR-D), and York (YK) were not analyzed, as inefficiencies in the digestion phase made particle counts and identification challenging. As such, eight replicates from each of Hespeler downstream (HE-D), Caledonia upstream (CD-U) and downstream (CD-D) and ten replicates from Hespeler upstream (HE-U) were analyzed.

2.5 Chemical Identification of Microparticles

Chemical confirmation is needed to determine if the particles found are plastic, as this cannot be determined with visual identification alone (Ivar do Sul, 2021). This analysis has not yet been completed, so suspected microplastics are referred to as microparticles herein. It is likely that a subset of these particles are composed of natural materials, although they may be anthropogenically modified, and that the abundance of microparticles discussed herein likely overestimates the abundance of microplastic particles.

2.6 Quality Assurance

Due to the ubiquitous nature of microplastics, caution was taken to limit contamination of samples (Miller et al., 2021; Munno et al., 2023). Surfaces were always sprayed with 70% ethanol and wiped down with a Kimwipe before handling any samples. Glass or metal materials were used instead of plastic whenever possible. Water used throughout was de-ionized water filtered through 0.45 μm GN-6 MCE filters (Pall) to ensure to no particles within our target size range were introduced. Tissues were stored in aluminum foil pouches that were ashed at 500ºC for 3 hours. Samples were kept covered as much as possible to limit deposition of particulate matter. A portable high efficiency purified air (HEPA) filter was used to limit airborne contamination in the microscope

room. To limit contamination from clothing, researchers wore natural clothing fibers as opposed to synthetic clothing when possible and recorded the colour and composition of clothing worn each day. Lab coats worn were 100% cotton and dyed a green colour to better trace contamination. If a green particle was found in a sample and was suspected to be from the lab coat, this particle was picked for chemical confirmation, but was subtracted from the total number of particles during the blank-correction step.

Blanks were used to account for contamination from the field and during sample processing (Miller et al., 2021). Blanks were collected as described above and underwent the same processing at the same time as the samples from the same sites. The total number of each particle type (morphology and colour) was determined for the blanks, and the same number of each particle type was subtracted from samples from the corresponding site and sample type. All particle counts presented herein have been blank-corrected. A summary of the particles subtracted from each site and sample type can be found in the Appendix (Table A2).

2.7 Data Analysis

Statistical analyses were completed using R software (version 4.2.0). All plots were generated using the ggplot2 package. During statistical analyses, mean particle counts were transformed (log(x+1)) due to low counts and frequent zeros in the dataset (Germanov et al., 2019).

Data were first analyzed for normality by visually examining both the density plot and QQ-plot of the values and checked using the Shapiro-Wilks test. An analysis of

variance (ANOVA) was used to determine if there was spatial variation across sites in transformed microparticle counts in each matrix when the data were normally distributed. If significant variation was observed, a post-hoc Tukey honest significance difference (HSD) test was performed to identify pairwise differences between sites. If the data were not normally distributed or there was not equal variance, a Kruskal-Wallis test and post-hoc Dunn's test (with Bonferroni correction) were done instead.

To assess differences in microparticle counts between upstream and downstream sites at each WWTP-impacted location, ANOVAs (for 3 sites including far-field; fingernail clams only) or independent *t*-tests (for 2 sites) were done for each sample matrix. The assumptions were confirmed by determining if the data were normally distributed (as above) and if there was equal variance by calculating the F-statistic. If the variance was not equal, Welch's *t*-test was used instead.

Log-linear regressions were used to examine the relationships between the number of microparticles found and size or age of the organism. Pearson's correlations were used to determine if there was a correlation between mean transformed microparticle counts (at each site) in each bivalve tissue type and in water samples.

3 RESULTS

3.1 Microparticles in Fingernail Clams

3.1.1 Size Distribution of Fingernail Clams

Mean fingernail clam length (overall mean 8.60 ± 0.80 mm) ranged from 6.45 mm downstream of Ayr (AR-D) to 10.37 mm at Caledonia far-field (CD-F), with significant

variation across all sites (ANOVA $F_{7,32}$= 5.326, p<0.0001; Figure 3.1A; Appendix Table A3). Fingernail clams were longer downstream of Caledonia (CD-D) compared to Hespeler downstream (HE-D) and Ayr downstream (AR-D), and longer at West Montrose (WMT), Caledonia upstream (CD-U), and York (YK) compared to Ayr downstream (AR-D); overall, clams tended to be larger in the main branch compared to the tributaries (p-values in Appendix Table A4). When examining differences in mean clam length at each WWTP, fingernail clams were shorter downstream of Ayr compared to upstream (p=0.01). There were no significant differences in length between upstream, downstream, and far-field sites at Hespeler (p=0.7) or at Caledonia (p=0.5).

Similar spatial patterns were observed in individual fingernail clam total weight (including shell; overall mean 186.51 ± 56.46 mg), ranging from 72.48 mg at Ayr downstream (AR-D) to 324.25 mg at Caledonia far-field (CD-F) (Figure 3.1B; Appendix Table A3). There was significant variation across sites (ANOVA $F_{9.40}$=6.682, p<0.0001). As for length, clams collected from downstream of Caledonia (CD-D and CD-F) were heavier than those at Hespeler upstream (HE-U) and Ayr downstream (AR-D), and clams at West Montrose (WMT), Caledonia upstream (CD-U), and York (YK) were heavier than at Ayr downstream (AR-D), again indicating clams tended to be larger in the main branch (p-values in Appendix Table A4). In contrast to clam length, there were no significant differences in weight at upstream, downstream, and far-field sites of each WWTP (p=0.7, 0.08, and 0.8, respectively for Hespeler, Ayr, and Caledonia), although there appeared to be more variation in clam size downstream of Ayr and Caledonia.

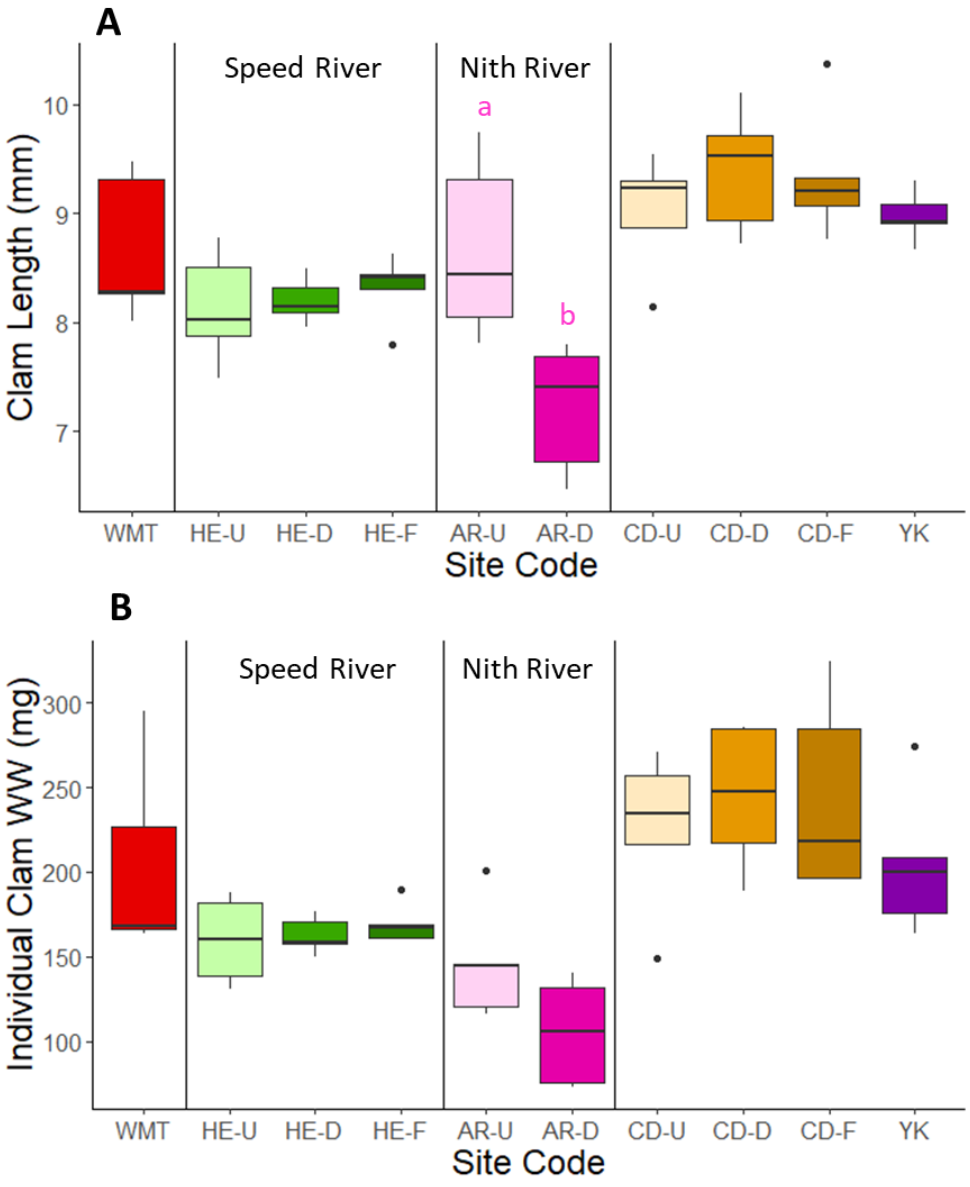


Figure 3.1 Average lengths (A, in mm) and wet weights (B, in mg including shells) of individual fingernail clams collected from sites in the Grand River watershed, ON, in August 2021 (AR-U and AR-D were sampled September 2022). Weight reported as mass per individual clams (total wet weight/number of clams). Sites are ordered generally from most upstream (left) to downstream (right), including panels to denote tributaries. Lighter colours represent the upstream sites, darker colours the downstream sites, and the darkest colours the far-field sites. Different letters denote statistical differences between upstream (U), downstream (D), and far-field (F) sites at each WWTP (n=5 composite samples of 10 clams/site, unpaired t-test and ANOVA).

3.1.2 Microparticle Abundance and Spatial Distribution in Fingernail Clams

All but one replicate of fingernail clams contained microparticles (98%), with a maximum of 50 found in a single replicate (5 per clam) at Hespeler upstream (HE-U; Figure 3.2). On average, 2.13 ± 1.76 particles/60 mg wet tissue mass (approximate # of particles per clam) were found (Table 3.1) with a total of 740 particles found after blank correction. There was significant variation in particle counts across sites (ANOVA $F_{9,40}$=5.371, p<0.0001). Counts tended to be higher in the tributaries, Hespeler downstream and far-field (HE-D and HE-F) as well as Ayr upstream and downstream (AR-U and AR-D), compared to other sites (p-values in Appendix Table A5). At each WWTP, there were no differences between particle counts at the upstream, downstream, or far-field sites (p=0.9, 0.1, and 0.2, respectively, at Hespeler, Ayr, and Caledonia).

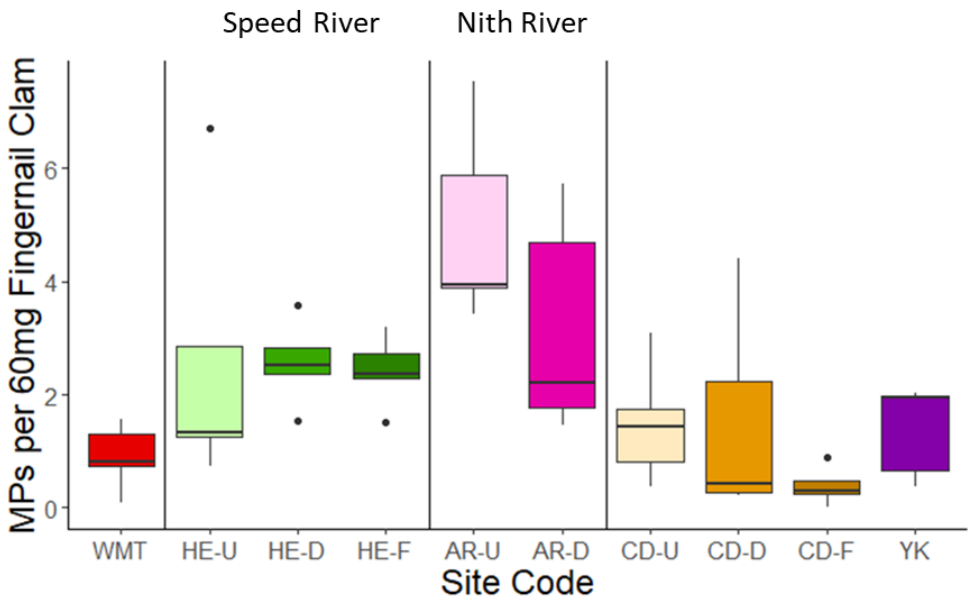


Figure 3.2 Spatial patterns of microparticle counts in fingernail clams collected from sites in the Grand River watershed, ON, in August 2021 (AR-U and AR-D were sampled September 2022). Microparticle counts shown in count/60 mg, as 60 mg was the approximate wet mass of soft tissue in a single fingernail clam. Sites are ordered generally from most upstream (left) to downstream (right), including panels to denote tributaries. Lighter colours represent the upstream (U), darker colours represent the downstream (D), and the darkest colours represent the far-field (F) sites. No statistical differences were observed between upstream, downstream, and far-field at each WWTP (n=5 composite samples of 10 clams/site, unpaired t-test and ANOVA).

Table 3.1 Summary of microparticles observed across all sample types collected from the Grand River watershed, ON, in August 2021 (some water samples were collected November 2021 and some fingernail clams September 2022). Summary includes total number of particles found, the percentage of samples containing microparticles, the range in raw counts of plastics observed, the average number of particles ± SD per g or mL in fingernail clams (n=5/site), mussel gills (n=8-10/site), mussel digestive gland (n=10/site), mussel hemolymph (n=9-10/site), and surface water samples (n=3/site), and the range of particles found corrected for tissue mass/volume. Surface water samples are further categorized into four size fractions (italicized), in decreasing size from 500 µm to 40 µm.

Sample Type	Total Particles Found	% Samples with Particles	Range (total number of particles in sample)	Average Number of Particles (± SD)	Range (number of particles corrected for size)
Fingernail Clams	740	98%	0 – 50	35.5 ± 29.4 particles/g	0 – 125.4 particles/g
Mussel Gills	601	94.1%	0 – 74	4.3 ± 4.4 particles/g	0 - 19.9 particles/g
Mussel Digestive Gland	836	100%	1 – 28	6.5 ± 8.1 particles/g	0.4 – 57.8 particles/g
Mussel Hemolymph	485	89.7%	0 – 36	4.3 ± 4.2 particles/mL	0 – 24 particles/mL
Surface Water Total	577	100%	25 – 210	0.0055 ± 0.0028 particles/mL (5.5 ± 2.8 particle/L)	0.0013 – 0.0105 particles/mL (1.3 – 10.5 particles/L)
533.40 µm	*134*	*95.8%*	*0 – 55*	*0.0010 ± 0.00080 particles/mL*	*0 – 0.0028 particles/mL*
228.60 µm	*125*	*91.7%*	*0 – 75*	*0.0012 ± 0.0011 particles/mL*	*0 – 0.0038 particles/mL*
116.84 µm	*203*	*100%*	*5 – 140*	*0.0020 ± 0.0017 particles/mL*	*0 – 0.0070 particles/mL*
35.56 µm	*115*	*83.3%*	*0 – 90*	*0.0012 ± 0.0014 particles/mL*	*0 – 0.0045 particles/mL*

3.1.3 Microparticle Morphology, Colour, and Size in Fingernail Clams

Particle morphology across all samples was dominated by fibers (93.2%), with some fragments (5.7%), and few films and fiber bundles (<1% respectively). The relative abundance of morphologies was similar among sites, with the exception of Ayr (AR-U and AR-D) where samples were composed of almost entirely fibers (Figure 3.3A). Particle colours were dominated by clear (58.8%) and blue (18.4%), but many grey, black, pink, and red particles were also found across all sites (between 2-7%; Figure

3.3B). Within sites, between 6 and 11 colours were found, with the most diversity in colours observed at Caledonia downstream (CD-D).

Mean particle length and width across all samples was 1290.0 ± 1256.6 μm and 36.7 ± 79.8 μm, respectively (Table 3.2). There was no significant variation in particle length across sites (Kruskal-Wallis, χ^2=11.79 p=0.2), but particles at Hespeler and Caledonia downstream (HE-D and CD-D) were significantly wider than particles at Ayr upstream (AR-U; Kruskal-Wallis, χ^2= 24.216, p=0.004). Despite these differences, there were no differences observed between microparticle lengths or widths upstream and downstream at each WWTP (p=0.09, 0.1, 0.5 for length; p=0.3, 0.2, 1 for width, respectively, at Hespeler, Ayr, and Caledonia).

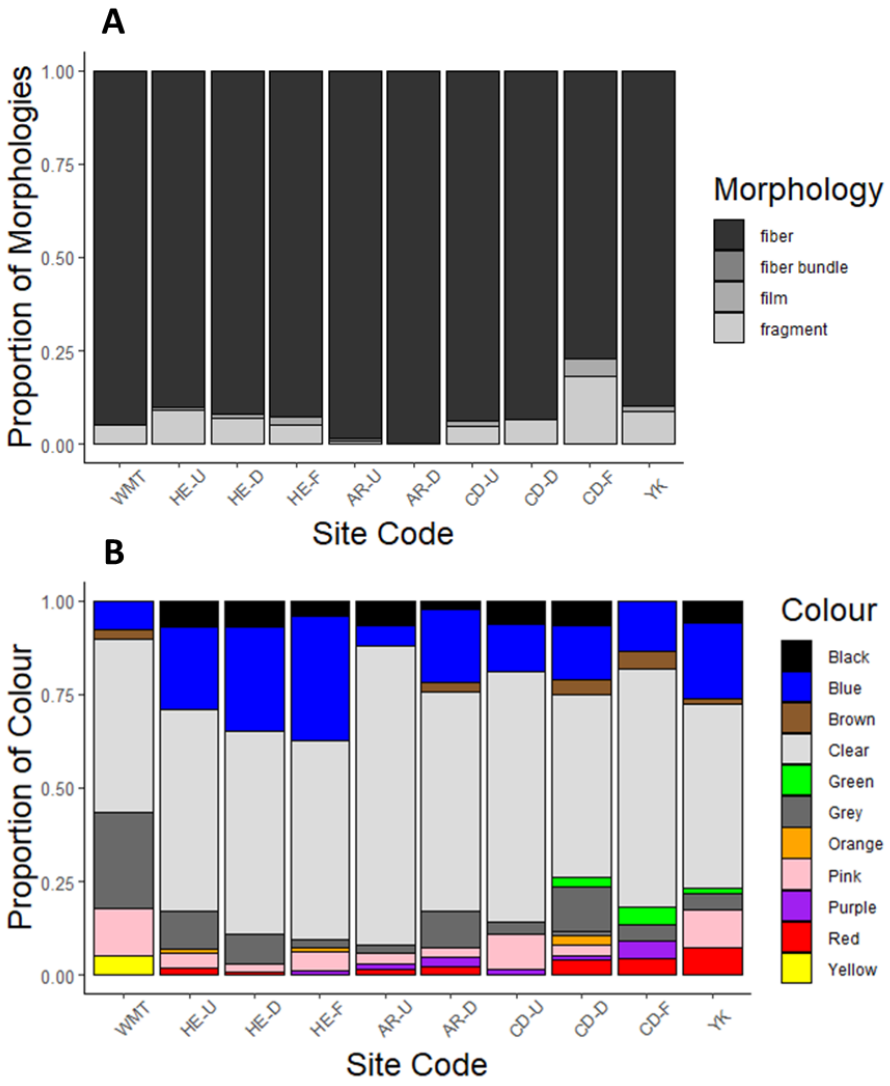


Figure 3.3 Proportion of each particle morphology (A) and colour (B) across sites in fingernail clam samples collected from sites in the Grand River watershed, ON, in August 2021 (AR-U and AR-D were sampled September 2022). Sites are ordered from most upstream (left) to downstream (right). Each morphology and colour are represented as a proportion of the total found at each site (n=5 composite samples of 10 clams/site).

Table 3.2 Mean particle size in fingernail clam tissues collected from sites in the Grand River watershed, ON, in August 2021 (AR-U and AR-D sampled September 2022). Mean particle length ± standard deviation (in µm), mean particle width ± standard deviation (in µm), and percent of particles measured at each site as well as overall mean. Sites are ordered generally from most upstream (top) to downstream (bottom).

Site Code	Particle Length (µm)	Particle Width (µm)	Percent Measured (%)
WMT	1231.8 ± 1027.3	30.3 ± 43.8	100
HE-U	1099.5 ± 784.0	41.8 ± 79.3	100
HE-D	1502.3 ± 1608.9	43.8 ± 94.7	100
HE-F	1352.7 ± 1023.6	46.7 ± 111.6	100
AR-U	1066.4 ± 734.8	18.6 ± 18.0	100
AR-D	1317.0 ± 966.2	26.9 ± 42.6	100
CD-U	1313.6 ± 1050.8	42.1 ± 102.5	98.4
CD-D	1146.5 ± 889.0	32.4 ± 50.7	100
CD-F	1922.7 ± 3445.2	99.6 ± 168.9	100
YK	1560.9 ± 1689.2	29.4 ± 56.5	98.6
Mean	**1290.0 ± 1256.6**	**36.7 ± 79.8**	**99.7**

3.1.4 Relationship between Microparticle Count and Size of Fingernail Clam

There were no significant log-linear relationships between total microparticle counts and the mean length or weight of fingernail clam in each composite sample ($p=0.4$ for length; $p=0.1$ for weight; Figure 3.4; Table A6). Microparticle counts showed a slight decrease with increasing size of the clam.

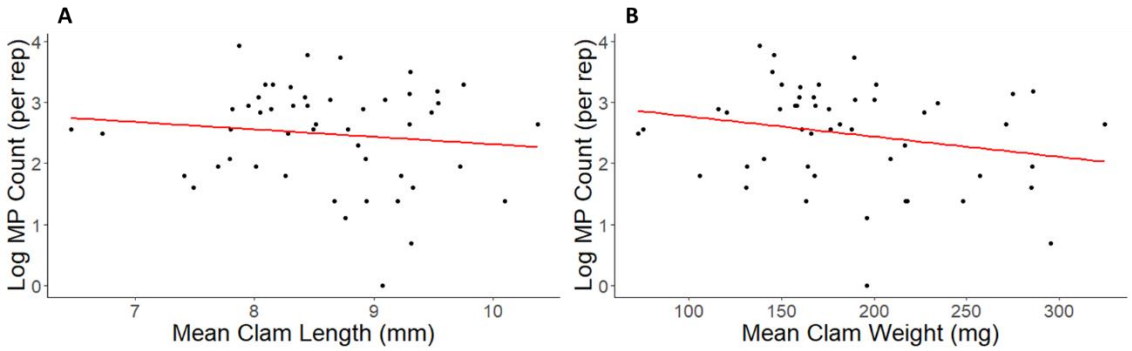


Figure 3.4 Log-linear relationship between microparticle counts and mean length (A) and wet weight (B, including shells) of fingernail clams collected from the Grand River watershed, ON, in August 2021 (some were sampled September 2022). Total microparticle counts (transformed log(x+1)) per replicate are shown, not corrected for mass. Relationships were not significant (Linear Model).

3.1.5 Correlation of Particle Counts in Fingernail Clams, Mussel Tissues, and Water Samples

Mean microparticle counts per site in fingernail clams were negatively correlated with those in mussel digestive gland tissues (p=0.03), but no correlation was observed when compared to mussel gills (p=0.8) or hemolymph (p=0.1; Appendix Table A7). No correlation was found between mean particle counts per site in fingernail clams and surface water samples (p=0.5).

3.2 Characteristics of Freshwater Mussels

Flutedshell mussel length ranged from 75.8 mm at Ayr downstream (AR-D) to 138.43 mm at West Montrose (WMT), with significant variation across sites (ANOVA $F_{7,72}$=6.098, p>0.0001; Appendix Table A8; Figure 3.5A). Mussels were longer at Caledonia (CD-U and CD-D) and York (YK) compared to West Montrose (WMT), Hespeler (HE-U and HE-D), and Ayr downstream (AR-D); overall, there were larger

mussels further downstream in the watershed (p-values can be found in Appendix Table A9). When comparing upstream and downstream at each WWTP, there were no significant differences in mussel length (p=0.7, 0.6, and 0.2, respectively, at Hespeler, Ayr, and Caledonia), although there was more variation in length downstream at all treatment plants.

Mussel wet weight ranged from 39.2 g at Ayr downstream (AR-D) to 402.5 g at West Montrose (WMT), with significant variation across sites (Kruskal-Wallis χ^2=34.757, p>0.0001; Appendix Table A8; Figure 3.5B). Mussels were heavier at Caledonia (CD-U and CD-D) and York (YK) compared to West Montrose (WMT) and Hespeler (HE-U and HE-D); overall, results indicated that mussels also weighed more at sites further downstream in the watershed (p-values can be found in Appendix Table A9). Interestingly, mussels at Ayr did not differ in weight compared to other sites (p>0.05) which was seen in mussel length. When comparing upstream and downstream at each WWTP, there were no significant differences in mussel weight (p=0.5, 1.0, and 0.2, respectively, at Hespeler, Ayr, and Caledonia), although there was more variation in size downstream at Hespeler and Ayr.

Mussels ranged in age from 6 years at several sites to 21 at West Montrose (WMT), although there was no significant variation across sites (Kruskal-Wallis χ^2=11.021, p=0.1; Appendix Table A8; Figure 3.5C), nor any differences in age when comparing upstream to downstream at each WWTP (p=0.8, 0.9, and 0.7, respectively, at Hespeler, Ayr, and Caledonia). No linear relationship between mussel age and length was observed (LM, $F_{1,77}$=3.008, p=0.09, R^2=0.0251). A slightly positive relationship was

observed between age and length, however the slope of this relationship differed by site when mussels were grouped (Appendix Figure A1).

At most sites, approximately 50% of the mussels collected were gravid and brooding glochidia, with Caledonia upstream (CD-U) having the most gravid mussels at 70% (Figure 3.5D). In contrast, only 1 gravid mussel was collected at Ayr downstream (AR-D), although this sampling was done later in the season.

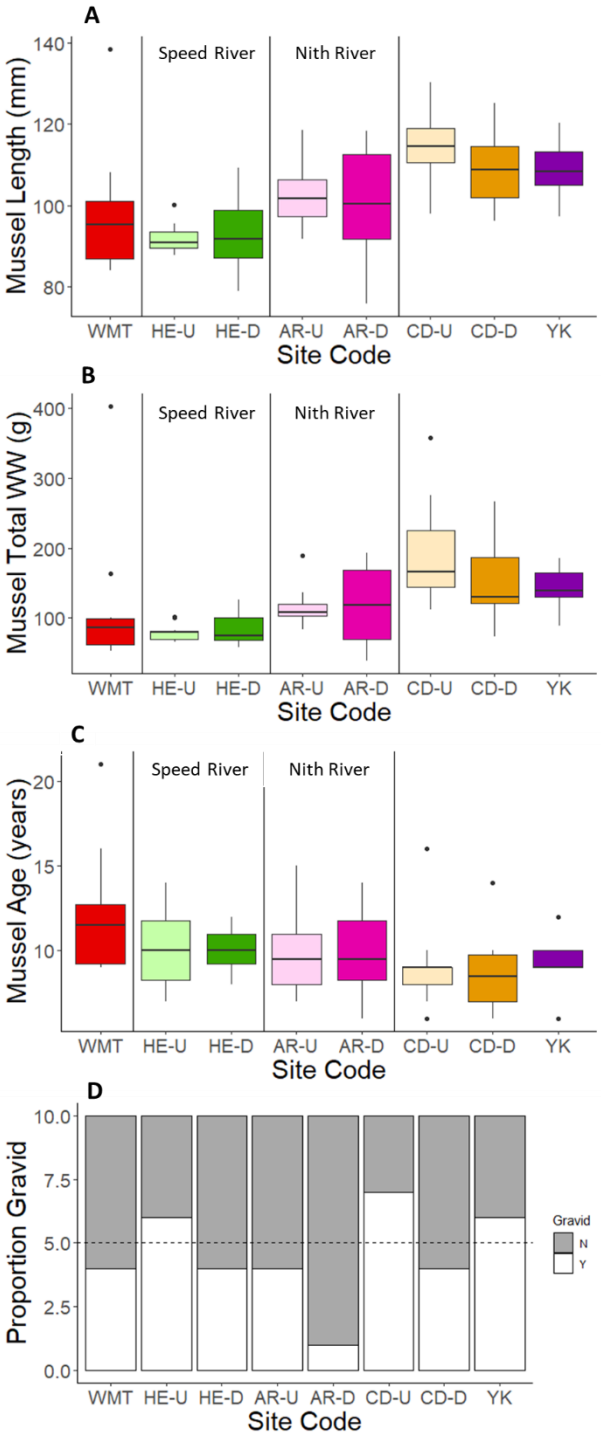


Figure 3.5 Characteristics of mussels collected from sites in the Grand River watershed, ON, in August 2021. A. Mean length of mussels in mm, **B**. Mean wet weight of mussels in g (including shell), **C**. Mean age of mussel in years, and **D**. Proportion of

gravid mussels. Sites are ordered generally from most upstream (left) to downstream (right), including panels to denote tributaries. In panels A-C, lighter colours represent the upstream sites (U) and darker colours represent the downstream sites (D). In panel D lighter colours represent gravid mussels. There were no statistically significant differences between upstream and downstream at each WWTPs in panels A-C (n=10/site, unpaired t-test).

3.3 Microparticles in Mussel Gill Tissues

3.3.1 Microparticle Abundance and Spatial Distribution in Mussel Gill

Microparticles were found in all but 2 gill samples (94%), with a maximum of 74 found in a single sample at the Caledonia downstream (CD-D) site for a total of 602 particles found across all samples analyzed. On average, 4.31 ± 4.37 particles/g were found per replicate (Table 3.1). There was significant variation in microparticle counts across sites (Figure 3.6; ANOVA $F_{3,30}$=7.947, p=0.0005), where Caledonia downstream had significantly higher counts than Hespeler upstream (Tukey's HE-U vs CD-D, p=0.0002), and Hespeler downstream had higher counts than upstream (Tukey's HE-U vs HE-D, p=0.04); showing an increase in particle counts further downstream in the watershed. When comparing sites at each WWTP, microparticle counts were higher downstream compared to upstream at both Hespeler (p=0.04) and Caledonia (p=0.002).

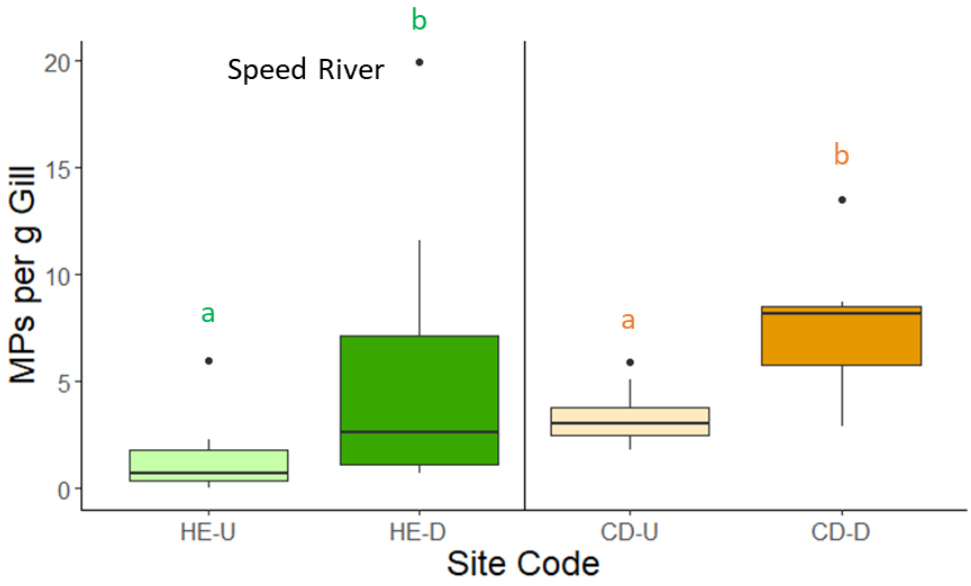


Figure 3.6 Spatial patterns of microparticles (counts/g) in mussel gill tissues collected from sites in the Grand River watershed, ON, in August 2021. Sites are ordered from most upstream (left) to downstream (right), including a panel to denote the Speed River tributary. Lighter colours represent the upstream site (U) while darker colours represent the downstream sites (D) at each WWTP. Different letters represent statistical differences between upstream and downstream at each WWTP (n=8-10/site, unpaired t-test).

3.3.2 Particle Morphology, Colour, and Size in Mussel Gill

Across all gill samples, particle morphology was dominated by fibers (95.5%) with few fragments (2.8%) and films (1%) (Figure 3.7). There was more variety in the morphologies at both Hespeler sites (HE-U and HE-D), including fragments and some films and fiber bundles, when compared to the Caledonia sites (CD-U and CD-D) which were composed almost entirely of fibers. When all samples were combined, microparticle colours were dominated by clear (65.2%) and blue (22.5%), with few red, black, and grey (1-5%) ones. Within sites, between 5 and 7 colours were found, with more variety of

colours found at Hespeler compared to Caledonia, which was dominated by clear particles.

The particles found in gill tissues had a mean length of 1481.5 ± 1366.4 μm and width of 27.4 ± 35.2 μm (Table 3.3). There were no among-site significant differences in particle length (Kruskal-Wallis χ^2= 2.0084, p=0.6). However, there were differences in particle widths across sites (Kruskal-Wallis χ^2=9.2389, p=0.03) where particles were widest at Hespeler downstream compared to Hespeler upstream (Dunn's HE-D vs HE-U, p=0.03) and Caledonia upstream (Dunn's HE-D vs CD-U, p=0.03). When comparing particle size upstream to downstream, no differences in length were observed at either WWTP (p=0.8 and 0.4, respectively, at Hespeler and Caledonia). There were no differences in width at Caledonia (p=0.2), but particles were wider at Hespeler downstream compared to upstream (p=0.02).

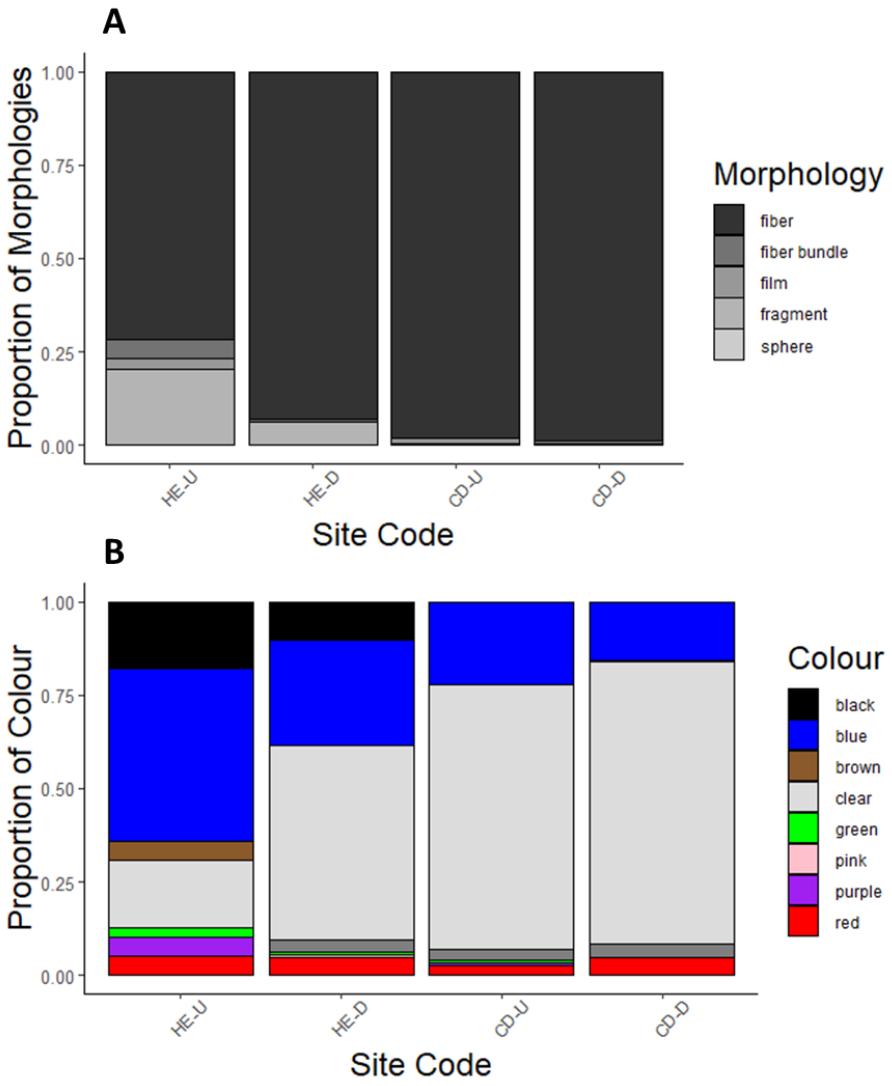


Figure 3.7 Proportion of each particle morphology (A) and colour (B) across sites in mussel gills collected from sites in the Grand River watershed, ON, in August 2021. Sites are ordered from most upstream (left) to downstream (right). Each morphology and colour are represented as a proportion of the total found at each site (n=8-10/site).

Table 3.3 Mean particle size in mussel tissues collected from sites in the Grand River watershed, ON, in August 2021. Mean particle length ± standard deviation (in µm), mean particle width ± standard deviation (in µm), and percent of particles measured at each site as well as overall mean. Particle sizes separated based on mussel tissue type (gill, digestive gland, and hemolymph). Sites ordered generally from most upstream (top) to downstream (bottom).

Site Code	Mussel Gills			Mussel Digestive Gland			Mussel Hemolymph		
	Particle Length (µm)	Particle Width (µm)	Percent Measured (%)	Particle Length (µm)	Particle Width (µm)	Percent Measured (%)	Particle Length (µm)	Particle Width (µm)	Percent Measured (%)
WMT	N/A	N/A	N/A	884.4 ± 960.6	30.3 ± 30.7	92.3	969.8 ± 914.9	32.0 ± 45.9	94.4
HE-U	1442.4 ± 1744.2	48.4 ± 58.5	97.4	1372.4 ± 2017.3	40.5 ± 47.3	86.6	1294.6 ± 1950.4	29.7 ± 19.6	97.1
HE-D	1511.9 ± 1377.1	25.5 ± 21.2	74.0	1148.6 ± 1085.5	29.4 ± 29.6	95.5	729.5 ± 543.32	46.5 ± 48.2	95.8
AR-U	N/A	N/A	N/A	1212.5 ± 1242.5	33.7 ± 40.8	97.9	1070.6 ± 1947.0	48.4 ± 58.7	91.9
AR-D	N/A	N/A	N/A	1719.3 ± 2829.2	25.8 ± 23.8	100	756.3 ± 983.4	45.2 ± 53.3	97.4
CD-U	1560.3 ± 1467.4	28.8 ± 52.2	58.0	1009.5 ± 1495.6	33.7 ± 49.3	99.4	1137.3 ± 2006.7	30.1 ± 26.3	90.7
CD-D	1420.7 ± 1196.2	22.9 ± 10.4	73.6	1175.1 ± 1546.6	36.8 ± 40.1	91.9	836.2 ± 1018.3	38.0 ± 33.29	79.8
YK	N/A	N/A	N/A	1020.5 ± 1993.4	51.2 ± 54.5	99.3	830.4 ± 771.2	37.6 ± 63.3	61.7
Mean	**1481.5 ± 1366.4**	**27.4 ± 35.2**	**70.0**	**1107.1 ± 1636.2**	**36.3 ± 43.1**	**95.5**	**931.9 ± 1265.7**	**37.5 ± 48.0**	81.4

3.3.3 Relationships between Microparticle Count in Gills and Size of Mussel

Microparticle counts increased with mussel lengths or weights, although this relationship was only significant for mussel length and the fit was poor (length $p=0.02$, $R^2=0.12$; weight $p=0.07$; Figure 3.8; Appendix Table A6). A slight negative relationship was observed between particle counts and mussel age, although it was not significant ($p=0.3$).

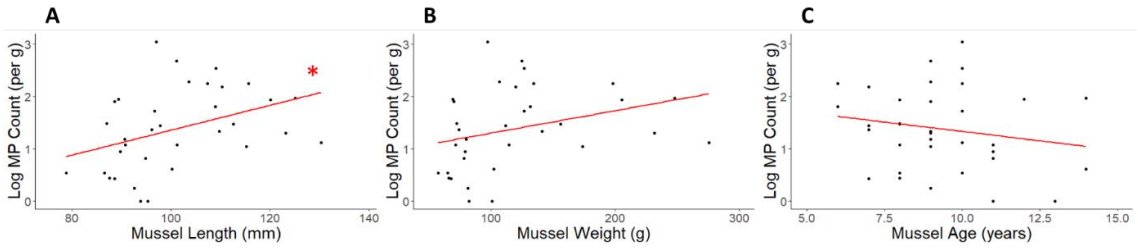


Figure 3.8 Log-linear relationship between microparticle counts (counts/g) in mussel gills and mussel lengths (A), total wet weight (B), and age (C) collected from sites in the Grand River watershed, ON, in August 2021. Microparticle counts (transformed (log(x+1)) per g of gill tissue are shown. Significant relationships denoted with * (Linear Model).

3.3.4 Correlation of Particle Counts in Mussel Gill and Water Samples

There was no significant correlation between mean particle counts per site in mussel gill tissues and surface water samples (p=0.3; Appendix Table A7).

3.4 Microparticles in Mussel Digestive Gland Tissues

3.4.1 Microparticle Abundance and Spatial Distribution in Mussel Digestive Gland

All digestive gland samples contained microparticles and 28 was the most found in a single sample at the Caledonia upstream (CD-U) site. On average, samples contained 6.54 ± 8.12 particles/g, the highest counts observed across all three mussel tissues examined, for a total of 836 particles (Table 3.1). Particle counts varied across sites (Figure 3.9; ANOVA $F_{7,72}$= 4.831, p=0.0002), where Ayr upstream and downstream had significantly lower counts than West Montrose (Tukey's, AR-U vs WMT, p=0.0002; AR-D vs WMT, p=0.0001). There were no differences between counts upstream and downstream when looking at each individual WWTP (p=0.8, 0.9, and 0.7, respectively, at Hespeler, Ayr, and Caledonia).

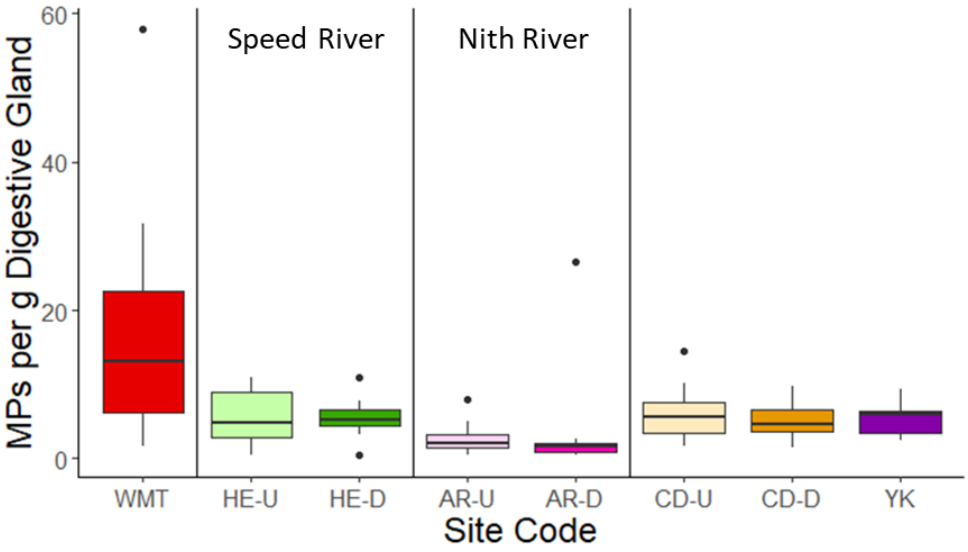


Figure 3.9 Spatial patterns of microparticles (counts/g) in mussel digestive gland tissues collected from sites in the Grand River watershed, ON, in August 2021. Sites are ordered generally from most upstream (left) to downstream (right), including panels to denote tributaries. Lighter colours represent the upstream site (U) while darker colours represent the downstream sites (D). There were no statistical differences between upstream and downstream at any of the WWTPs (n=10/site, unpaired t-test).

3.4.2 Particle Morphology, Colour, and Size in Mussel Digestive Gland

Across all digestive gland samples, particle morphology was dominated by fibers (79.8%), with some spheres (9.6%), fragments (7.8%), and films (2.6%). Patterns of microparticle morphologies were similar among sites, although there tended to be more fibers downstream of WWTPs (Figure 3.10A). As seen in the gill samples, colours were dominated by clear (61.2%) and blue (18.9%), but higher percentages of black (9.3%), brown (3.6%), and red (3.1%) microparticles were also found in the digestive gland (Figure 3.10B). At each site, between 6 to 10 colours were found, with more variability in the proportion of colours at sites upstream in the watershed whereas particles were mostly clear and blue further downstream at Caledonia and York. Clear spheres were only found

in mussel digestive gland samples, and they were present at all sites except Ayr downstream (AR-D).

Particles had a mean length of 1107.1 ± 1636.2 μm and a width of 36.3 ± 43.1 μm. There was significant variation in particle length (Kruskal-Wallis χ^2=23.357, p=0.001) and particle width (Kruskal-Wallis χ^2=24.79, p=0.0008) across all sites (Table 3.3). Particles were longest at Ayr downstream compared to Caledonia upstream (Dunn's AR-D vs CD-U, p=0.02), West Montrose (Dunn's AR-D vs WMT, p=0.005), and York (Dunn's AR-D vs YK, p=0.004). The spatial patterns differed for particle width, where Caledonia upstream had significantly smaller particle width compared to Hespeler upstream (Dunn's CD-U vs HE-U, p=0.01) and York (Dunn's CD-U vs YK, p=0.004). However, there were no pairwise differences in particle length or width between upstream/downstream sites at each WWTP (length p=0.4, 0.3, and 0.4; width p=0.1, 0.3, and 0.2, respectively, at Hespeler, Ayr, and Caledonia).

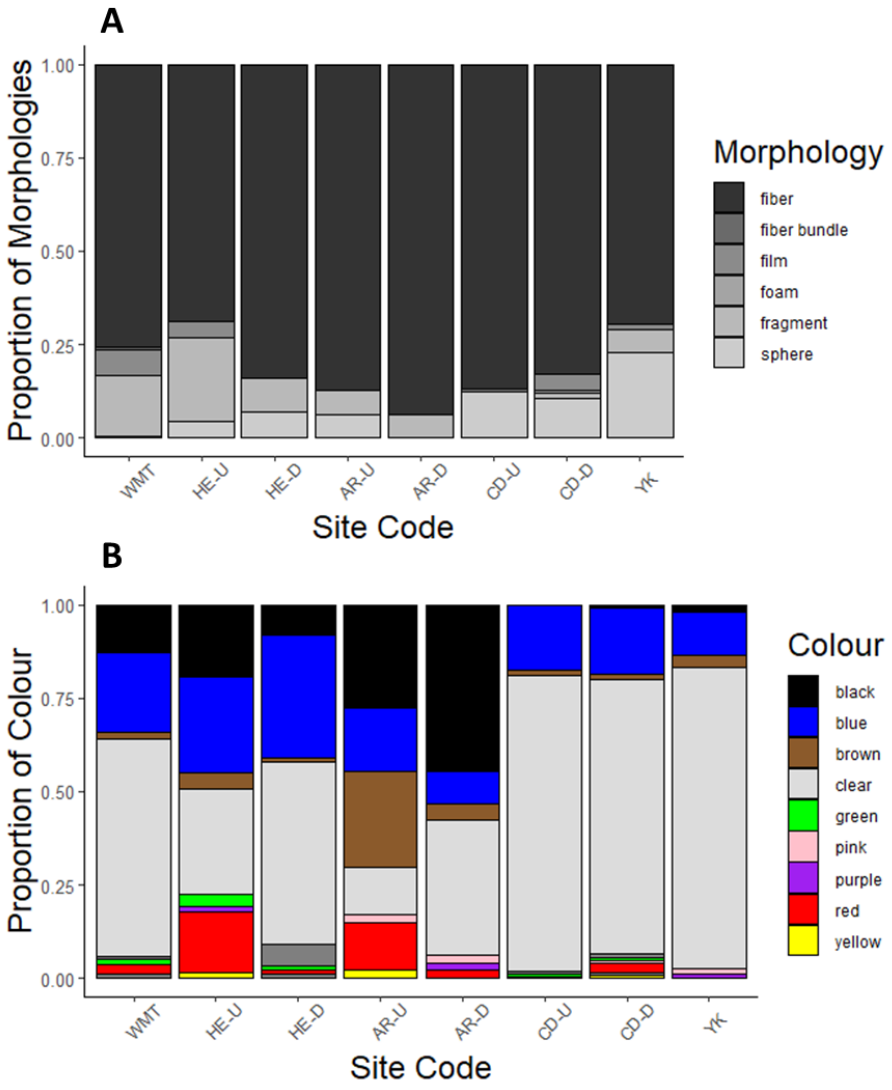


Figure 3.10 Proportion of each particle morphology (A) and colour (B) in mussel digestive gland samples collected from sites on the Grand River, ON, in August 2021. Sites are ordered generally from most upstream (left) to downstream (right). Each morphology and colour are represented as a proportion of the total found at each site (n=10/site).

3.4.3 Relationship between Particle Count in Digestive Gland and Size of Mussel

There was a significant negative relationship between particle counts in the digestive gland and mussel lengths or widths (Figure 3.11); however, there were poor fits

for these models (mussel length, p=0.04, R^2=0.04; mussel weight, p=0.03, R^2=0.07; Appendix Table A6). A very slight, but not significant, negative relationship between microparticle counts and mussel age was also observed (p=0.4).

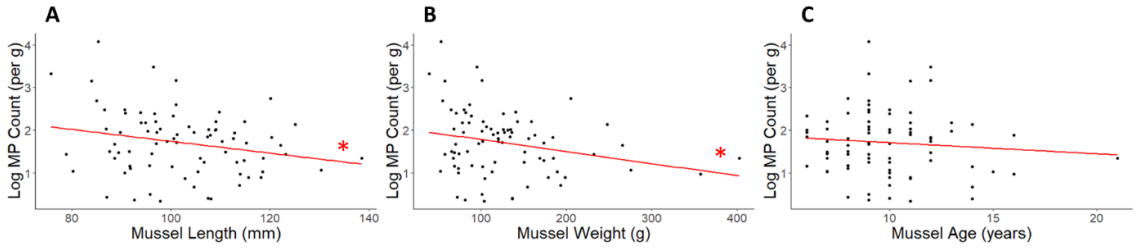


Figure 3.11 Log-linear relationships between microparticle counts (counts/g) in mussel digestive gland and length (A), weight (B), and age (C) of mussels collected from sites on the Grand River, ON, in August 2021. Microparticle counts (transformed (log(x+1)) per g of digestive gland tissue are shown. Significant relationships denoted with * (Linear Model).

3.4.4 Correlation of Particle Counts in Mussel Digestive Gland and Water Samples

There was no significant correlation between mean particle counts per site in mussel digestive gland and surface water samples (p=0.9, Appendix Table A7).

3.5 Microparticles in Mussel Hemolymph

3.5.1 Particle Abundance and Spatial Distribution in Mussel Hemolymph

Most hemolymph samples contained microplastics (89.7%), with 36 being the maximum number observed in a single sample at the Caledonia downstream site (CD-D; Table 3.1). On average, 4.28 ± 4.22 particles/mL were observed in each sample for a total of 485 particles (Figure 3.12). Particle counts varied across sites (Kruskal-Wallis χ^2=20.601, p=0.005), but the only pairwise differences were between Hespeler downstream or Ayr upstream to York (Dunn's HE-D vs YK, p=0.006; AR-U vs YK,

p=0.04). When comparing upstream to downstream at each WWTP, no significant differences were observed in microparticle counts (p=0.5, 0.8, and 0.5, respectively, at Hespeler, Ayr, and Caledonia).

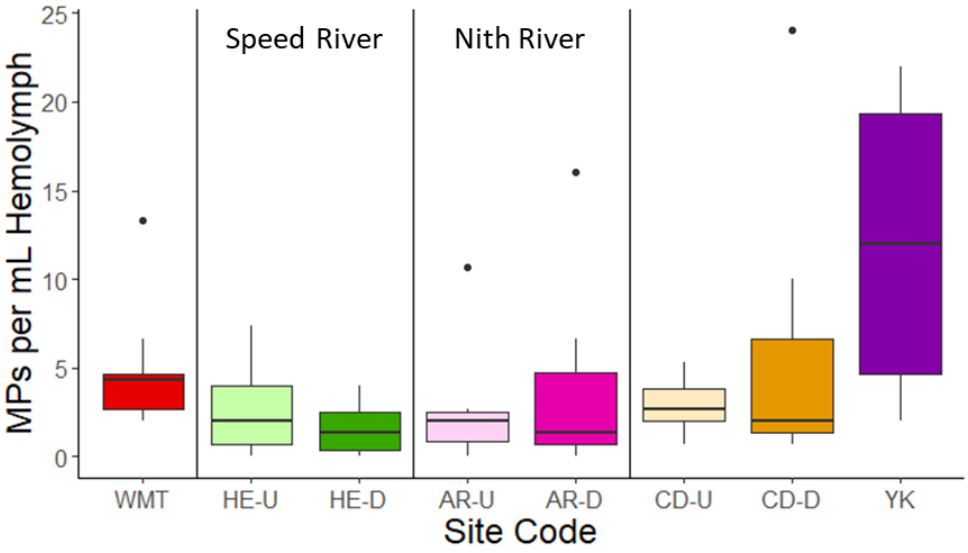


Figure 3.12 Spatial patterns of microparticles (counts/mL) in mussel hemolymph samples collected from sites in the Grand River watershed, ON, in August 2021. Sites are ordered generally from most upstream (left) to downstream (right), including panels to denote tributaries. Lighter colours represent the upstream site (U) while darker colours represent the downstream sites (D). There were no statistical differences between upstream and downstream at any of the WWTPs (n=9-10/site, unpaired t-test).

3.5.2 Particle Morphology, Colour, and Size in Mussel Hemolymph

Across all sites, particle morphology in mussel hemolymph was dominated by fibers (78.7%) and fragments (14.8%), with some films (4.9%) and fiber bundles (1.2%; Figure 3.13A). Within sites, fibers were dominant, but higher proportions of other morphologies were observed than in mussel gill and digestive gland samples, especially at the sites near WWTPs (both upstream and downstream) compared to West Montrose and York. As in other mussel tissues, microparticles were dominated by clear (48.9%),

blue (21.0%), and black (17.9%), with lower proportions of brown, red, white, and green (1-5%) across all sites (Figure 3.13B). Between 5 and 9 colours were observed at each site. At Hespeler, there were higher proportions of black and blue with fewer clear particles downstream compared to upstream, with an opposite trend at Caledonia with fewer blue, black, and brown particles and more clear particles. The proportions of colours were similar up- and downstream at Ayr.

Mussel hemolymph contained the smallest microparticles of all bivalve tissues with a mean length of 931.9 ± 1265.7 µm and width of 37.5 ± 48.0 µm (Table 3.3). There was no significant variation in particle length among sites (Kruskal-Wallis χ^2= 5.568, p=0.6). However, there was significant variation in particle width across sites (Kruskal-Wallis χ^2= 24.026, p= 0.001), where particles were less wide at York compared to Ayr upstream (Dunn's YK vs AR-U, p=0.02) and Caledonia downstream (Dunn's YK vs CD-D, p=0.005). No pairwise differences between upstream/downstream sites at each WWTP were observed for either particle length (p=0.1, 0.4, and 0.4, respectively, at Hespeler, Ayr, and Caledonia) or width (p=0.1, 0.8, 0.2, respectively, at Hespeler, Ayr, and Caledonia).

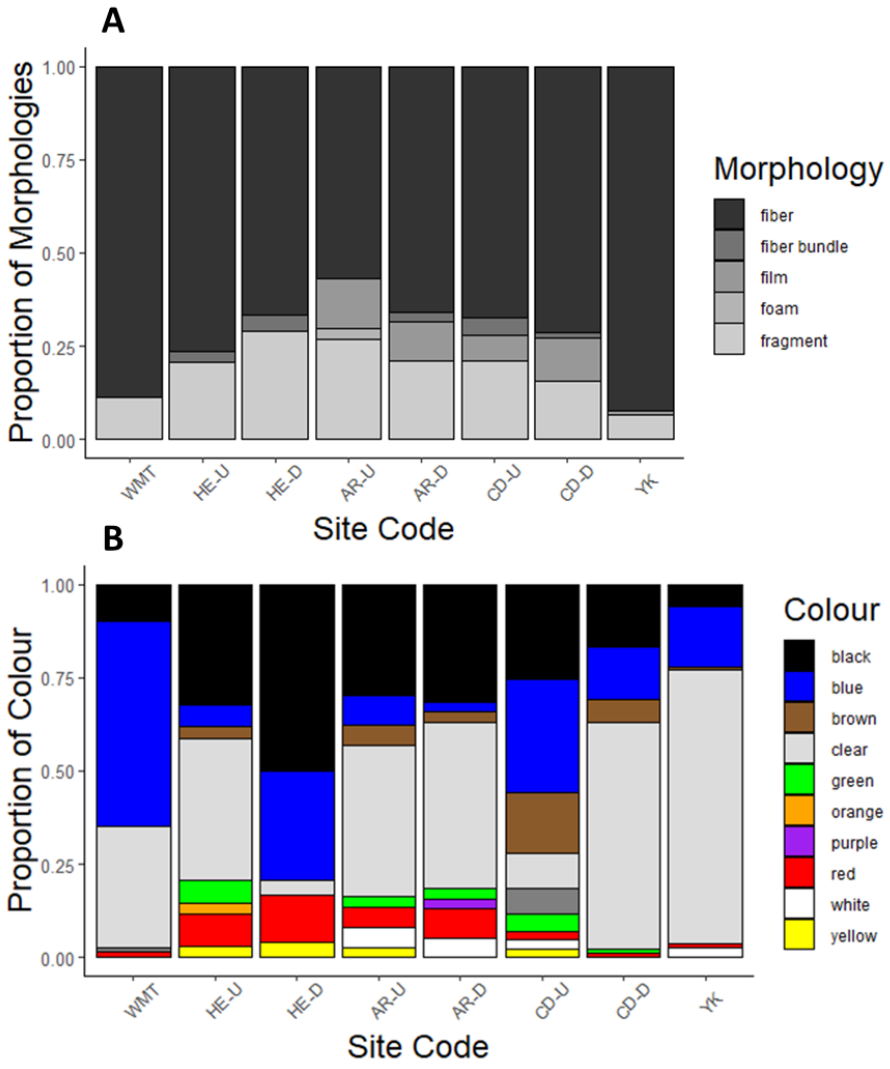


Figure 3.13 Proportion of each particle morphology (A) and colour (B) across sites in mussel hemolymph samples collected from sites in the Grand River watershed, ON, in August 2021. Sites ordered generally from most upstream (left) to downstream (right). Each morphology and colour are represented as a proportion of the total found at each site (n=9-10/site).

3.5.3 Relationship between Particle Count in Hemolymph and Size of Mussel

Although particle counts in hemolymph tended to be greater in longer and heavier mussels, these relationships were not significant (p=0.4 and 0.5, respectively; Figure 3.14;

Appendix Table A6). No relationship was observed between particle counts and mussel age (p=0.9).

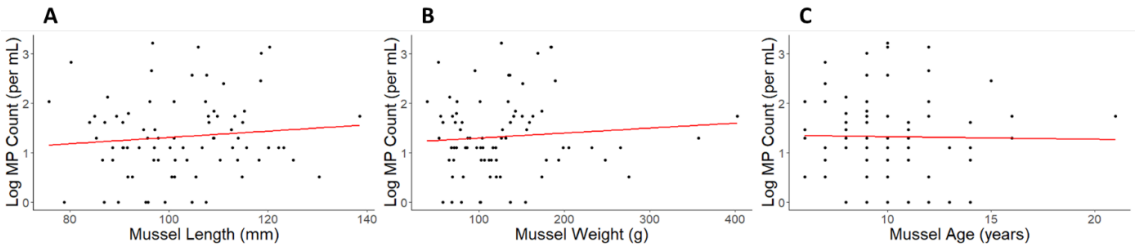


Figure 3.14 Log-linear relationship between microparticle counts (counts/mL) in hemolymph and length (A), weight (B), and age (C) of mussel samples collected from sites on the Grand River, ON, in August 2021. Microparticle counts (transformed (log(x+1)) per mL of hemolymph are shown. No significant relationships were observed (Linear Model).

3.5.4 Correlation of Particle Counts in Mussel Tissues and between Hemolymph and Water Samples

There was no correlation between particle counts in any of the three mussel tissue types (digestive gland and gill, p=0.8; digestive gland and hemolymph, p=0.06; hemolymph and gill, p=0.4; Appendix Table A7). There was also no correlation between mean particle counts per site in surface water samples and any of the three mussel tissues (gill p=0.3; digestive gland p=0.9; hemolymph p=0.4).

3.6 Microplastics in Surface Water

3.6.1 Particle Abundance and Spatial Distribution in Surface Water

All surface water samples contained microparticles in at least one of the size fractions examined, with 210 being the highest number observed in a pooled 20 L sample from the West Montrose site (WMT; Table 3.1). On average, 5.47 ± 2.81 particles/L were

found in water samples, with each size fraction contributing 1-2 particles/L. In total 577 were counted after blank correction. There was no significant variation in particle counts across the sites when size fractions were pooled (Figure 3.15; ANOVA $F_{7,16} = 2.142$, p=0.1). At each WWTP, there were no significant differences between counts upstream and downstream at each of the individual WWTPs (p=0.9, 0.3, and 0.4, respectively, for Hespeler, Ayr, and Caledonia), although counts were elevated at Ayr downstream (AR-D) compared to upstream (AR-U).

When comparing results for the four size fractions, the highest number of particles observed was 140 in the 100 µm size fraction at West Montrose (WMT). The 100 and 200 µm size fractions consistently contained the highest number of microparticles. There was no variation in particle counts across sites in any of the size fractions (p=0.2 for 500 µm, p=0.1 for 200 µm, p=0.2 for 100 µm, and p=0.2 for 40 µm size fractions). When examining each WWTP, the trends in particle counts between upstream and downstream depended on the size fraction examined; however none of these differences were statistically significant (Appendix Figure A2).

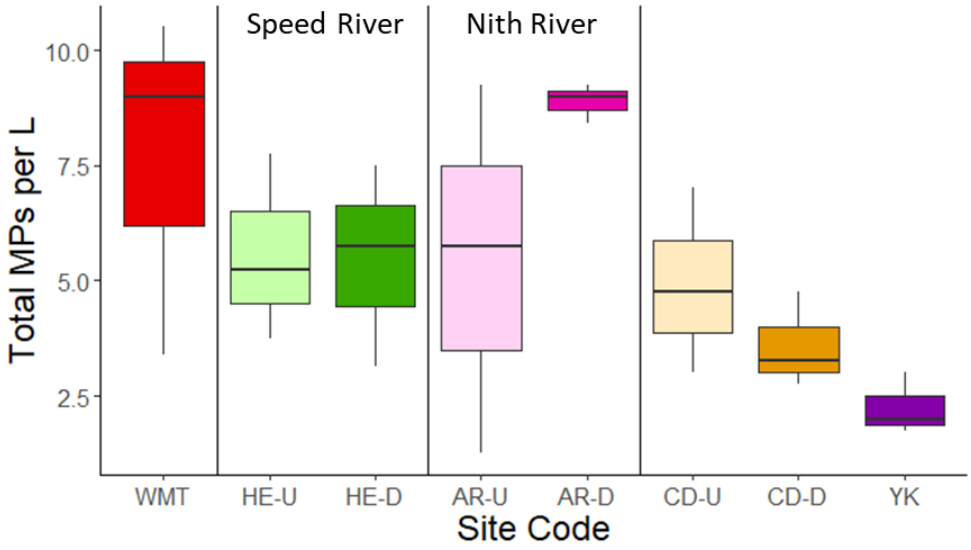


Figure 3.15 Spatial variation in microparticles (counts/L) in all size fractions of surface water samples collected from sites in the Grand River watershed, ON, in August 2021 (AR-D sampled November 2021). Sites are ordered generally from most upstream (left) to downstream (right), including panels to denote tributaries. Lighter colours represent the upstream site (U) while darker colours represent the downstream sites (D). There were no statistical differences between upstream and downstream at any of the WWTPs (n=3/site, unpaired t-test).

3.6.2 Particle Morphology, Colour, and Size in Surface Water

Across all size fractions, particle morphology of the water samples was dominated by fibers (84.9%), followed by fragments (8.3%) and films (5.9%; Figure 3.16A). The proportion of fibers was much lower at HE-D compared to HE-U, while the proportions of each morphology were relatively similar up- and downstream at the other WWTPs. Particle colours were dominated by clear (53.9%), followed by black (19.2%) and blue (18.7%), with some red (4.2%) and brown (1.6%); all other colours represented less than 1% of the total (Figure 3.16B). Between 5 and 8 colours were observed at each site. Mean particle length was 976.8 ± 1388.0 µm and particle width 34.3 ± 49.6 µm (Table 3.4).

There was no significant variation in particle length across sites (Kruskal-Wallis χ^2= 2.3891, p=0.9), but there was significant variation in particle width (Kruskal-Wallis χ^2= 21.806, p=0.003) as particles at Hespeler downstream were significantly wider than those at Ayr downstream (Dunn's HE-D vs AR-D, p=0.005). There were no differences in particle length (p=0.5, 0.9, and 0.08, respectively, at Hespeler, Ayr, and Caledonia) or width (p=0.1, 0.6, and 0.3, respectively, at Hespeler, Ayr, and Caledonia) when comparing upstream to downstream at each WWTP.

Fibers were also the dominant morphology within each size fraction, however foams and fiber bundles were only observed in the 100 and 500 µm size fractions (Appendix Figure A3A; Table A10). The proportions of each morphology differed depending on the size fraction, with the 500 and 40 µm size fractions typically having the highest proportion of fibers. In addition, higher proportions of fibers were consistently observed at downstream sites across all size fractions, except at Hespeler where relatively fewer fibers were observed in all size fractions at the downstream site. Within sites, the proportion of each colour differed greatly among the size fractions (Appendix Figure A3B; Table A11). The 500 µm size fraction tended to have the greatest number of colours at 9, while the 40 µm size fraction had the least number of colours at 5. The proportion of each colour differed considerably between the upstream and downstream site at each WWTP. The 500 µm fraction had the highest mean particle length at 1200.9 ± 1662.4 µm, but surprisingly the smallest mean length was in the 100 µm fraction (859.3 ± 1335.1 µm) and not the 40 µm fraction (1045.7 ± 1588.8 µm). The same trend was not seen in the mean particle width where the 100 µm fraction contained the widest particles (42.1 ± 57.7

μm) and the 40 μm fraction contained the narrowest particles (24.4 ± 26.0 μm; Table 3.4). There was no significant variation in particle length across sites in any of the size fractions (p=0.2, 0.3, 0.9, and 0.2, respectively, for the 500, 200, 100, and 40 μm size fractions). However, there was significant variation in particle width across sites in the 100 μm size fraction (p=0.002), although no paired differences were found when followed up with a Dunn's test. All other size fractions had no significant differences in particle width across sites (p=0.2, 0.6, and 0.3, respectively, for the 500, 200, and 40 μm size fractions).

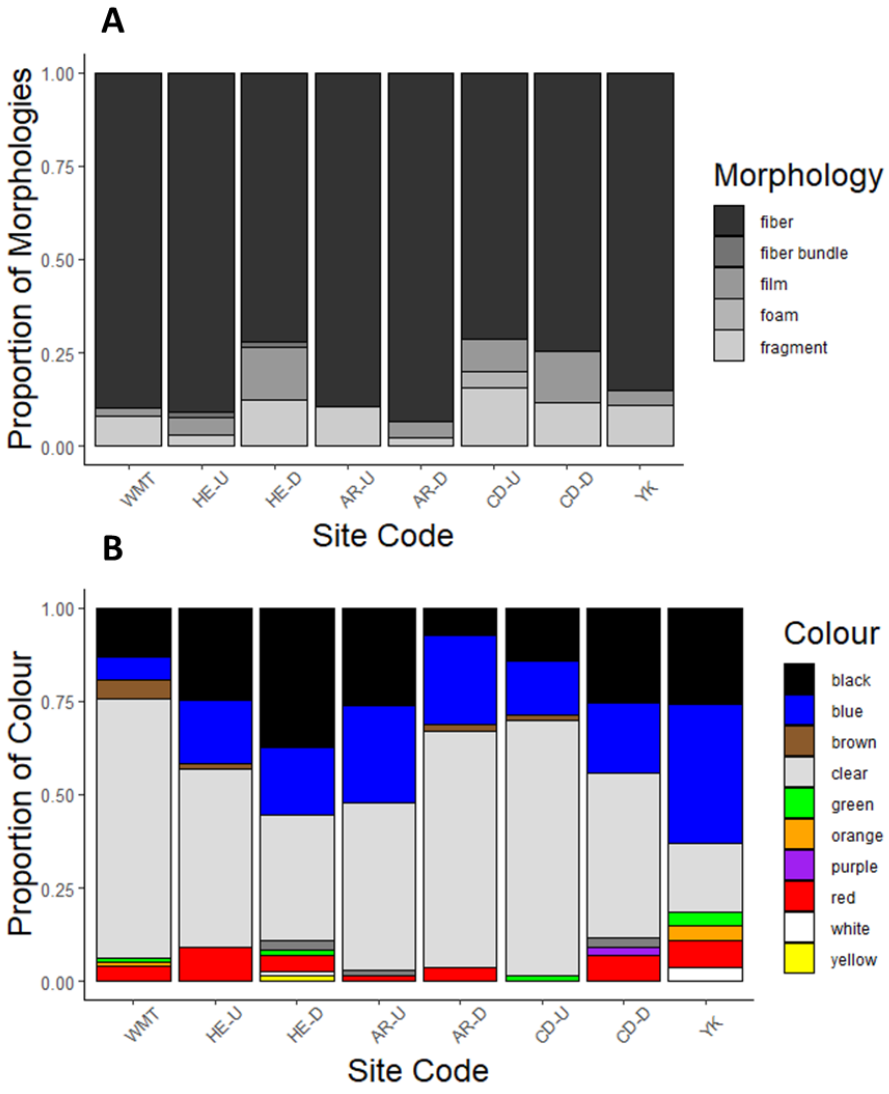


Figure 3.16 Proportion of each particle morphology (A) and colour (B) across sites in surface water samples collected from the Grand River, ON, in August 2021 (AR-D sampled November 2021). Sites are ordered from most upstream (left) to downstream (right). Each morphology and colour are represented as a proportion of the total found at each site (n=3).

Table 3.4 Mean particle size in surface water samples collected from sites on the Grand River, ON, in August 2021 (AR-D collected November 2021). Mean particle length ± standard deviation (in µm), mean particle width ± standard deviation (in µm), and percent of particles measured at each site as well as overall mean. Pooled water samples can be found on the left, followed by the particle size in each of the four size fractions examined (500, 200, 100, and 40 µm). Sites are ordered generally from most upstream (top) to downstream (bottom).

Site Code	Surface Water Samples			500 µm Size Fraction		200 µm Size Fraction		100 µm Size Fraction		40 µm Size Fraction	
	Particle Length (µm)	Particle Width (µm)	Percent Measured (%)	Particle Length (µm)	Particle Width (µm)	Particle Length (µm)	Particle Width (µm)	Particle Length (µm)	Particle Width (µm)	Particle Length (µm)	Particle Width (µm)
WMT	906.4 ± 1050.9	25.5 ± 27.7	96.9	1196.7 ± 1066.4	26.3 ± 31.9	910.4 ± 893.1	29.0 ± 37.5	824.9 ± 1322.1	23.1 ± 20.0	748.4 ± 568.1	23.1 ± 9.8
HE-U	1053.3± 1468.7	31.6 ± 50.0	98.5	1068.5 ± 883.5	21.7 ± 7.7	1107.7 ± 967.7	21.5 ± 9.2	894.5 ± 1549.8	37.2 ± 61.0	1626.5 ± 1583.6	20.1 ± 7.4
HE-D	1287.9 ± 2302.5	50.2 ± 77.8	98.6	1674.4 ± 2842.5	37.9 ± 36.2	676.5 ± 603.3	58.5 ± 108.8	1333.5 ± 2178.8	52.9 ± 75.3	2111.3 ± 4236.0	41.5 ± 42.7
AR-U	848.4 ± 803.7	31.2 ± 35.4	93.8	1122.5 ± 1087.3	36.8 ± 39.3	982.4 ± 751.2	30.9 ± 37.0	881.2 ± 781.9	43.1 ± 52.8	414.9 ± 185.6	19.7 ± 7.5
AR-D	830.2 ± 851.3	28.2 ± 40.1	95.0	753.0 ± 789.7	27.0 ± 27.3	898.3 ± 596.3	56.8 ± 108.6	770.8 ± 875.4	25.8 ± 30.2	945.8 ± 943.9	26.1 ± 36.4
CD-U	820.7 ± 1043.5	46.5 ± 66.6	97.1	1364.2 ± 1792.0	55.9 ± 92.7	680.5 ± 527.9	22.8 ± 13.5	669.7 ± 979.7	76.9 ± 84.1	1074.2 ± 1127.1	15.9 ± 4.6
CD-D	1459.8 ± 2222.8	36.5 ± 39.7	100	2534.5 ± 3164.5	24.4 ± 21.4	1707.1 ± 1351.0	18.5 ± 4.6	623.2 ± 540.1	50.8 ± 51.4	2211.3 ± 3553.4	24.0 ± 5.3
YK	840.3 ± 1017.6	31.5 ± 34.3	96.3	969.4 ± 373.7	17.7 ± 7.7	1002.3 ± 1824.5	22.3 ± 4.2	683.2 ± 638.5	51.2 ± 54.4	800.9 ± 592.2	21.9 ± 5.6
Mean	**976.8 ± 1388.0**	**34.3 ± 49.6**	**96.7**	**1200.9 ± 1662.4**	**31.4 ± 40.1**	**871.7 ± 846.1**	**33.5 ± 58.2**	**859.3 ± 1335.1**	**42.1 ± 57.7**	**1045.7 ± 1588.8**	**24.4 ± 26.0**

4 DISCUSSION

4.1 Microparticle Abundance and Characteristics

Although microparticles are found in many habitats and freshwater species, it was unclear whether organisms living downstream of WWTPs have elevated exposures to them. To assess this, fingernail clams, freshwater mussel tissues (gills, digestive gland, and hemolymph), and surface water samples were collected at sites impacted by WWTP outfalls as well as at a site upstream and downstream of the study area for comparison. Microparticles were found in nearly all replicates from each sample type collected from

sites along the Grand River. The dominant particle morphologies were fibers, and most were clear, blue, and black in colour. Nearly half the particles in each sample type were in the 80 μm – 1 mm size range. Although particle counts in bivalve samples differed among sites, spatial trends were not consistent across tissue types and elevated levels of microparticles downstream of WWTPs were only seen in mussel gill tissues. Overall, counts in mussel tissues tended to be lower in tributary sites (Hespeler and Ayr) compared to those on the main branch of the Grand River, while the opposite trend was seen in fingernail clams. Overall, these results suggest widespread microparticle contamination of the Grand River and that WWTPs do not result in elevated levels in most sample types examined herein.

4.1.1 Microparticle Morphology

Fibers were the dominant morphology in all bivalve and water samples, making up 79 to 96% of the total in each sample type, and they tended to be at higher proportions in samples from wastewater-impacted sites. The dominance of fibers in bivalve samples from the Grand River agrees with a recent review, which found that fibers were the dominant morphology across all studies of bivalves (Khanjani et al., 2023). This is likely because fibers dominate the morphologies observed in WWTP effluent (Ramasamy et al., 2022) and tributaries to the Great Lakes (Baldwin et al., 2016). Previous work on microparticle abundance in flutedshell mussels in the Grand River also found fibers, but chemical analysis confirmed that they were not plastic and were therefore not included in total counts (Wardlaw and Prosser, 2020). A high proportion of fibers found herein are also likely non-plastic, although they may be anthropogenically-derived and may also

impact biota. The high prevalence of fibers in all sample types herein highlights the importance of incorporating microfibers (both plastic and non-plastic) into laboratory toxicity studies and ensuring that field sampling techniques capture small and thin microfibers.

Fragments were also common in this study, making up at least 5% of the morphologies in fingernail clams, mussel digestive gland and hemolymph, and surface water samples. This was much lower than reported in a previous study on flutedshell mussels in the Grand River, where all but one microplastic was identified as a fragment (Wardlaw and Prosser, 2020), and this may be due to differences in the methods for tissue digestion, filter sizes that were used (50 μm compared to 38 μm herein), or chemical composition as mentioned above. No pellets and few microbeads were found herein, contrasting to previous studies in Great Lakes surface waters and beaches which found that microbeads and plastic pellets were the dominant particle morphologies (Eriksen et al., 2013; Zbyszewski et al., 2014; Zbyszewski and Corcoran, 2011); however, these studies were completed on large lakes before the microbead ban in Ontario (2015) and Canada (2016; Xanthos and Walker, 2017), and they targeted larger particles (>333 μm in Eriksen et al., 2013) which likely underestimated the number of small fibers present. The low occurrence of fragments, films, foams, pellets, and spheres herein suggests the effectiveness of wastewater treatment and the microbead ban in reducing inputs of these morphologies to freshwater rivers.

A small number of spheres were found in mussel digestive glands and not in the surface water or other bivalve samples with the exception of a single occurrence in

mussel gill tissue from Caledonia. These larger particles likely quickly settle out of the water column into sediments (Eriksen et al., 2013), so their presence in mussels suggests some uptake of sediment-derived microparticles. The spheres (mean 147.3 ± 49.2 μm; range 32.9 – 446.2 μm) are likely too large to be ingested by fingernail clams or to transfer into the mussel hemolymph (particles typically <20 μm; Browne et al., 2008). The composition and origin of these spheres is unknown, but potential sources include reflectance beads used in road marking (Burghardt et al., 2022), airblasting applications (Cole et al., 2011), and absorbent beads used in wastewater treatment (Hamidon et al., 2022). These are likely not the only sources, as clear spheres were found at West Montrose, Hespeler upstream, and Ayr upstream where there is no road or WWTP immediately impacting these sites. The inputs of microparticles, especially spheres, to the Grand River needs to be further characterized to better understand the relative importance of these sources to organisms.

4.1.2 Microparticle Colour

Many colours of microparticles were observed in all sample types, with 11 being the highest number found in fingernail clam samples, and the majority were clear (49 to 65%) followed by blue and black (typically >10% each). Clear and white particles were also the most common in flutedshell mussels previously collected from the Grand River (Wardlaw and Prosser, 2020), and blue, clear, and black particles were abundant in other studies of freshwater mussels (Almeshal et al., 2022; Doucet et al., 2021). The clear particles in the current study are likely a mix of plastic and non-plastic (cellulose) particles, and chemical analysis is needed to understand their composition. Dyes used to

colour textiles (natural and synthetic) affect the surface properties of fibers, leading to increased biofilm formation and changes in density; this may cause these particles to settle more quickly than undyed particles that remain in the water column and are more readily ingested by filter feeding bivalves (Almeshal et al., 2022). Berglund et al. (2019) suggest that mussels may have colour preferences for their food as phytoplankton are also a dark colour, but this was not supported herein as dark coloured particles were not dominant in their tissues. Lab toxicity studies should investigate whether colour influences bivalve feeding preferences and microparticle bioaccumulation.

4.1.3 Microparticle Size

In the bivalves sampled herein, the longest and shortest particles were observed in mussel gills (1481 ± 1366 μm) and hemolymph (932 ± 1266 μm), respectively, and particle widths were similar in all samples except mussel gills, which were the narrowest (27 ± 35 μm). Interestingly, particles found in water samples were shorter, but of a similar width, than those in bivalves (mean length 977 ± 1388 μm, width 34.3 ± 49.6 μm). Freshwater bivalves are thought to mostly feed on particles <20 μm, however these small particles were not quantified herein because we used a sieve size of 38 μm during microparticle isolation. Very few particles (<4% in each sample type) were shorter than 80 μm, a size known to translocate into cells in marine bivalves (Von Moos et al., 2012), however the lower end of this range was not targeted herein. Over half of the particles in fingernail clams (50.1%), mussel digestive gland (64.4%) and hemolymph (68.2%) were between 80 μm and 1 mm; in contrast, half of the particles in mussel gills (50.6%) were between 1 mm and 5 mm. These particles were much larger than the dominant size range

in another study on whole freshwater mussels (48% of all particles were <0.1 mm), however their lower size limit was 1.2 µm, allowing for detection of much smaller particles than herein (Atici, 2022). A recent review of microplastics in WWTPs found that agitation during the treatment process led to a high proportion of small fibers remaining in the effluent, and over 90% of microparticles in wastewater effluents were <500 µm (Ramasamy et al., 2022; Sun et al., 2019); this falls within the size range most prevalent in bivalves collected from the Grand River, despite the size cutoff used herein. The high proportion of large microparticles in fingernail clams is surprising due to their small size, but these particles may not have been within the tissues but instead trapped inside their shells. These particles were unlikely external as each clam shell was thoroughly rinsed when collected. In contrast to fingernail clams and other mussel tissues, mussel gills contained a high proportion of larger microparticles. Previous work on microplastic uptake in marine bivalves showed that particles >1 mm tended to be rejected upon sorting (Ward et al., 2019), therefore larger particles are more prevalent in the gills where sorting occurs than in other tissues. The differences in sizes among mussel tissues herein suggests selective uptake of smaller particles. In addition, some large particles in mussel gills may have been broken down into smaller pieces during mussel digestion but were not detected herein due to this size limitation. Future studies should incorporate methods to lower the size limit of microparticle detection to better understand the abundance of small particles in biota and understand particle sorting and rejection in the gills.

4.1.4 Spatial Trends in Microparticle Abundance

Microparticle count in mussel gill and hemolymph samples from the Grand River tended to increase further downstream in the watershed, similar to other studies in the same watershed (Wardlaw and Prosser, 2020), the Höje river in Sweden (Berglund et al., 2019), and the Karasu River in Turkey (Atici, 2022). What was most surprising was the decreasing microparticle counts in surface water samples downstream, although this was not significant. This may be due to higher dilution at downstream sites where the Grand River was wider. It was also surprising that nearly all samples from the furthest upstream site had high particle counts despite no nearby inputs from a WWTP, potential sources to this site are discussed further below.

4.2 Wastewater Treatment Plants as a Source of Microparticles in the Grand River

4.2.1 Wastewater Treatment Plants as Sources of Microparticles to Freshwater Bivalves

Higher microparticle counts downstream of WWTP outfalls were only observed in mussel gills and not seen in the other mussel tissues, fingernail clams, or water samples at the three treatment plants examined. Elevated particle counts were also seen immediately downstream of a major WWTP outfall (population served ~250,000 people) discharging to the Grand River in flutedshell mussel gills, although this difference was not significant from the upstream site (Weir et al., 2023). This may be because gills are the first organs in contact with surface water during filter feeding and where particle sorting occurs (Cummings and Graf, 2009). Marine bivalves can selectively reject >20% of fibers during laboratory exposures (Ward et al., 2019), and freshwater bivalves are likely to do the same. Particle sorting in the gills leads to high rates of microparticle rejection in

pseudofeces which is then expelled from the shell and may be ingested by other aquatic organisms (Cummings and Graf, 2009). Overall, previous studies in freshwater mussels showed little difference in particle counts between individuals caged upstream and downstream of the WWTP outfalls (Domogalla-Urbansky et al., 2019; Weir et al., 2023), providing further support that municipal wastewaters do not increase microparticle accumulation in mussels, especially in the digestive and circulatory systems. However, more chronic field and lab studies are recommended to better understand the long-term effects of microparticle exposures and whether microparticles in pseudofeces pose a risk to other benthic macroinvertebrates.

It is important to note that the number of particles being released from each WWTP was not characterized in the current study, and that the impacts of wastewater effluent on environmental levels was determined solely with a one-time surface water sample. Much of the literature on microplastics in municipal wastewater treatment plants focuses on plastic removal efficiency and characterizing which steps of the treatment process led to the highest removal rates (Horton, Walton, et al., 2017). Investigation of the abundance and characteristics of microparticles found in wastewater effluent is needed to better assess the contribution of WWTPs to microparticle pollution in surface waters; this analysis is ongoing for many of the WWTPs examined herein. In addition, more regular water sampling would be helpful for understanding potential daily and seasonal changes in microparticle releases from WWTPs.

4.2.2 Additional Sources of Plastics

The ubiquitous presence of microparticles in all samples, limited evidence of higher counts at effluent-exposed sites, and elevated counts at the most upstream site (West Montrose) suggest that there are other important sources of microparticles to the Grand River. Urban areas have been recently identified as the major source of plastics to surface waters in in North America, in part due to high populations, urbanization, and high levels of wastewater treatment (Li et al., 2023). Higher microparticle counts are found closest to urban areas (Mani et al., 2015; Yonkos et al., 2014), including surface waters near the large cities of Detroit and Cleveland in the Great Lakes basin (Eriksen et al., 2013). Road and stormwater runoff is another important source of microparticles, especially in urban areas and on well-travelled country roads. These particles are from tire wear, vehicle debris, and road-markings (Eriksen et al., 2013; Horton, Svendsen, et al., 2017; Horton, Walton, et al., 2017). In addition, precipitation can deposit high numbers of particles in urban areas, especially during large storms (Dris et al., 2016).

West Montrose is downstream of a primarily agricultural area, and the microparticles in this reach of the river are likely coming from agricultural runoff. Agricultural practices involve the use of many plastic materials which may fragment and be carried into surface waters through overland runoff. Plastic mulch, plant coverings, seed coatings, baling twine, and packaging are just some sources of plastics from agriculture (Kasirajan and Ngouajio, 2012; Kyrikou and Briassoulis, 2007; Scarasci-Mugnozza et al., 2001; Steinmetz et al., 2016); of these, plastic mulch has been used in agricultural fields near West Montrose (S. Gewurtz, personal communication, June 2,

2023). Additionally, biosolids from wastewater treatment are often used in agriculture as fertilizers (Carr et al., 2016; Habib et al., 1998), and they typically contain very high concentrations of microparticles, especially fibers from textiles and fragments and microbeads from personal care products (Habib et al., 1998; Zubris and Richards, 2005). Microparticles have been found in agricultural lands where biosolids have been applied as fertilizer, even 15 years after their application (Zubris and Richards, 2005). A better understanding of agricultural practices and their impact on environmental concentrations of microparticles is needed, especially within the Grand River watershed.

4.3 Bivalves as Bioindicators for Microparticles

4.3.1 Microparticle Accumulation in Bivalves

Microparticle counts in fingernail clams (mean 35.5 ± 29.4 microparticles/g, range 0 – 125.4 microparticles/g) and mussel tissues (gills mean 4.3 ± 4.4 microparticles/g, range 0 - 19.9 microparticles/g; digestive gland mean 6.5 ± 8.1 microparticles/g, range 0.4 – 57.8 microparticles/g; and hemolymph mean 4.3 ± 4.2 microparticles/mL, range 0 – 24 microparticles/mL) had much higher ranges than reported in previous studies on freshwater bivalves. More specifically, microparticle counts were lower in mussels from the Grand River (range 0-0.16 microparticles/g; pore size 50 µm; Wardlaw and Prosser, 2020) and the St. John River in Canada (range 0-10.9 microparticles/g; pore size 90 µm; Doucet et al., 2021), the Tisza River in Hungary (range 0.2-0.38 microparticles/g; pore size 1.2 µm; Almeshal et al., 2022), and the Karasu River in Turkey (range 0.81-6.69 microparticles/g; pore size 1.2 µm; Atici, 2022) even though some studies used a lower particle size limit than herein. Since microparticles have not yet been confirmed as

plastics herein, particle counts are likely an over-estimation of microplastics. Once such analyses are done, microplastic counts may be more comparable to those in other studies.

On a per g basis, fingernail clams had higher microparticle counts than freshwater mussels. It is important to remember that fingernail clam samples were a composite sample and each clam had low counts per individual. It is difficult to directly compare clams and freshwater mussels, as not all tissues in the mussel were analyzed and total microparticles/mussel could not be calculated. However, there are likely taxa differences in the characteristics of particles ingested as a study in freshwater mussels found nearly twice the number of fragments in one species compared to another (Almeshal et al., 2022).

Within mussels, digestive gland tissues had the highest mean particle count of all tissues examined. A study of caged flutedshell mussels also found more particles in the digestive gland compared to other tissues, suggesting there may be bioaccumulation during digestion (Weir et al., 2023). Overall counts in the current study were higher which is unsurprising as mussels collected herein were chronically exposed to wastewater effluent as opposed to the 28-day exposure in the cages (Weir et al., 2023).

There was little evidence for microparticle bioaccumulation (increasing counts with size/age) in bivalves. Mussel gills showed a significant increase in microparticle counts (per g) in longer mussels, while the digestive gland showed a significant decrease in counts in longer and heavier mussels and hemolymph showed no relationship. In addition, no relationship between microparticle counts and fingernail clam size was

observed. Other studies have found no effect of size on microparticle counts in whole flutedshell mussels from the Grand River (Wardlaw and Prosser, 2020), while whole freshwater mussels from the Höje river in Sweden had higher microparticle counts in larger individuals (Berglund et al., 2019). These differ from the trends observed herein, but each tissue may accumulate particles and grow at different rates showing varying trends between each tissue and the whole animal. Particles may not be bioaccumulating as high clearance rates have been observed in multiple species of freshwater bivalve (Weber et al., 2021). Laboratory studies are needed to better understand the fate of ingested microparticles in freshwater bivalves and should focus on individual tissues as well as whole animals.

A potential confounder for the comparison of particle counts across sites herein is the differences in clam and mussel sizes, as some sites had larger and older individuals than others, and size can influence microparticle counts, as discussed above. This suggests that future monitoring should, at a minimum, standardize the sizes of animals collected and processed for microparticles. However, wastewater treatment plants are known to negatively impact the condition factor of mussels (Gillis, 2012), which was observed herein for fingernail clams at Ayr downstream, and the impacts of effluent may make it difficult to have standardized sampling of animals that are the same size and age across sites.

4.2.2 Bivalves are Potential Bioindicators for Microparticle Pollution

Although microparticle counts in bivalves and water samples were not related, particle characteristics were similar across all sample types, suggesting that freshwater

bivalves can be used as indicators of microparticle pollution. Holt and Miller (2011) define the traits of good bioindicator species as one with the ability to indicate the contaminant of concern with moderate tolerance to disturbance, that are abundant, common, well-studied, and economically or commercially important. Freshwater bivalves are currently used as bioindicators for many contaminants as they possess most of these traits, but to serve as good bioindicators for microparticle pollution their uptake must be further characterized through additional field studies and lab experiments to better understand the relationship between microparticle exposure and tissue uptake.

Fingernail clams contained more than 5x more microparticles per g of tissue when compared to individual tissues in freshwater mussels and these data were correlated with microparticle counts in mussel digestive gland, making them a strong candidate for use as an indicator taxa. Fingernail clams are easily collected and can be used as a surrogate for freshwater mussels, which are described as North America's most imperiled groups of organisms (Metcalfe-Smith et al., 2000; Williams et al., 1993). The high occurrence of microparticles in small bivalves has been observed in zebra mussels, where smaller individual mussels contained more microplastics than larger ones, likely due to their higher filtering rates and larger relative gill areas (Weber et al., 2021). Similar trends are likely occurring in fingernail clams and mussels, where fingernail clams and juvenile mussels are at risk for greater uptake of microparticles, and this should be further investigated in laboratory studies.

4.4 Limitations of Study and Future Directions

As in most microparticle studies, the mesh size used during the microparticle isolation step limits the size of particles collected, and particles <38 μm were not quantified herein. These smaller particles are more likely to be ingested as most bivalves feed on particulates <20 μm (Strayer, 2008; Vaughn et al., 2008) and can more easily translocate through tissues and impact function in the organism (Browne et al., 2008; Haag, 2012; Von Moos et al., 2012); future studies should aim to target these smaller particles. In contrast, a total of 57 particles across all sample types were >5 mm, greater than the definition of microplastics. These larger particles were found in all sample types and are potentially a source of contamination, despite numerous measures to limit this. The chemical analysis of all particles will determine the proportion that are plastic and the types of polymers present and offer insights into potential sources of these microparticles (Rochman et al., 2019).

Although most tissues examine herein suggest that bivalves downstream of WWTP outfalls do not take up more microparticles, further sampling is needed to determine if individuals at downstream sites had higher environmental exposures. Water samples did not show elevated particle counts downstream of wastewater treatment plants, however effluent directly from the WWTP was not analyzed herein, but this work is ongoing. Although bivalves are mostly filter feeding organisms, microparticles in the sediment should also be examined since freshwater bivalves are known to ingest sediment-bound particles (Cummings and Graf, 2009; Haag, 2012; Way, 1989). Most microparticles will eventually end up in the sediment due to biofouling and aggregation

that cause them to sink (Atugoda et al., 2020), and sediment had much higher microparticle counts than surface water in the Grand River (Weir et al., 2023). As such, characterizing both matrices gives a better understanding of the magnitude and spatial patterns of microplastic pollution (Talbot and Chang, 2022), and is strongly recommended for future studies, especially those focused on understanding the risks of microparticle exposures to aquatic organisms.

Data herein were only collected at a single timepoint, and biotic and abiotic samples were collected on different days at these sites. In addition, water samples were collected 2 months apart at Ayr due to technical challenges and it is impossible to conclude if elevated particle counts at the downstream site were due to wastewater effluent or seasonal changes. Seasonality has a large impact on microparticle concentrations in surface waters, with higher concentrations observed in spring and summer than other seasons (Lasee et al., 2017). This variable exposure may influence the uptake of microparticles into bivalves over time. Future work in freshwater systems should incorporate sampling under different conditions to understand longer-term trends in microplastic characteristics and abundance in environmental matrices and biota.

5 CONCLUSIONS

This study determined the abundance and characteristics of microparticles in two groups of freshwater bivalves collected near wastewater treatment plants and compared them to microparticles in surface waters from the same sites. All sample types contained microparticles with similar characteristics, and clear, blue, and black fibers from 80 µm to 1 mm were dominant. Bivalves collected downstream of WWTPs only showed elevated

microparticle counts in freshwater mussel gills but not in mussel digestive gland or hemolymph, fingernail clams, or surface water samples. The few differences between samples collected upstream and downstream of WWTPs supports the growing evidence that this point source is not a significant source of microparticles despite contributing many other contaminants. However, small particles (<38 μm) were not targeted herein and may be abundant in WWTP effluent due to particle breakdown during wastewater treatment. Particle abundance in bivalve tissues was higher than previously reported, and fingernail clams contained more microparticles per mass when compared to all mussel tissues. Within mussel tissues, the digestive gland contained the highest microparticle counts. Although I found no correlation between particle counts in water and bivalve samples, similarities in their particle size, morphology and colour indicate the potential for these organisms to act as bioindicators of plastic pollution. Future work in fresh waters should expand on the potential to use fingernail clams as bioindicators and on characterizing non-wastewater sources of microparticles to rivers. In addition, these results can be used to design environmentally-relevant lab studies to better understand the uptake and impact of microparticles on freshwater bivalves.

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APPENDIX

Appendix Table A1. Relative percent difference between sub-sampled and complete microplastic counts on surface water filters. The percent difference between the number of particles counted in the 20% sub-sample multiplied by 5 to give calculated number of particles in 100% sample was compared to the actual number of particles found in the 100% sample. RPD ranges from 22.2 to 102%.

Sample ID	Number of MPs in 20% Sub-Sample	Calculated Number of MPs in 100% Sample	Actual Number of MPs in 100% Sample	Relative Percent Difference (%)
YK-D-WSB-100	21	105	52	67.5
YK-D-WSB-200	15	75	35	72.7
CD-D-WSB-100	37	185	60	102.0
CD-D-WSB-200	25	125	41	101.2
YK-D-WSB-40	12	60	35	52.6
CD-D-WSB-40	13	65	31	70.8
HE-D-WSB-40	9	45	27	50.0
AR2-D-WSB-40	6	30	18	50.0
AR2-U-WSB-40	5	25	20	22.2
HE-U-WSB-40	9	45	24	60.9

Appendix Table A2. Summary of particle types collected in blanks from each matrix at each site collected on the Grand River, ON, in August 2021 (AR-D water samples collected November 2021, AR fingernail clam samples September 2022). Sites ordered from most upstream (top) to downstream (bottom). The number of each particle type collected in fingernail clams, mussel gills, mussel digestive gland, mussel hemolymph, and surface water samples blanks are shown. For each replicate, the equivalent number (or less) of each particle type were subtracted in samples of the same matrix from the same site.

Site	Fingernail Clams	Mussel Hemolymph	Mussel Tissues	500 Fraction Water Sample	200 Fraction Water Sample	100 Fraction Water Sample	40 Fraction Water Sample
WMT	Clear fiber: 22 Blue fiber: 5 Black fiber: 1 **Total: 28**	Clear fiber: 33 Blue fiber: 1 Green fragment: **Total: 35**	Blue fiber: 5 Clear fiber: 17 **Total: 22**	Clear fiber: 3 Blue fiber: 3 **Total: 6**	Clear fiber: 6 Clear film: 2 Black fiber: 1 Blue fiber: 1 **Total: 10**	Clear fiber: 15 Blue fiber: 2 Black fiber: 1 **Total: 18**	Clear fiber: 5 Blue fiber: 1 Blue fragment: 1 Black fiber: 2 **Total: 9**
HE-U	Clear fiber: 8 Blue fiber: 2 Purple fiber: 1 **Total: 11**	Clear fiber: 22 Blue fiber: 5 Clear fragment: 1 Clear film: 2 **Total: 30**	Clear fiber: 14 Clear film: 1 Gray fragment: 1 Blue film: 1 Blue fiber: 1 **Total: 18**	Clear fiber: 9 Clear film: 1 **Total: 10**	Clear fiber: 15 Clear film: 1 Clear fragment: 1 Black fiber: 2 Blue fiber: 2 **Total: 21**	Clear fiber: 12 Blue fiber: 1 Black fiber: 2 Brown fiber: 1 Green fiber: 1 **Total: 17**	Clear fiber: 6 Blue fiber: 1 Black fiber: 1 Red fragment: 1 **Total: 9**
HE-D	Clear fiber: 8 Black fiber: 2 Brown fiber: 1 Blue fiber: 1 **Total: 12**	Clear fiber: 22 Blue fiber: 5 Clear fragment: 1 Clear film: 2 **Total: 30**	Clear fiber: 14 Clear film: 1 Gray fragment: 1 Blue film: 1 Blue fiber: 1 **Total: 18**	Clear fiber: 7 Blue fiber: 1 **Total: 8**	Clear fiber: 14 Blue fiber: 2 **Total: 16**	Clear fiber: 12 Blue fiber: 1 Black fiber: 2 Brown fiber: 1 Green fiber: 1 **Total: 17**	Clear fiber: 15 Clear film: 2 Clear sphere: 1 Blue fiber: 5 Red fiber: 3 Black fiber: 1 **Total: 27**
HE-F	Clear fiber: 7 Blue fragment: 1 Black fiber: 1 **Total: 9**	N/A	N/A	N/A	N/A	N/A	N/A
AR-U	Clear fiber: 8 Blue fiber: 1 Gray fiber: 3 **Total: 12**	Clear fiber: 35 Blue fiber: 3 Blue fragment: 2 Gray fiber: 2 Black fiber: 1 **Total: 43**	Clear fiber: 25 Clear film: 2 Clear sphere: 1 Blue fiber: 3 Silver fragment: 1 **Total: 32**	Clear fiber: 6 Black fiber: 1 **Total: 7**	Clear fiber: 14 Clear film: 1 Black fiber: 2 **Total: 17**	Clear fiber: 28 Clear film: 7 Blue fiber: 1 Black fiber: 1 Red fiber: 1 Brown fiber: 1 **Total: 39**	Clear fiber: 8 Blue fiber: 2 **Total: 10**
AR-D	Clear fiber: 12 Black fiber: 3 Gray fiber: 1 Gray fiber bundle: 1 **Total: 17**	Clear fiber: 35 Blue fiber: 3 Blue fragment: 2 Gray fiber: 2 Black fiber: 1 **Total: 43**	Clear fiber: 25 Clear film: 2 Clear sphere: 1 Blue fiber: 3 Silver fragment: 1 **Total: 32**	Clear fiber: 5 Red fiber: 1 Blue fragment: 1 **Total: 7**	Clear fiber: 14 Clear film: 2 Black fiber: 3 Blue fiber: 1 **Total: 20**	Clear fiber: 10 Clear film: 2 Green fiber: 1 Black fiber: 6 Blue fiber: 1 **Total: 20**	Clear fiber: 5 Black fiber: 1 **Total: 6**

Appendix Table A3 (continued).

Site	Fingernail Clams	Mussel Hemolymph	Mussel Tissues	500 Fraction Water Sample	200 Fraction Water Sample	100 Fraction Water Sample	40 Fraction Water Sample
CD-U	Clear fiber: 9 Gray fiber: 2 Blue fiber: 5 Black fiber: 4 **Total: 20**	Clear fiber: 24 Red fiber: 1 Clear film: 1 Gray fiber: 1 Blue fiber: 2 **Total: 29**	Clear fiber: 9 Clear fiber bundle: 1 Clear film: 1 Black fiber: 3 Blue fragment: 3 Blue fiber: 1 **Total: 18**	Clear fiber: 6 Blue fiber: 1 Red fiber: 2 **Total: 9**	Clear fiber: 9 Red fiber: 1 Black fiber: 2 **Total: 12**	Clear fiber: 14 Clear film: 2 Blue fiber: 1 Black fiber: 1 Brown fiber: 1 **Total: 19**	Clear fiber: 8 Blue fiber: 1 Black fiber: 1 **Total: 10**
CD-D	Clear fiber: 12 Blue fiber: 6 Red fiber: 1 Black fiber: 1 **Total: 20**	Clear fiber: 24 Red fiber: 1 Clear film: 1 Gray fiber: 1 Blue fiber: 2 **Total: 29**	Clear fiber: 9 Clear fiber bundle: 1 Clear film: 1 Black fiber: 3 Blue fragment: 3 Blue fiber: 1 **Total: 18**	Clear fiber: 8 Blue fiber: 3 **Total: 11**	Clear fiber: 17 Clear film: 1 Blue fragment: 1 Black fiber: 6 **Total: 25**	Clear fiber: 21 Brown fiber: 5 Black fiber: 4 Red fiber: 2 Blue fiber: 3 **Total: 35**	Clear fiber: 9 Blue fiber: 3 Black fiber: 1 **Total: 13**
CD-F	Clear fiber: 15 Black fiber: 3 Blue fiber: 5 Pink fiber: 1 **Total: 24**	N/A	N/A	N/A	N/A	N/A	N/A
YK	Clear fiber: 6 Blue fiber: 4 Purple fiber: 1 Black fiber: 2 Gray fiber: 2 **Total: 15**	Clear fiber: 22 Black fiber: 2 Black fragment: 1 Blue fiber: 2 Brown fiber: 1 **Total: 28**	Black fiber: 3 Clear fiber: 11 Blue fiber: 4 **Total: 18**	Clear fiber: 8 Blue fiber: 1 Black fiber: 2 **Total: 11**	Clear fiber: 13 Red fiber: 1 Black fiber: 1 **Total: 15**	Clear fiber: 16 Clear film: 1 Red fiber: 1 Black fiber: 1 **Total: 19**	Clear fiber: 8 Clear film: 1 Blue fiber: 3 **Total: 12**

Appendix Table A4. Mean size of fingernail clams across sites collected on the Grand River, ON, in August 2021 (AR-U and AR-D collected September 2022). Characteristics include clam length in mm and wet weight in g. Sites ordered from most upstream (top) to downstream (bottom).

Site	Mean Length (mm)	Mean Wet Weight per Clam (mg)
WMT	8.67 ± 0.67	204.01 ± 57.58
HE-U	8.14 ± 0.51	159.67 ± 25.18
HE-D	8.20 ± 0.21	162.47 ± 10.66
HE-F	8.32 ± 0.31	169.35 ± 11.90
AR-U	8.67 ± 0.83	145.58 ± 33.82
AR-D	7.22 ± 0.60	105.09 ± 31.12
CD-U	9.016 ± 0.55	225.65 ± 47.62
CD-D	9.40 ± 0.57	244.96 ± 42.40
CD-F	9.35 ± 0.61	243.82 ± 57.84
YK	8.98 ± 0.23	204.53 ± 43.24
Total	**8.60 ± 0.80**	**186.51 ± 56.46**

Appendix Table A5. Tukey HSD significant differences in fingernail clam length (left) and wet weight (right) across sites on the Grand River, ON, collected summer 2021 (AR-U and AR-D collected September 2022). Bolded sites indicate differences between upstream, downstream, and far-field at a WWTP.

Site 1	Site 2	p-value
AR-U	WMT	0.002
CD-F	HE-D	0.02
CD-F	HE-F	0.03
CD-U	AR-U	0.03
CD-D	AR-U	0.01
CD-F	AR-U	0.0001
YK	AR-U	0.02
CD-F	AR-D	0.007

Appendix Table A6. Tukey HSD significant differences in microparticle counts in fingernail clams collected on the Grand River, ON, collected summer 2021 (AR-U and AR-D collected September 2022). Bolded sites indicate differences between upstream, downstream, and far-field at a WWTP.

Site 1	Site 2	p-value
AR-U	WMT-U	0.002
CD-F	HE-D	0.02
CD-F	HE-F	0.03
CD-U	AR-U	0.03
CD-D	AR-U	0.01
CD-F	AR-U	0.0001
YK-D	AR-U	0.02
CD-F	AR-D	0.007

Appendix Table A7. Significance of linear relationship between microparticle counts and various characteristics of bivalves collected on the Grand River, ON, August 2021 (some fingernail clams collected September 2022). For each sample type, particle counts were compared to bivalve length and wet weight. For mussel samples, particle counts were also compared to age. Both log-log and log-linear relationships were recorded. Significant relationships were bolded.

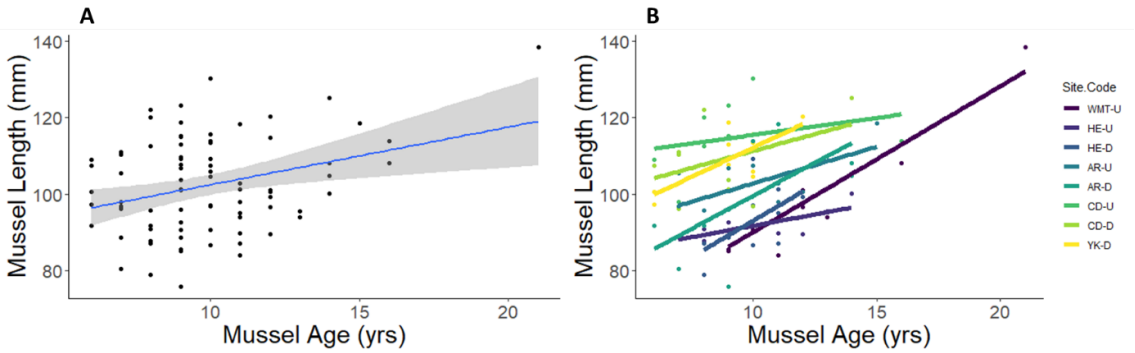
Sample Type	Characteristic	Log-Log			Log-Linear		
		Equation	Adjusted R^2	p-value	Equation	Adjusted R^2	p-value
Fingernail Clam	Length	y = -0.01282x + 2.17870	-0.008249	0.4427	y = -0.1144x + 8.8795	-0.006617	0.4144
	Weight	y = -0.07677x + 5.37233	0.01982	0.1647	y = -15.54x + 225.13	0.03171	0.1131
Mussel Gill	Length	**y = 0.06331x + 4.52120**	**0.1364**	**0.01804**	**y = 6.257x + 92.450**	**0.1224**	**0.02413**
	Weight	**y = 0.20882x + 4.39675**	**0.1199**	**0.02546**	y = 22.42x + 88.00	0.06753	0.07488
	Age	y = -0.04982x + 2.27919	0.003175	0.301	y = -0.4753x + 9.9788	0.003839	0.2963
Mussel Digestive Gland	Length	**y = -0.03888x + 4.68616**	**0.04492**	**0.03292**	**y = -3.868x + 108.822**	**0.04149**	**0.03875**
	Weight	**y = -0.17558x + 5.02904**	**0.07062**	**0.009842**	**y = -21.435x + 162.796**	**0.04839**	**0.02794**
	Age	y = -0.02436x + 2.29979	-0.007433	0.5203	y = -0.3217x + 10.4240	-0.004335	0.4194
Mussel Hemolymph	Length	y = 0.01484x + 4.60132	-0.00376	0.4016	y = 1.595x + 100.243	-0.002833	0.3792
	Weight	y = 0.04652x + 4.67508	-0.006588	0.4834	y = 6.810x + 118.254	-0.006226	0.4715
	Age	y = -0.009571x + 2.275068	-0.01219	0.7879	y = -0.05677x + 9.98530	-0.01286	0.8806

Appendix Table A8. Spearman's correlation of the mean particle count per site between mussel tissues, fingernail clams, and water samples collected from sites on the Grand River, ON, in August 2021 (some water samples collected November 2021 and some fingernail clams September 2022). Values for ρ (rho) and p-value are shown for each pair of sample types including fingernail clams (n=5/site), mussel gills (n=8-10/site), mussel digestive gland (n=10/site), mussel hemolymph (n=9-10/site), and surface water samples (n=3/site). Statistically significant correlations are shown in bold.

Sample to Compare	Tissue Type	ρ (Rho)	p-value
Mussel Digestive Gland	Mussel Gill	0.05088631	0.775
Mussel Hemolymph	Mussel Digestive Gland	0.2153365	0.05831
Mussel Gill	Mussel Hemolymph	0.1643256	0.3608
Fingernail Clams	Mussel Gill	-0.4	0.75
	Mussel Digestive Gland	**-0.7857143**	**0.02793**
	Mussel Hemolymph	-0.6190476	0.115
Water Samples	Fingernail Clam Tissues	0.3095238	0.4618
	Mussel Gill	-0.8	0.3333
	Mussel Digestive Gland	0.04761905	0.9349
	Mussel Hemolymph	-0.3571429	0.3894

Appendix Table A9. Characteristics of flutedshell mussels across sites collected on the Grand River, ON, in August 2021. Characteristics include mussel length in mm, weight in g, age in years, and the proportion of gravid females. Sites ordered from most upstream (top) to downstream (bottom).

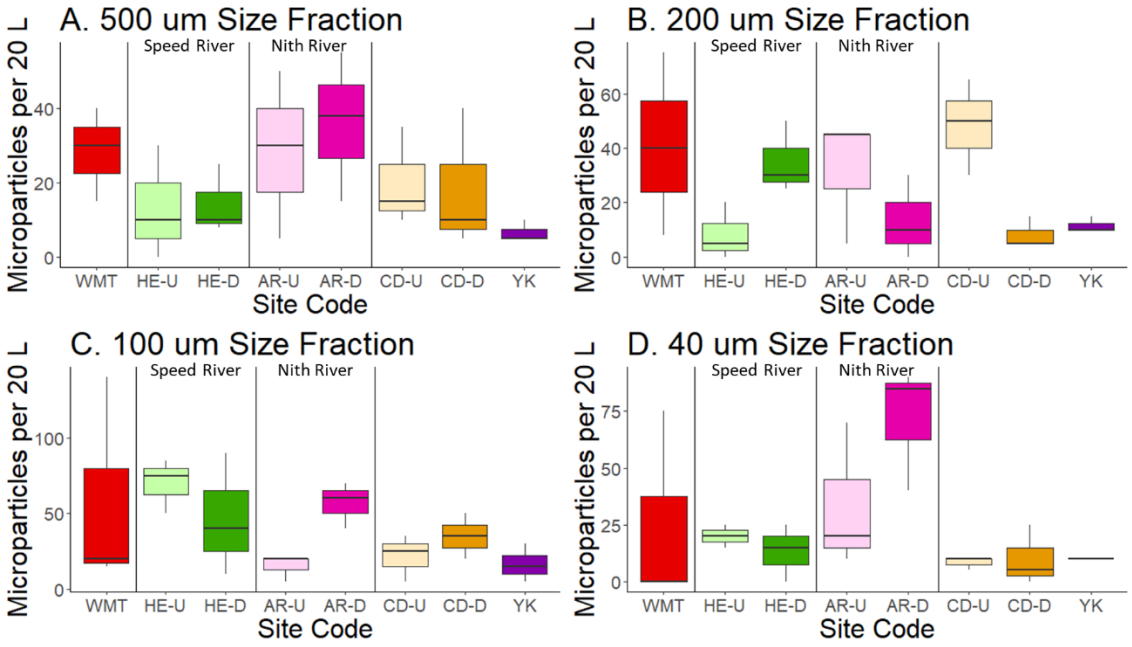
Site	Mussel Length (mm)	Mussel Wet Weight (g)	Mussel Age (years)	Proportion Gravid (%)
WMT	98.4 ± 16.1	117.6 ± 105.1	12.2 ± 3.8	40
HE-U	91.9 ± 3.8	80.0 ± 12.9	10.2 ± 2.3	60
HE-D	93.0 ± 8.9	85.2 ± 23.3	10.0 ± 1.3	40
AR-U	102.4 ± 7.7	116.1 ± 29.9	9.8 ± 2.5	40
AR-D	99.5 ± 14.7	117.4 ± 57.1	10.0 ± 2.7	10
CD-U	114.7 ± 8.8	194.5 ± 75.7	9.1 ± 2.7	70
CD-D	108.9 ± 9.9	155.1 ± 62.4	8.7 ± 2.3	40
YK	109.0 ± 7.4	143.9 ± 29.8	9.0 ± 1.8	60
Total	**102.2 ± 12.5**	**126.2 ± 65.3**	**9.88 ± 2.63**	**36**



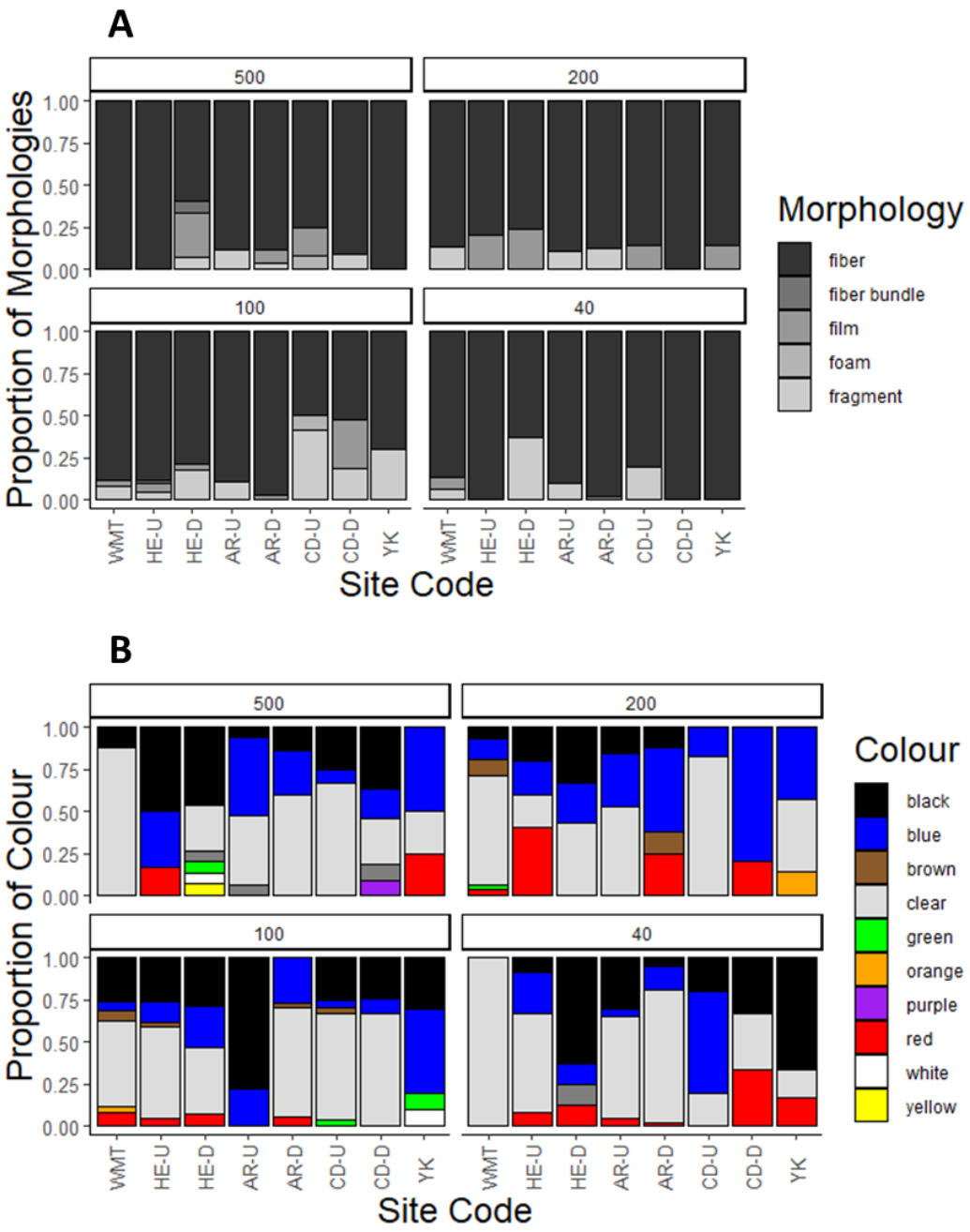
Appendix Figure A1. Linear relationship between mussel length and age of flutedshell mussels collected on the Grand River, ON, August 2021. A. Across all sites, **B.** Separated by site.

Appendix Table A10. Tukey HSD (length) and Dunn's Test (weight) significant differences in mussel length (left) and wet weight (right) across sites on the Grand River, ON, collected in August 2021. Bolded sites indicate differences between upstream and downstream at a WWTP.

Mussel Length			Mussel Weight		
Site 1	**Site 2**	**p-value**	**Site 1**	**Site 2**	**p-value**
CD-U	WMT	0.02	WMT	CD-U	0.01
CD-U	HE-U	0.001	HE-U	CD-U	0.0004
CD-D	HE-U	0.01	HE-U	CD-D	0.03
YK	HE-U	0.01	HE-U	YK	0.02
CD-U	HE-D	0.003	HE-D	CD-U	0.001
CD-D	HE-D	0.02	HE-D	YK	0.05
YK	HE-D	0.02			
CD-U	AR-D	0.03			



Appendix Figure A2. Abundance of microparticles in surface water across sites, separated by size fraction, collected from sites on the Grand River, ON, in August 2021 (AR-D sampled November 2021). The size of the filter used to capture microparticles separates each panel and is presented in decreasing order. **A.** 500 µm filter, **B.** 200 µm filter, **C.** 100 µm filter, **D.** 40 µm filter. Microparticle counts were multiplied by 5 to represent the total number of microparticles on a sub-sampled filter. Sites ordered generally from most upstream (left) to downstream (right), including panels to denote tributaries. Lighter colours represent upstream sites (U) while darker colours represent downstream sites (D). No statistically significant differences were observed across sites (n=3/site; ANOVA and Kruskal-Wallis Tests).



Appendix Figure A3. Particle morphology (A) and colour (B) of microparticles in surface water across sites, separated by size fraction (AR-D sampled November 2021). The size of the filter used to capture microparticles is presented in decreasing order, from 500 µm to 40 µm. Sites ordered generally from most upstream (left) to downstream (right).

Appendix Table A11. Proportion of each particle morphology across each of the four size fractions in surface water samples. The size of the filter used to capture microparticles is presented in decreasing order, from 500 µm to 40 µm.

Morphology	500	200	100	40
Fiber	86.6	85.6	79.3	92.2
Fragment	4.5	5.6	13.8	6.2
Film	7.5	8.8	5.4	1.7
Foam	0.7	0	1.0	0
Fiber Bundle	0.7	0	0.5	0
Sphere	0	0	0	0

Appendix Table A12. Proportion of each particle colour across each of the four size fractions in surface water samples. The size of the filter used to capture microparticles is presented in decreasing order, from 500 µm to 40 µm.

Colour	500	200	100	40
Clear	51.5	53.6	50.7	62.6
Blue	21.6	25.6	16.3	12.2
Black	20.1	11.2	24.1	18.3
Red	1.5	4.8	4.4	6.1
Gray	2.2	0	0	0.8
Purple	0.7	0	0	0
Brown	0	3.2	2.5	0
Orange	0	0.8	0.5	0
Green	0.7	0.8	0	0
White	0.7	0	0.5	0
Yellow	0.7	0	0	0