7-19-2018

Effect of Microplastics on the Accumulation of POPs in Fish

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by

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A Thesis

Submitted to the Faculty of Graduate Studies

Through the Great Lakes Institute for Environmental Research

In Partial Fulfillment of the Requirements for

The Degree of Master of Science at the

University of Windsor

Windsor, Ontario, Canada

2018

EFFECT OF MICROPLASTICS ON THE ACCUMULATION OF POPS IN FISH

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May 18, 2018

DECLARATION OF CO-AUTHORSHIP/ PREVIOUS PUBLICATION

I. Co-Authorship Declaration

I hereby declare this thesis incorporates material that is result of joint research, as follows:

I was the primary author and person responsible for the experimental implementation and majority of writing for each chapter included in this thesis. Chapter 2 was co-authored by Dr. Ken G. Drouillard and Dr. Sherri A. Mason. Ken G. Drouillard and Sherri A. Mason provided intellectual guidance, and editorial support and it is published in the journal, Chemosphere. Chapter 3 was co-authored by Dr. Ken G. Drouillard. Ken G. Drouillard provided intellectual guidance, statistical guidance, and editorial support and is being prepared for submission to the journal, Environmental Science and Technology.

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II. Declaration of Previous Publication

This thesis includes three original papers that have been previously published/will be submitted for publication in peer reviewed journals, as follows:

Thesis Chapter	Publication title/full citation	Publication status
Chapter 2	Grigorakis, S.; Mason, S. A.; Drouillard, K. G.	Published (Chemosphere)

	Determination of the gut retention of plastic microbeads and microfibers in goldfish (Carasius auratus). Chemosphere. 2017, 169, 233-238.	
Chapter 3	Effect of microplastic amendment to food on dietary PCB assimilation efficiency by fish	To be submitted

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ABSTRACT

Microplastic are ubiquitous in aquatic habitats and commonly found in the gut contents of fish yet relatively little is known about the retention of microplastic particles by fish. Microplastics also contribute to an anthropogenic organic phase in the environment capable of absorbing hydrophobic organic compounds including persistent organic pollutants (POPs). Relatively little is known about the potential interactions between microplastics and persistent organic pollutant (POP) exposures to fish. In order to determine how microplastic particles affect the accumulation of POPs in fish, I first determined the gut retention of two types of microplastic particles (microbeads and microfibers) in goldfish. Although a small number of microplastic particles were retained in fish GI-tracts after 6 days (0-3 particles/50), the retention of microplastics was generally similar to the retention of bulk digesta contents. According to a breakpoint regression model fitted to digesta contents and microplastic particles, the 50% and 90% evacuation times were 10 h and 33.4 h, respectively. The results of this study indicate that neither microbeads nor microfibers are likely to accumulate within the gut contents of fish over successive meals. In Chapter 3 of this thesis, I applied a duel-tracer design to quantify polychlorinated biphenyl (PCB) dietary assimilation efficiencies (AE) in goldfish to compare microplastic-associated PCB AEs with diet matrix-associated PCB AEs. Microplastic-associated PCBs showed a 13.36% (12.27-14.49%) assimilation efficiency in goldfish while food matrix-associated PCBs showed 51.64% (48.97-54.32%) assimilation efficiency; which is 3.9 fold higher than measured for microplastic-associated PCBs. The joint findings from this thesis indicate that microplastic particles, and POPs associated with them, are unlikely to significantly enhance POPs bioaccumulation by fish.

DEDICATION

To my parents who always encourage me to dream big and to my girlfriend Heather for never letting me give up.

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor, Dr. Ken Drouillard, for providing me the opportunity to work as a MSc student under his tutelage. Dr. Drouillard not only provided me with excellent supervision and guidance, but also compassion and patience when I needed it most. I am endlessly grateful for the opportunity he presented me.

I would also like to thank Dr. Dennis Higgs and Dr. Chris Weisener for being a part of my advisory committee. I appreciate their valuable input and discussion to my research projects.

I want to thank all of the members of the Drouillard Lab that I have had the pleasure to work with: Bronson Goodfellow, Joe Lafontaine, Xin Sun, Jenna O'Brien, Joe Robinet, and Shifeng Yao. A special thank you to Bronson and Joe L for our endless discussions, the vast amount of time they spent helping me, and for their motivation they gave me to work hard and never give up.

Whether it was soccer or school, my parents did everything in their power to ensure I was always provided the best opportunities possible. Their endless supply of support and advice carried me through this MSc project. I love them and do not know how to possibly thank them enough for all they have done for me throughout my life.

Lastly, I would like to thank my girlfriend Heather. As my mental and physical health took a toll on me, she was a rare source of happiness and motivation in my life. When I was ready to quit, Heather picked me up by the bootstraps and carried me until I could carry myself again. Thank you for always standing by my side. I love you.

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General Introduction

1.1 General Introduction

In his review paper, Pruter (1987) made note of studies finding "tiny pellets" of plastic in the environment. The first study to explicitly refer to these small plastic particles as microplastics was published in 2004 (Thompson et al., 2004). The field of microplastic research dramatically expanded after 2011 as demonstrated by a literature search. A Scopus database search performed using the term "microplastic" yielded 444 publications (excluding non-environmentally relevant database hits) over the years 1967—2017 (see Figure 1.1).

Microplastics are characterized as plastic particles under 5mm in size. As a result of this broad definition, microplastics can be further categorized into plastic type, shape and degree of weathering. Plastic types include Polyethylene (PE), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC) which reflect some of the most commonly produced plastics on a global basis. Microplastics can be further classified into 5 shapes (Table 1.1) (Eriksen et al., 2013). Weathered categories of microplastics are sometimes designated as primary microplastics which refer to plastics that have been intentionally manufactured as < 5mm in size and released to the environment or secondary microplastics which were manufactured as macroplastics and have broken down into microplastic size classes through UV radiation and mechanical stress such as wave action or abrasion occurring in the environment (Cole et al., 2011).

Eriksen and colleagues estimate that there are at least 5.25 trillion microplastic particles in marine waters (Eriksen et al., 2014). The shorelines of urban areas tend to have more microplastic pollution than shorelines in more remote areas (Leite et al., 2014). Sediment as deep

as 4843m, from the Porcupine Abyssal Plain, have been found to be contaminated with microplastics (Van Cauwenberghe et al., 2013). Lake Hovsgal, northern Mongolia, is a government protected park that has less than 10,000 people inhabiting a vast area surrounding the lake. Despite this, Lake Hovsgal contains as much plastic as some heavily polluted areas (Free et al., 2014). Any one particular area/body of water exhibits a great deal of heterogeneity in terms of microplastic dispersion (Goldstein et al., 2013). A 2015 review of microplastics shows that microplastics have contaminated both marine and freshwater systems; all while interacting with various organisms (Eerkes-Medrano et al., 2015). This latter review of studies showcases the extent of knowledge available in the field of microplastics: presence/distribution, transport pathways, and proper methodology associated with environmental sampling for microplastics detection and characterization.

Interactions between microplastics and aquatic organisms are an area of special concern, considering the relative abundance and apparent global distribution of microplastic in water systems. Various species of zooplankton are capable of ingesting microplastics and can further transfer those plastic particles to the predators that consume them (Frias et al., 2014; Browne et al., 2013; Setala et al., 2014). Mussels have also been shown to not only passively uptake and accumulate microplastics, but also to transfer those plastics to higher trophic levels (Farrell and Nelson 2013; von Moos et al., 2012; Collignon et al., 2012; Santana et al., 2017). While the results of these studies are interesting, it is worth noting that the studies were designed to test the possibility of the trophic transfer of microplastics. In essence, the prey items (zooplankton/mussels) were exposed to high concentrations of microplastic and were fed to their predators as soon as they were seen egesting microplastics. Such designs may not be indicative of a natural system. Nonetheless, microplastic exposure to organisms in their natural habitat has

been verified. Zooplankton, mussels, lugworms, and whales have been found to have microplastics in their gut tracts upon capture (Van Cauwerberghe et al., 2015; Lusher et al., 2012; Lusher et al., 2015). Thus, ingestion of microplastics by organisms has been documented to occur but there remains a lack of knowledge in regard to how plastic exposures may affect the health of organisms that consume them. Table 1.2 illustrates examples of potential direct and indirect effects of microplastics on organism's health.

Direct toxicity of microplastic on organisms in various trophic levels has been reported. Cole and his colleagues put various zooplankton species in 20 mL of sea water and added increasing amounts of microplastics that were in the size range of natural food items (Cole et al., 2013). Feeding rate of algae was not lowered until the concentration of microplastics reached 4000 particles/mL and did not show a significant difference until a concentration of 7000 particles/mL. It was noted that microplastic particles were sticking to exterior appendages of zooplankton; which impedes with the organism's ability to swim and find food items. Concentrations of microplastics higher than 4000 particles/mL are not environmentally relevant. Zooplankton mitigate their risk of consuming microplastics by coupling two factors: Zooplankton will avoid non-food items in search of preferred items and the encounter rate of microplastics compared to prey items in the environment are very low (Lima et al., 2014). Microplastics can be transferred up the planktonic food web in a lab setting (Setala et al., 2014). Mysid shrimp were shown to uptake microplastics from zooplankton that were previously exposed to high concentrations of microplastics. The Setala study highlights the possibility of indirect microplastic exposure on organisms in higher trophic levels. Only 65% of Acartia species exposed to 10000 particles/mL ingested microplastic particles; further showcasing zooplankton's ability to avoid non-food items. *Dapnia magna* were capable of ingesting

microplastic spheres of different sizes (20 nm and 1000 nm), but excreted the 1000 nm spheres significantly more than the 20 nm spheres (Rosenkranz et al., 2009). This indicates that the 20 nm spheres may have translocated into tissues but adequate resolution was not attained to verify such claims. Direct effects of microplastic toxicity has been better documented in lugworms (*Arenicola marina*). The presence of microplastics, even at concentration as low as 0.074% dry weight, in sediments caused a significant decrease in feeding activity of lugworms compared to the control group (Besseling et al., 2012). With a diet of 7.4% dry weight microplastic in sediments, lugworms had 50% less tissue energy content than lugworms not exposed to microplastics. Lugworm fitness in natural environments could therefore be lowered in the presence of microplastics due to reduction in growth. Lugworms chronically exposed to unplasticized PVC (UPVC) had increased phagocytic cell activity; which is an expensive metabolic task and also caused a 50% reduction in available energy (Wright et al., 2013).

A potential secondary toxicity effect associated with microplastics may be related to how these particles interact with toxic chemicals present in the environment. Phenanthrene and other environmentally persistent, hydrophobic contaminants have a high affinity for plastic and the fate of these chemicals in a natural environment can be influenced by the fate and distribution of plastic (Teuten et al., 2007). Besseling and colleagues spiked microplastics with polychlorinated biphenyls (PCBs) and observed significant uptake of PCBs by lugworms when in the presence of contaminated microplastics (0.074, 0.74, and 7.4%) amended to sediments. Lugworms exposed to microplastic spiked with triclosan had reduced survival and feeding activity (Browne et al., 2013). Browne and his colleagues also showed that polybrominated diphenyl ethers (PBDEs) dosed microplastics lowered feeding, while nonyphneyl- lowered immune response, and direct exposure to polyvinyl chloride (PVC) microplastics lowered antioxidant capacity. This was

determined to be due to a combination of reduced feeding, longer gut retention times of ingested material (egestion events took 1.5 times longer during chronic exposure to UPVC), and inflammation. Medaka fish (*Oryzias latipes)* exposed to microplastics naturally contaminated with polycyclic aromatic hydrocarbons (PAHs), PCBs, and PBDEs had concentrations higher than control and negative control groups; though it was only significant for PBDEs (Rochman et al., 2013). In another study using medaka, Rochman and colleagues found that chronic exposure to microplastics at environmentally relevant concentrations had endocrine disruption effects (Rochman et al., 2014).

1.2 Polychlorinated Biphenyls as Model of Persistent Organics Pollutants

PCBs are among the most common and widely distributed contaminants in the category of persistent organic pollutants (POPs) and tend to be found in higher concentrations around industrialized areas (Beyer and Biziuk, 2009). Their resistance to degradation from metabolism and high degree of hydrophobicity make PCBs capable of bioaccumulation and food web biomagnification in aquatic ecosystems (Hornebuckle et al., 2006). PCBs were banned over 30 years ago, but their presence in biota and sediment still persists today (Hornebuckle et al., 2006). There are 209 possible congeners of PCB; all with varying physical-chemical properties, such as hydrophobicity (Hornebuckle et al., 2006). The degree of hydrophobicity that a chemical exhibits is measured by how the chemical partitions between octanol and water (Kow). As Kow increases, so does the degree of hydrophobicity. Humans exposed to PCBs can suffer from both acute and chronic symptoms. Human populations in Kyushu (Japan) and Yu-Cheng (Taiwan) were both accidentally contaminated by large amounts of PCBs released into the food supply via contaminated rice oil during industrial accidents. This caused Yusho disease with symptoms that included lesions on the skin and eyes (Onuzuka et al., 2008). Chronic symptoms are often related

to child birth. Pregnant woman with Yusho disease had babies with a lower birth weight (~160-250g) and at age 4, those children continued to exhibited lower weights in a dose-dependent fashion (Jacobson and Jacobson, 1993). Human exposure to PCB is 90% from the diet with fish contributing the bulk of human PCB exposures (Liem et al., 2000). Sub-populations of humans can be exposed to higher levels of PCB as a result of high fish consumption in contaminated areas (Liem et al., 2000). Considering the potential for microplastics to alter the bioavailability of hydrophobic contaminants such as PCBs and ubiquitous presence of both microplastics and PCBs in the environment, it is imperative that microplastic-PCB interactions be further studied.

1.3 Microplastic/POPs Interactions on POPs Bioaccumulation by Fish

Microplastics have the potential to influence both the uptake and elimination of hydrophobic contaminants, yet they are not accounted for in current bioaccumulation models. The potential of microplastics altering the bioavailability of contaminants, such as PCBs, can be explained by using the following equation for bioaccumulation:

$$\frac{dC_b}{d_t} = (Gv \cdot Ew \cdot C_{wd} + Gfeed \cdot AE_d C_d) - \left(\frac{Gv \cdot Ew}{BCF} + \frac{Gfeed(1 - AEfood) \cdot AE}{Kb,ex} + k_m + k_g\right) C_b$$

The variables C_b, C_d, and C_{wd} represent the concentration of chemical in the fish (ug/g), in the diet (ug/g), and dissolved in water (ug/mL), respectively. G_v and G_{feed} represent gill ventilation rate ($mL/g_{bw}/d$) and feeding rate ($g_{feed}/g_{bw} \cdot d$), respectively. BCF represents the animal/water partition coefficient (L/d) and $K_{b.ex}$ represents the animal/feces partition. Metabollic biotransformation (k_m) and growth dilution (k_g) have units of 1/d. Chemical exchange efficiency across gills (E_w) and chemical assimilation efficiency from food (AE) are both

unitless. A modified bioaccumulation model that considers microplastic/POPs interactions could be constructed whereby microplastics are treated as a non-assimilated 3rd phase of organic material that flows through the GI-tract via feeding and fecal egestion. Microplastics presence within the GI-tract can be hypothesized to also alter multiple toxicokinetic parameters within Equation 1. For example, microplastics could affect the magnitude of AE of hydrophobic chemicals by acting as a non-assimilated sorptive pool or organic material in the digestive tract. This would occur if PCB-microplastic sorptive interactions are capable of resisting PCB solubilisation and transport across unstirred water layers as facilitated by mixed micelles (bile salt/fatty acid vesicles generated in the small intestine). If POP/microplastic sorptive capacity was high relative to miscelle vesical partition capacities, we would expect that high microplastic contents in the diet would decrease the AE of POPs chemicals. The second toxicokinetic parameter likely impacted by microplastic presence in the GI-tract is $K_{b,ex}$. This parameter refers to the organism/feces partition coefficient and is estimated as the magnitude of the lipid equivalent content of the organism compared to its feces and/or lower intestinal digesta contents. Under the GI-magnification model, $K_{B,ex}$ progressively decreases from food to feces as lipids and organic carbon from ingested food is assimilated by the organism. However, because microplastics are considered a non-absorbable 3rd phase of high PCB sorptive capacity, $K_{B,ex}$ will be expected to be higher when animals are fed a microplastic containing diet compared to when they are given non-microplastic diets. This would be theoretically similar to the effect mineral oil has on the elimination of mirex/DDT (Rozman, 1983). If microplastics in food cause decreases in both AE_d and $K_{B,Ex}$, then the POPs bioaccumulation and biomagnification potential in the animal will be expected to decrease. Microplastics could also lower C_{WD} because freely dissolved chemical in water would scavenge onto plastic, thus decreasing chemical exposures

across the gill surface. Finally, microplastic-adsorbed PCBs incorporated into food could increase the C_{food} of ingested food. This would potentially increase animal exposures to PCBs and potentially counteract alterations in AE_d or $K_{B,Ex.}$

In order to assess the impact of microplastics on bioaccumulation of POPs by fish, the effect of microplastics on POPs AE, K_{BEX} and C_{diet} need to be determined. Before we can study all of the above, some baseline studies need also be completed. First, microplastic exposure needs to be shown to be food web based. Considering the various organisms shown to naturally ingest microplastic particles in their native environments, it can be safely concluded that microplastic particles are a part of the aquatic food web (Eerkes-Medrano et al., 2015). Second, the partition capacity of microplastics for POPs compounds and demonstration of POP-microplastic contamination in natural environments need to be demonstrated. Several studies have, to date, quantified partition capacity of different plastic types including polyethylene and polystyrene for POPs compounds such as PCBs (Lee et al., 2013, 2017; Smedes et al., 2017). Other studies have documented the presence of PCBs, PBDEs and PAHs present in environmental samples of microplastics (Kalogerakis et al., 2014; Rios and Jones 2015). Third, the gut retention times of different microplastic types (by shape and plastic type) need to be determined to establish if microplastic retention in the gut tract is essentially the same as digesta, or if microplastics have the capacity to accumulate within the gut tract over successive meals. The latter is necessary to understand the potential size of the microplastic pool that can be accumulated within a fish's gut tract and whether this can be determined based directly on the microplastic content of ingested food or if a separate microplastic GI-tract bioaccumulation model would be necessitated. Fourth, AE values of microplastic-associated POPs need to be measured and compared with AEs of POPs presented in diet unaffected by microplastic particles. To date, only two studies have measured

POPs AEs after loading chemicals on to microplastics (Granby et al., 2018; Wardrop et al., 2016). Last, K_{BEX} should be directly quantified in fish fed food containing microplastic particles and compared with diets not containing microplastics (Drouillard et al. 2012). The latter would be necessary to appropriately model enhanced elimination of POPs compounds by fish when fed microplastic containing diets.

1.4 Thesis Objectives

The objectives of this thesis were to characterise microplastic gut retention times and to determine microplastic-diet matrix interactions on POPs dietary assimilation efficiencies by fish. Chapter 2 of this thesis quantified the gastro-intestinal retention of two types of microplastics (microbeads and microfibers) in gold fish after feeding fish food amended with each microplastic type. The following specific hypotheses were tested in Chapter 2:

Hypothesis 1: microbeads, derived from personal care products, are retained and lost from the gastrointestinal tract of fish at the same rate as food/digesta

Hypothesis 2: microfibers, derived from laundered textiles, are retained and lost from the gastrointestinal tract of fish at the same rate of food/digesta

Hypothesis 3: microbeads have similar retention in the gastrointestinal tract of fish as microfibers

Chapter 3 of this thesis determined the dietary assimilation efficiencies (AE) of polychlorinated biphenyls (PCBs) by fish in the presence and absence of microplastics (personal care product derived microbeads) added to their food at five different microplastic treatment concentrations. This study applied a unique duel tracer design whereby non-environmental PCBs were adsorbed onto microbead particles and added to fish food pellets which had been previously dosed with a different set of environmental PCBs. This enabled simultaneous determination of

microplastic-associated PCB AEs and diet matrix associated PCB AEs and their potential interactions across diet treatments. The specific hypotheses tested in Chapter 3 included the following:

Hypothesis 1: microplastic-associated PCBs have similar AE values as diet matrix-associated PCBs

Hypothesis 2: diet matrix-associated PCB AEs are unaffected by the presence of microplastic particles in the diet across different treatments that vary microplastic contents (0 to 25% microplastic content in food by weight)

Hypothesis 3: PCB AEs from microplastics or the diet matrix demonstrate similar relationships with chemical hydrophobicity

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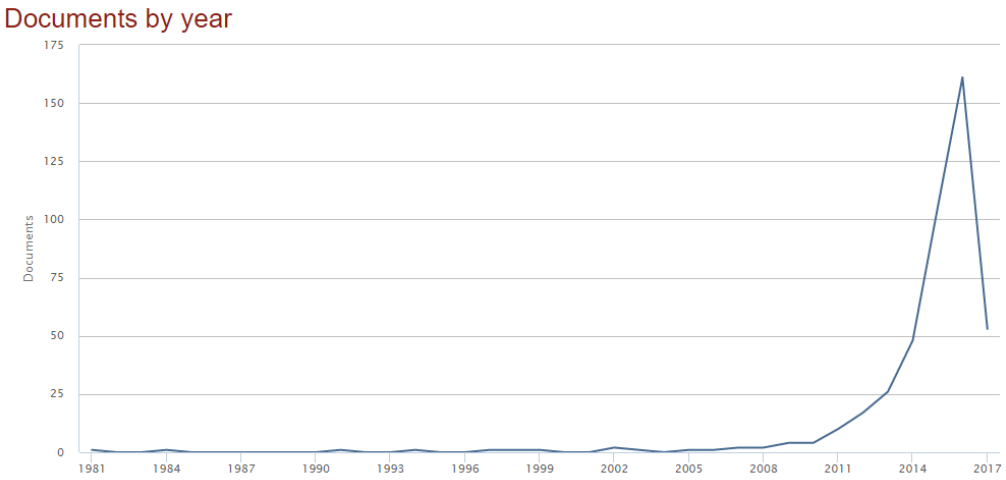

Figure 1.1. Documents explicitly mentioning microplastics by year (2017 data is up to date as of April 5, 2017).

Microplastic shape	Source
Pellet	Microbead exfoliates (face wash)
Fibre/line	Effluent of washing machines (clothes)
Fragment	Hard plastics (chairs, tables, etc)
Foam	Cushions
film	Plastic bags

Table 1.1. Different shapes of microplastics and their sources (Eriksen et al., 2013)

Direct Toxicity	Indirect Toxicity
Clogging feeding apparatus Gastric blockage Gastric distention (leading to lower feeding)	Exposures to toxic substances associated with microplastics • Leachates from plastic interior • Absorbed/partitioned chemicals picked up from the environmnet by microplastic particles
➔ Reduced growth and fitness	➔ Oxidative stress, endocrine disruption, reproductive toxicity

Table 1.2. Examples of direct and indirect effects of microplastics on organisms.

CHAPTER 2

Determination of the gut retention of plastic microbeads and microfibers in goldfish (*Carassius auratus*)

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2.1 Introduction

Microplastics are a diverse array of synthetic polymer particles that vary in chemical composition, size (from low micrometre scale to an upper size range variously defined between 1 nm and 5 mm), density and shape (Andrady, 2011). They have been observed in most freshwater and marine environments (Eriksen et al., 2014; Corcoran, 2015; Eerkes-Medrano et al., 2015) to such an extent that they were included as sedimentary geochemical markers of the Anthropocene (Waters et al., 2016). Microplastics are often distinguished between those that are synthesized at the defined sizes for an intended application (primary microplastics) relative to particles derived from the breakdown of macroplastics (secondary microplastics). Microbeads are defined as primary microplastics that range in size between 0.1 μm to <5 mm (Environment Canada, 2015) and are used in a wide variety of industrial and consumer applications including personal care products (PCPs). Legislation banning the production of microbeads in PCPs comes into effect in 2017 as passed by the U.S. federal government and similar legislation is under review in Canada. While much of the legislative focus has been on microbeads used in PCPs, other common sources of microplastics to municipal wastewaters include abraded particles from synthetic

textiles such as nylon and acrylics, henceforth referred to as microfibers, used in clothing (Browne, 2011).

Concerns have been raised about the ecotoxicology of microplastics in the environment, including their potential to bioaccumulate in organisms and subsequent transfer through food webs (Sánchez et al., 2014). Zooplankton are capable of ingesting microplastics, potentially mistaking them for food, and can further transfer these to tertiary consumers (Frias et al., 2014; Browne et al., 2013; Setala et al., 2014; Rehse et al., 2016). Mussels have been shown to accumulate microplastics and transfer them to higher trophic levels (Browne et al., 2013; von Moos et al., 2012; Collignon et al., 2012). In a study examining 504 fish from the English Channel that included benthic and pelagic species, 36.5% of specimens had microplastics in their gastrointestinal (GI-) tracts (Lusher et al., 2013). Microplastics in the gut contents of field collected fish have subsequently been widely reported in coastal and freshwaters (Sanchez et al., 2014; Neves et al., 2015; Avio et al, 2015; Phillips and Bonner, 2015; Biginagwa et al., 2016; Bellas et al., 2016). Considering microplastics are being found in fish, there are relatively few studies focussing on the potential of microplastics to bioaccumulate.

Exposure to microplastics in water and food can interfere with normal digestive processes due to intestinal blockage, causing reductions in animal feeding rates and energy assimilation (Besseling et al., 2012), lead to histopathological alteration to intestinal and hepatic tissues of fish (Pedà et al. 2016; Lu et al., 2016) and lower hatching success of fish eggs (Lönnstedt and Eklöv, 2016). Translocation of microplastics from gut to the circulatory system has been demonstrated in mussels (Browne et al., 2008; von Moos et al., 2012; Avio et al. 2015a) implying that retention of microplastics beyond entrainment in the GI-tract may be possible in

some animals. Avio et al., (2015b) and Lu et al., (2016) confirmed microplastics accumulation in hepatic tissues of fish exposed to microplastics at elevated concentrations in water.

Although microplastics are commonly detected in the intestinal tracts of fish, there is limited information characterizing the retention of microplastics by fish. Particle size and shape are likely to influence factors such as GI-retention but limited information is available comparing microplastic types. Neves et al. (2015) observed a higher frequency of fibers in commercial fish gut contents compared to plastic fragments. The above study further reported differences in plastic types in benthic fish, which tended to accumulate a greater proportion of fibers, compared to pelagic fish which contained more fragments. It is not known whether these differences are related to emission patterns and fate of different particle types or whether particle shape might influence the gut retention characteristics of these microplastic types. In this study, the GI-tract retention of two microplastic types, microbeads and microfibers, was determined in goldfish with the objective to determine if i) retention of microplastics by fish exceeds that of food digesta, i.e. exhibits net accumulation in the GI-tract of fish, and ii) to determine if microfibers are retained to a greater or lesser degree than PCP derived microbeads.

2.2 Methods

Microplastic source

Microfibers were extracted from clothing (35cm x 12cm cut out of a commercial polyester fleece scarf) by mechanical agitation in hot water. Following agitation, the water was sieved through stacked 500 µm, 250 µm and 63 µm sieves. Fibers retained on the 63µm sieve were removed by tweezer under magnification and size graded to between 50-500 µm fiber lengths under a dissecting microscope. Microplastic beads were extracted from a commercial cosmetic product (facial cleanser labelled with polyethylene). The contents of the product was

poured onto a 63 sieve and the soluble matrix associated with the product washed with water until only microplastics remained. Microbeads were removed from the sieve under magnification. Figure 2.1 provides images of isolated microbeads and microfibers under 5x magnification.

Experimental

Goldfish were selected as a model fish species because they have been routinely used in many bioaccumulation/toxicokinetic studies owing to the ease of husbandry, tolerance to handling and willingness to accept artificial diets. In their wild state, goldfish are benthic feeders and thus might be expected to accumulate microplastics similar to those reported for other benthic feeders. Fish were exposed to microplastics via food. Commercial fish pellets (0.18-0.21g, ~3 mm size) were placed in warm water to soften them. Treatment pellets were amended with 50 microbeads or 50 microfibers per pellet by manual insertion of macroplastic particles into each pellet under microscope. Pellets were air dried after manipulation. Control pellets were wetted and dried in an identical manner but not amended with microplastics. The food was prepared in this manner to ensure that every experimental fish consumed exactly 50 microplastic particles to increase precision of gut retention characterization.

Twenty-Eight sexually mature goldfish were fasted for 48 h prior to exposing them to prepared food in order to ensure complete evacuation of gut contents from previous meals and to increase the likelihood that they would accept the microplastic amended pellet provided to them. After fasting, fish were removed from their communal tank and placed in individual fish bowls. Twenty four fish were allocated to the microbead and microfiber treatments, respectively. Five fish were allocated as controls and fed non-amended pellets. Each fish was presented with a single treatment pellet and observed until it was verified that the fish consumed the pellet. After

the fish consumed the treatment pellet, non-amended fish pellets were added to the bowl and the fish was allowed to consume to satiation for up to 60 minutes. Any remaining fish food in the bowl was subsequently removed. Fish were fasted for the remainder of the experimental period. Control fish were sacrificed after 1.5 h from feeding the control pellets. Triplicate animals from each treatment were sacrificed after 1.5, 4, 8, 16, 32, 48, 96, and 144 h. The mean ± SE of water temperatures was 14.2 ± 0.21 ^{o}C and exhibited no changes over the fasting duration. The mean ± SE body weights of fish from the microbead and microfiber treatments were 24.80 ± 2.77 g and 27.07 ± 3.40 g and were not significantly different from one another ($p>0.4$; ANOVA). On sacrifice, fish were euthanized by immersion in a solution of MS-222 (100 mg/L) and stored frozen until subsequent analysis. This research was performed under ethics approval from the University of Windsor's Animal Care Committee.

Microplastic analysis

On analysis, the gut tract of each fish was dissected and removed. The gut contents were pushed thought the intestine using tweezers and a probe onto a pre-weighed aluminum weight boat and the gut tract tissues were retained for further analysis. The weigh boat was dried at 110^{o}C for 1 h and reweighed to determine dry food digesta weight. Subsequently, the dried digesta and gut tissues were re-combined and placed into a 10% KOH solution on a hot plate set at its lowest setting for 1 hour. The solution was taken off of the hot plate and after 2 additional hours, 5mL of 30% H_2O_2 was added to the solution. The solution was poured through a vacuum filtered Buchner funnel using Whatman^{TM} (55mm) filter papers (1 µm glass fiber filters). Fish carcass samples were also digested in a similar manner. Filter papers from each digestion were analyzed under a stereomicroscope to quantify the number of microplastics remaining in the GI-

tract/contents, fish carcass or digested food pellets. Quality control of the method was established by measuring and verifying microbeads and microfibers in 5 amended pellets. The mean ± standard deviation of recoveries of microplastic particles for the digested pellets was 98.8±1.8%.

Data analysis

Digesta contents weights were standardized to the mean body weight according to:

$$X_{DG(ss)} = X_{DG(s)} \cdot \frac{BW_{(mean)}}{BW_{(s)}} \quad (1)$$

where $X_{DG(ss)}$ is the size standardized digesta weight (g), $X_{DG(s)}$ is the digesta weight measured in an individual fish, $BW_{(mean)}$ is the mean body weight of fish from the treatment and $BW_{(s)}$ is the body weight of the individual fish. The % remaining of digesta contents was calculating by dividing $X_{DG(ss)}$ by the mean $X_{DG(ss)}$ generated for fish sampled at the first time point (1.5h) and multiplying by 100. For microbeads and microfibers, %remaining was calculated by dividing the number of microplastics measured in a fish's digestive tract by 50 and multiplying by 100.

Statistical analysis was performed using a general linear model (GLM) according to:

Model = Time + Group + Time * Group + Constant (1)

Where time is the time since feeding (h), group represents a categorical variable specified as digesta retention treatment 1, digesta retention treatment 2, microfibers and microbeads. Under cases where the interaction term (Time * Group) was non-significant, analysis of covariance (ANCOVA) was performed to adjust for time as a covariate and increase the statistical power of the group comparison test. Where the interaction term was found to be significant, GLMs were performed on subsets of the data to test for differences between selected group comparisons.

GLM(1) tested for differences in digesta retention time between treatment 1 and treatment 2. GLM(2) tested for differences in digesta retention time and microfiber retention from measurements taken in treatment 1. GLM(3) tested for differences in digesta retention time and microbead retention from measurements taken in treatment 2. Finally, GLM(4) tested for differences in microfiber and microbead retention. Data transformation was necessary owing to failure of normality of the % retention data on the combined data (digesta, microbeads and microfibers). However, when the fist time point (1.5 h) was removed, transformation of % retention data by natural log transformation yielded a normal data set ($p>0.05$; Lillefor's test). Thus, statistical comparisons by GLM were performed with the 1.5 h time point removed and applying a ln transformation. Non-transformed digesta retention data (inclusive of the 1.5 h time point) for individual fish were subsequently fit to an exponential model using non-linear least squares regression according to:

$$\%Retained = 100 \cdot e^{-B \cdot time} \quad (2)$$

Where 100 is constant forcing 100% of gut contents retention at time 0, b is the fitted coefficient and time is time since feeding (h). The ability of Eq. 2 calibrated independently to gut contents to predict microplastic retention was evaluated using goodness of fit tests by performing a linear regression on observed (microplastic) vs model (Eq. 2) predicted digesta retention. The goodness of fit result was evaluated by determining if the slope was significantly different from 1, the constant was significantly different from 0 and by evaluating the magnitude of the coefficient of determination. All statistics were performed using Systat 13 statistical software. Except where otherwise noted, measures of central tendency and variation are expressed as mean and standard error (SE).

2.3 Results and Discussion

Digesta retention

During experimental trials, all fish were observed to consume the microplastic amended treatment pellet. No fish mortalities occurred nor were there apparent signs of distress following exposure to the amended food pellet. Fish sacrificed at the 1.5 h time point had a mean $X_{DG(ss)}$ weight of 0.60±0.04 g. This corresponds to a food consumption of 2.32% body weight across the treatments and is consistent with expected food consumption rates in fasted fish.

A general linear model (GLM(1) as described in methods) was performed to compare % retention of digesta between the two treatments. The GLM and ANCOVA revealed a non-significant ($F_{1,39}$ = 0.92; p>0.3; ANCOVA) difference in digesta retention between the treatments after adjusting for time as a covariate. Given that digesta retention did not significantly differ between the two treatments, the data were combined and fit to the exponential model yielding the following solution:

$$\%Retained = 100 \cdot e^{-0.069 \cdot time}; \ R^2 = 0.69 \quad (3)$$

Based on Eq. 3, the time to evacuate 50% and 90% of digesta was 10.0 and 33.4 h, respectively. Overall, the exponential model fit described the temporal trends of digesta contents well during the first 24 h but tended to underestimate observed digesta contents at longer time points (Figure 2.2). This may be related to the method of separating gut contents from the intestinal tissues which could have included residual gut secretions and/or sloughed cells/tissues generated from the GI-tract processing method itself. However, the fitted model produced retention estimates that were generally consistent with other studies on digesta retention in fish of similar size and temperature. Stehlik et al., (2014) reported full clearance of gut contents from clearnose skate (*Raja eglanteria*) by 48 h when held at 15^{o}C. Yellow perch held at 17.1^{o}C exhibited a gut

evacuation coefficient of 0.035·time (h^{-1}) based on a log linear model which implies a 50% digest retention of 19.8 h and 90% retention of 65 h (Gringas and Boisclair, 2000).

Microplastic retention in GI-tracts

Control fish sacrificed after 1.5 h were examined for evidence of microplastics in gut contents and carcass samples. No microplastics were found in control fish or within their gut contents. In addition, 10 control fish pellets were examined for presence of microplastics. Similar to control fish, microplastics were not observed in non-ammended food pellets.

During the first sampling point (1.5 h), there was good recovery of microplastics within the gut contents of treatment fish. For microfibers, 2 fish had 50 microfibers recovered (100% recovery) and the third fish had 48 fibers recovered in the GI-tract. For the microbeads, 40 to 44 particles (80-84% recovery) were recovered from fish during the first time point. Small numbers of microplastics were recovered at the 144 h time point (1 to 3 microfibers in replicate 144h sampled fish and 0 to 3 microbeads in triplicate fish).

A general linear model (GLM) was applied to test percent retention of all treatments (digesta from each treatment, microbeads and microfibers) within the study. Both Time ($F_{1,76}$ = 88.1; $p<0.001$) and the Group x Time ($F_{3,76}=3.09$; $p<0.05$) interaction terms were significant but group was not significant ($F_{3,76}$ =0.212; $p>0.8$) in the overall GLM. Due to the significant interaction terms, additional GLMs were applied to subsets of the data to evaluate for differences in retention on selected measurements. GLM(2) and the ANCOVA revealed no significant differences ($F_{1,39}=0.959$; $p>0.5$; ANCOVA) in microfiber and digesta retention. Similarly, GLM(3) and ANCOVA revealed non-significant ($F_{1,39}=4.00$; $p>0.05$; ANCOVA) differences in microbead retention from gut digesta retention. Finally, a comparison of microfiber and

microbead retention yielded non-significant differences ($F_{1,39}=0.678$; $p>0.4$; ANCOVA) from one another. Microplastic and microfiber retention with time along with digesta contents trends are presented in Figure 2.2.

For microfibers, the linear regression between %microfiber retention and gut digesta model (Eq. 3) prediction yielded a slope of 0.96±0.09, constant of (7.33±4.07) and coefficient of determination (R^2) of 0.85. The above slope was not significantly different from unity ($t_{1,22}=0.042$; $p>0.5$; t-test) and the constant was not different from zero ($t_{1,22}=1.80$; $p>0.05$; t-test). For microbeads, the goodness of fit test produced a similar slope (0.94±0.04) that was not significantly different from unity ($t_{1,22} = 1.44$; $p>0.1$; t-test) and constant (3.67±2.06) not significantly different from zero ($t_{1,22}=1.79$; $p>0.05$; t-test) with an R^2 of 0.95. It is perhaps notable that the digesta retention model (Eq. 3) which was calibrated only to digesta retention data explained even more variation in microplastic retention than digesta contents itself. This was mainly related to the better fit of model predictions to microplastic retention at the later time points (Figure 2.2). The reason for the differences in model fit across measurements is attributed to the fact that microplastic exposure was controlled with a high degree of precision compared to gut contents. Although each fish was given exactly 50 microplastic particles, they were provided with food ad libitum after verifying their consumption of the microplastic amended pellet. Thus, digesta contents would have varied to a greater extent between fish compared to microplastic exposures. Overall the goodness of fit tests indicates that the gut digesta retention model adequately described the retention of both microplastic types.

Similar observations were generated for the marine isopod *Idotea emerginata* fed a diet spiked with microplastic particles and fibers (Hämer et al,. 2014). In the study by Hämer et al., (2014), microplastic particles appeared in the stomach and gut contents of isopods but were also

readily egested with the feces. Mazurais et al. (2015) examined microplastic retention in European sea bass (*Dicentrarchus labrax*) larvae when exposed to microplastics added to food. The above authors observed a correlation between microbeads in the gut of larvae with concentration of microbeads added to the diet. However, the authors noted that microbeads were fully cleared from the gut of larvae after 2 days post exposure and could be identified in feces suggesting passive retention in the gut contents of fish. While fish are regularly feeding in their native environments, microplastic particles retrieved from the gut contents of fish are still likely to be a result of the most present feeding rather than an accumulation across successive meals.

Microplastics were also examined in carcass samples of treatment fish but were not observed apart from the gut tissue and gut contents analyzed separately and discussed above. This differs from the results of Avio et al., (2015b) who observed translocation of polyethylene and polystyrene microplastics to liver of laboratory held mullet (*Mugil cephalus*) exposed to microplastics in water (nominal microplastic dose was $2.5x10^3$ particles/L of polyethylene or polystyrene particles sized from 100 to 1000 µm) for 7 days. Between 1-2 microplastic particles per individual were detected in liver of exposed fish, although the presence of microplastics in liver was two orders of magnitude lower than what was observed in gut contents of fish. Similarly, Lu et al. (2016) exposed zebrafish (*Danio rerio*) to solutions containing 5 or 20 µm diameter polystyrene microplastics at concentrations of between 4.5×10^6 to 2.9×10^8 particles/L for 7 days. The above authors reported that 5 µm microplastics accumulated in fish gills, liver and gut, whereas larger microplastics (20 µm in diameter) accumulated only in fish gills and gut but not in liver. Time to steady state of microplastics in zebra fish was reported to be 48 h, implying rapid clearance from animals consistent with the gut retention data presented here (Lu et al., 2016). While the present study failed to identify microplastic translocation in fish tissues

apart from their detection in the GI tract, this could be a function of exposures to different microplastic types, different dosing strategies, levels of exposures and differences in the method of detection of microplastics in exposed animals. The lack of translocation of larger (20 µm plus) sized microbeads to liver in zebra fish as reported by Lu et al., (2016) is consistent with the present work given that particles greater than 63 µm were utilized but is not consistent with Avio et al. (2015b) who exposed fish to microplastics of comparable size to this research. Avio et al., (2015b) and Lu et al., (2016) provided continuous exposures of fish to microplastic contaminated water for up to 7 d days compared to a single dose from a microplastic amended meal applied in the present study. The above authors also used nominal microplastic concentrations in water that were considerably higher than what is present in natural waters. Avio et al., (2015b) used a more sensitive microplastic extraction/detection technique that employed a combination of density gradient separation and oxidant treatment which was shown to yield higher recoveries of microplastics from animal tissues then the oxidation treatment alone. Lu et al., (2016) utilized microplastic particles with encapsulated fluorescent dies to facilitate their detection in tissues which potentially yielded much lower detection limits then the visual method employed here. Thus, even though microplastics had very good recovery in pellets and gut contents of early time point sacrificed fish from the present work, translocation of smaller microplastic particles when exposed at higher concentrations or under long term exposures cannot be ruled out based on the results of this study.

2.4 Conclusions

Microplastics of two distinct particle shapes (microbeads and microfibers) exhibited similar retention in the GI-tract of goldfish compared to bulk food and digesta. Although a small number of particles were retained in fish after 6 days of fasting, there was no evidence for net

bioaccumulation of microplastics in the GI-tract or internal translocation to tissues of fish post exposure. This implies that the potential for long term entrainment and retention of textile derived microfibers or PCP-derived microbeads in fish is relatively low and the detection of microplastics in fish gut contents in the environment most likely represents recent exposures to microplastics in the diet as opposed to cumulative retention across multiple meals. However, this study was limited to evaluation of only two microplastic types and one species of fish. As such, further research to characterize microplastic retention by fish species over different plastic types, shapes and dietary concentrations may be warranted.

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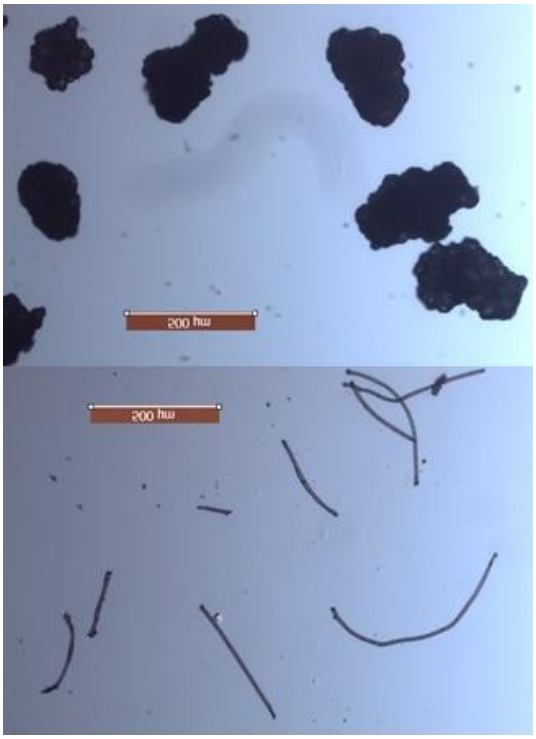

Figure 2.1. Image of microbeads (left) and microfibers (right) used for feeding trials (5 x magnification).

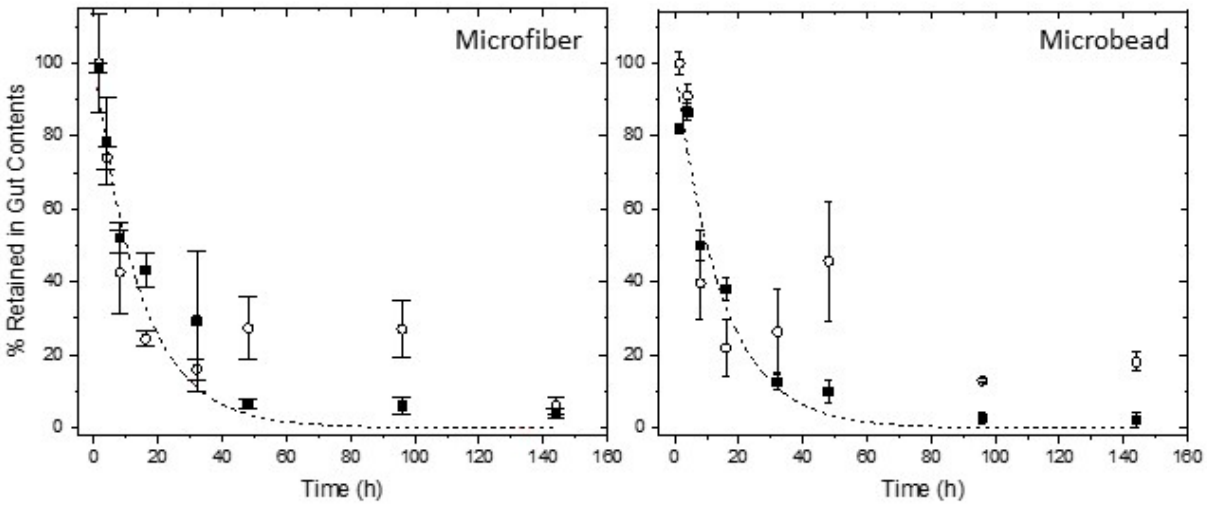

Figure 2.2. Gut retention of digesta and microplastics in gold fish post feeding. Left graphic presents mean microfiber (■) retention compared to digesta (O). Right graphic presents mean microbeads (■) retention compared to digesta (O). Dashed line is the exponential fit to the combined digesta retention data (Eq. 3). Error bars are standard error.

CHAPTER 3

Effect of microplastic amendment to food on dietary PCB assimilation efficiency by fish

3.1 Introduction

Microplastic pollution is a global phenomenon with their presence being reported in many freshwater and marine systems both in proximity to populated areas as well as in remote areas (Van Cauwenberghe et al., 2013; Eerkes-Medrano et al., 2015; Waters et al., 2016). Originally reported by Pruter in 1987 as "tiny pellets" floating in the ocean (Pruter, 1987), the term microplastic was coined by Thompson et al. (2004) and refers to plastic particles less than 5mm in size (Silva et al., 2018). Reviews of microplastic pollution indicate their concentrations in marine waters ranging from 0.067 particles/m^2 in open oceans to > 1000 particles/m^2 near populated beaches (Jiang 2018). In the Laurentian Great Lakes, microplastics have been reported in the range of 0.003-5.43 particles/m^2 (Driedger et al., 2015).

Concerns have been raised about the ecotoxicology of microplastic pollution due to direct and indirect effects of microplastics in aquatic organisms (Browne et al., 2013; von Moos et al., 2012; Collignon et al., 2012; Santana et al., 2017). Direct effects are anticipated for filter feeders, zooplankton and other planktivores as a result of physical interference with the feeding apparatus, digestion, gastrointestinal blockage and/or gastric distention leading to lower energy assimilation and growth (Xu et al., 2017; Foley et al., 2018). Indirect effects of microplastics include their trophic transfer and accumulation in gut tracts (or tissues) of secondary consumers such as fish and wildlife (Devriese et al., 2015; Frias et al., 2014; Lusher et al., 2012; Farrell and Nelson 2013) and the potential for toxicity/endocrine disruption related to microplastic leachates present in the plastic matrix or contaminants adsorbed/partitioned to microplastic particles

(Browne et al., 2007; Tuenten et al., 2009; Rochman et al., 2013). Trophic transfer of microplastics has been confirmed in studies characterizing microplastic presence in the gut tracts of fish in their native environments. Across such studies, microplastics abundances of 0.3 to 4.3 particles per fish have been typically reported, although their distribution across fish samples can be quite high with microplastic presence observed in upwards of 40 to 60% of sampled fish (Bessa et al., 2018; Boerger et al., 2010; Lusher et al., 2012; Cheung et al., 2018).

Microplastics are hydrophobic and have the capability of adsorbing and partitioning hydrophobic chemicals, including persistent organic pollutants (POPs), from the surrounding water or sediments (Lee et al., 2013, 2017; Smedes et al., 2017). For example, the partitioning capacity of polyethylene, one of the most common plastics found in aquatic environments (Andrady, 2011), for different POP compounds ranges from 2.8 to 30% of the partition capacity of biological lipids (Smedes et al., 2017). Considering that lipids generally comprise between 1 to 20% of animal body weight, this would imply that equilibrium partitioning of POPs to microplastics (assumed to be 100% polymer content) can approach or exceed POPs bioconcentration by organisms (Koelmans et al., 2016; Rios and Jones 2015). Given that fish ingest microplastics and their associated POPs, microplastics therefore have the potential to augment POPs bioaccumulation and biomagnification in aquatic ecosystems (Teuten et al, 2009).

There remains limited information regarding the bioavailability of microplastic-associated POPs to fish especially as compared to POPs ingested with common diet items. Bakir et al. (2014) observed elevated POPs-microplastic desorption in simulated gut fluids compared to POPs loss to seawater implying enhanced bioavailability of microplastic associated POPs via the dietary route. Rochman et al. (2013) observed increases in selected POPs compounds in zebrafish exposed to field derived microplastics in their diet and water compared to fish exposed

to virgin (non-environmental microplastics) and negative controls after two months of feeding. Two studies reported microplastic associated POPs dietary assimilation efficiencies (AEs) in fish. Wardrop et al. (2016) reported low values of AE's (0-12.5%) for polybrominated diphenyl ethers (PBDEs) introduced to fish tanks in conjunction with fish flakes. Granby et al., (2018) introduced contaminated microplastics directly into fish food and reported AEs for polychlorinated biphenyl (PCB) in the range of 38-55% and 12-107% for PBDEs that were similar in magnitude to chemically spiked diets free of microplastics.

In the present study, dietary assimilation efficiencies (AE) of polychlorinated biphenyls (PCBs) in goldfish (*Carassius auratus*) were determined after feeding fish a series of diet treatments amended with microplastics from a commercially available personal care product. The study applied a duel tracer design whereby microplastic particles were dosed with a set of non-environmental PCB congeners and amended to food previously dosed with a different set environmental PCBs. The design enabled simultaneous determination of AEs for microplastic-associated and diet-matrix associated-PCBs and testing their interactions across diets that varied in microplastic content.

3.2 Methods

Experimental Food Preparation

Approximately 50 g of commercial fish pellets were added to a round bottom flask containing ~200 ml of hexane to which was added 55 mg Aroclor 1254 (AccuStandard, New Haven, CT, USA). The hexane was mostly removed via roto-evaporator and then the food was air dried on solvent rinsed foil pans in a fume hood for 48 h.

Microplastic beads were obtained from a commercially available facewash product labelled with polyethylene. The cleanser was poured through a 63 µm stainless steel sieve and washed with soapy water to remove the detergent followed by rinsing with tap water. Microbeads were removed from the sieve manually under magnification. A set of 100 microbeads was weighted 5 times generating an estimate of a mean microbead weight of 0.037 mg/bead.

Microbeads were dosed with non-environmental polychlorinated biphenyls (hence referred to as performance reference compounds or PRCs; Sun et al., 2016). The PRC-PCBs were purchased as individual standards and consisted of PCBs 6, 13, 23, 21, 43, 62, 68, 57, 89, 112, 125, 166, 204 and 205 (AccuStandard, New Haven, CTt, USA) and chosen because of i) their rare occurrence in commercial Aroclor mixtures, ii) the ability to analytically distinguish them from Aroclor-PCBs by gas chromatography (GC) and iii) that they exhibit a wide range in chemical hydrophobicities comparable to the range of hydrophobicity of Aroclor PCBs. Similar to fish pellets, the microbeads were added to an excess of hexane along with 17.6 mg of sum PRCs. The excess solvent was removed by rotary evaporator and the beads allowed to air dry in the fumehood for 48 h.

Food was prepared across six experimental treatment groups (0%, 5%, 10%, 15%, 20%, 25% microplastic amendments). The PRC-dosed microplastic beads were added to the food less then 24 h before the experimental feeding in order to minimize redistribution PCBs between the two mediums (microplastics and food matrix of the pellet). Each food pellet was weighed as a dry pellet and then soaked in warm water until it loosened. After the pellet was softened, it was placed in a petri dish under a stereomicroscope and hollowed out using forceps and a teasing needle. An appropriate number of microplastic beads were manually placed into the hollow

portion of the pellet (ranging from 28 beads in the 5% treatment to 170 beads in the 25% treatment). The portion of the pellet removed during the hollowing process was placed back on top of the pellet and pressed in place. The amended pellet was placed in a solvent rinsed glass beaker and covered with aluminum foil until the experimental feeding. Ten food pellets were made for each treatment, five being used in experiments to feed the fish and the remaining five analyzed for Aroclor-PCB and PRC-PCB concentrations by GC.

Experimental Procedure

Thirty five goldfish were purchased from a local distributor and housed in a communal tank for 7 days while being fed to satiation on the same non-dosed commercial fish pellets used in experimental procedures. Initially, ten individual fish were fasted for 48 h to allow evacuation of their gut contents (Grigorakis et al. 2017) and five fish were sacrificed by immersion in an overdose solution of MS222 to serve as negative controls. The remaining fish were placed in isolation in a set of 10 L feeding tanks. They were each allowed 1 hr to normalize to their new surroundings and then presented with a single experimental pellet from one of the microplastic treatment groups. Each fish was observed to verify that it accepted the food pellet and watched for another 1 hr after feeding to ensure it did not reject the food item. After this period, replicate fish were placed into a 50 L aquaria and fasted for 5 days. After 5 days the fish were sacrificed by overdose with MS222 and stored frozen until chemical analysis. This process was repeated sequentially until groups of 5 fish for all the treatments were completed.

Chemical Analysis

Fish were individually homogenized and PCB extractions were performed on the tissue following the methods of Daley et al. (2009). Approximately 1 g of fish homogenate was ground

with ~15 g activated sodium sulfate and wet packed into a glass column containing ~15 mL of 50:50 (v/v) dichloromethane: hexane, a glass wool plug and fitted with a 1 µm glass syringe filter affixed to the bottom via luer lock. The column was spiked with 200 ng PCB 34 as a recovery standard and allowed to sit for 1 hr. The column was then slowly eluted under vacuum into a glass reservoir followed by further elution with 25 mL dichloromethane: hexane. Sample extracts were evaporated under a rotary evaporator to 10 mL of which 1 mL was removed for neutral lipid determination (Drouillard et al. 2004). The remaining 9 mL was evaporated to approximately 1 mL for clean-up by activated florisil chromotagraphy (Lazar et al., 1992). A modification to the Lazar procedure was that the first fraction was eluted with 50 mL hexane and the second fraction was eluted with 50 mL 15:85 (v/v) dichloromethane: hexane. Each fraction was separately collected and evaporated to a final volume of 1 mL.

PCBs were analyzed using an Agilent 5890 gas chromatograph equipped with ^{63}Ni electron capture detector (GC-ECD), 7673A autosampler and 60 m x 0.25 mm x 0.1 µm DB-5 column (J&W Scientific, CA, USA). The carrier gas was He adjusted to a flow of 30 mL/min and the makeup gas was Ar/CH_4 (95%/5%) with a flow rate of 50ml/min. Injections were 1 µL in splitless mode within an injector temperature of 250^{o}C. The oven program was initiated at 90^{o}C held for 0.5 min, ramped at 15^{o}C/min to a final temperature of 280^{o}C and held for 20 minutes. PCBs were identified by retention time and quantified by comparing to the area generated from a standard. For Aroclor PCBs, a certified standard (Quebec Ministry of Environment Congener mix; AccuStandard, New Haven, CT, USA) was used to identify and quantify 34 individual/co-eluting congeners. For PRC-PCBs, a working standard derived from the individual congener stock solutions was generated and calibrated based on the average GC-ECD homologue response factor derived from the certified standard. For each batch of 6 samples

analyzed, a method blank, Aroclor-PCB standard, PRC-Standard and in-house fish tissue homogenate (Detroit River Carp Homogenate) was injected. The average ± standard deviation recovery of the internal standard (PCB 34) was 90.3±12.5% and samples were not recovery corrected. Measurement of Aroclor-PCBs in the in-house reference tissue analyzed with each batch of 6 samples were always within 2 standard deviations of the mean laboratory control chart database generated for the reference tissue by the laboratory.

Data Analysis

Despite their presence in micro-plastic amended food, PRC-PCBs 6, 13 and 23 were infrequently detected in fish, potentially as a result of elimination occurring during the 5 day gut purging period post feeding. These congeners were subsequently excluded from the PRC-PCB analysis and expression of sum PRC-PCBs. Of the 34 environmental PCB congeners examined in Aroclor-dosed food samples, 28 were detected. For the negative control (non-dosed) fish there were 11 congeners detected, while treatment fish (0-25% microplastic amendments) typically contained 28 environmental PCBs. However, a number of the PCBs detected in treatment fish were infrequently detected (<50% of samples), while 3 congeners (PCBs 199, 205 and 209) were detected in controls at more than 30% of treatments. These congeners were subsequently censored from the dataset leaving 16 Aroclor 1254 PCBs (IUPAC # 52, 44, 95, 101, 99, 87, 110, 149, 118, 153, 105/132, 138, 158, 177, 180 and 170) that had high detection frequency and were present in treatment fish by more than 70% of those present in negative controls. All Aroclor PCB concentrations in treatment fish were control corrected by subtracting the equivalent geomean PCB congener concentration determined in negative control fish. References to sum Aroclor-PCBs in the text represent the sum of the above 16 congeners.

Assimilation efficiencies Aroclor-PCB and PRC-PCBs was calculated for each congener using the following formula:

$$\%AE = \frac{([PCB]_{fish} * W_{fish})}{([PCB]_{food} * W_{food})} \cdot 100 \quad (1)$$

Where $[PCB]_{fish}$ is the control corrected concentration of the PCB in the fish tissue in ng/g, W_{fish} is the whole body weight of the fish in g, $[PCB]_{food}$ is the geometric mean PCB concentration from the 5 replicate food pellets for the appropriate treatment, and W_{food} is the weight of the experimental pellet fed to the fish.

Analysis of variance (ANOVA) was used to test for differences in fish body weights, lipid contents and mass of pellet consumed across treatments. Raw data were not normally distributed (Lillefors test) but become normal after log transformation. Thus, measures of central tendency are reported in the text and tables as the geometric mean and 95% confidence interval (CI) around the geometric mean. Assimilation efficiencies were non-normal in their raw form but were normalized after arcsine transformation according to:

$$Arcsine\ AE = Arcsine\sqrt{\frac{\%AE}{100}} \quad (2)$$

Principle components analysis was applied on the combined (PRC and Aroclor-PCB) arcsine transformed AE data set. In order to satisfy the PCA requirements of a complete data matrix, missing values were substituted with the arcsine mean AE for the same congener and treatment group. Missing values of AEs consisted of individual sample/congener combinations where PCBs were either not detected in fish or were blank corrected to a zero value. AE values greater than 100% were removed/replaced in the same manner as missing values. A variance-covariance matrix was used with the PCA given that all AEs are scaled from 0-1. PCB congeners

with loadings (correlation coefficient) greater than 0.6 onto a given PCA axis were considered strongly associated with that axis.

Following interpretation of the PCA, general linear models (GLMs) were applied separately across PCBs grouped into PRC-PCBs and Aroclor-PCBs. GLMs were applied to test the following model:

$$Arcsine\ AE = A \cdot \%Microplastic + B \cdot \log K_{OW} + C \cdot (\%Microplastic \cdot logK_{OW}) + Constant \quad (3)$$

Under conditions where the interaction term was non-significant, it was removed and the GLM re-run. Back-transformation of arsine AEs to percentage values from the GLM model were calculated according to:

$$\%AE = \{Sine(Arcsine\ (AE)\}^2\ x\ 100 \quad (4)$$

3.3 Results

The overall geometric mean (95% CI) body weight of fish was 16.3 g (14.7-18.1g) and there were no significant ($F_{5,24}$=0.738; p>0.6; ANOVA) differences in fish body weights between treatments. Fish lipid contents were 1.04% (0.97-1.12%) and were significantly ($F_{5,24}$=2.998; p<0.05; ANOVA) different across treatment groups. However, the significant differences of fish lipid contents were only observed between the 10% and 25% microplastic amendment treatments (p<0.05; Tukey's HSD). Each fish was provided one dosed pellet with a geomean weight of 0.020g (0.0196-0.021g) or 0.12% of the geomean body weight of fish. All fish were observed to accept the dosed pellet and there were no significant ($F_{5,24}$=0.765; p>0.5; ANOVA) differences in the pellet weights given to fish between treatments. Table 3.1

summarizes geometric mean body weight, fish lipid contents and food consumed by each of the treatments.

Table 3.1 also summarizes sum Aroclor-PCB and sum PRC-PCB concentrations measured in 5 food pellets from each experimental treatment group. There were highly significant differences ($F_{5,24}$=5.834; p<0.001; ANOVA) in sum Aroclor-PCB concentrations between treatments that progressively increased across the microplastic treatments. Pairwise comparisons indicated significantly lower (p<0.05; Tukey's HSD) Aroclor sum PCB concentrations in the 5, 10, and 15% treatments compared to the 25% treatment group, although such differences were less than a factor of 2. PRC-PCBs also exhibited highly significant differences ($F_{4,19}$=56.098; p<0.001) between treatments and showed progressive increases in PRC-PCB concentrations with increasing microplastic amendments. Pairwise comparisons indicated a significant difference (p<0.01; Tukey's HSD) between each treatment combination except for the 20 and 25% treatment. The 4.6 fold difference in PRC-PCB concentrations between the 25% and 5% diet treatments was commensurate with the 5 fold difference in dosed microbeads added to the diets.

The geometric mean (95% CI) sum Aroclor PCB concentration for the 16 selected congeners in treatment and negative control fish was 13.77 ng/g wet weight (11.18 – 16.96 ng/g) and 0.36 ng/g wet weight (0.06 – 2.07) ng/g, respectively, and were highly significantly ($F_{1,33}$=157.059; p<0.001; ANOVA) different from one another. Sum Aroclor PCB concentrations in treatment fish did not significantly differ ($F_{5,24}$=1.650; p>0.1; ANOVA) across the treatments. None of the PRC-PCBs were detected in negative control or the 0% microplastic amendment treatment groups. For the 5-25% treatments, the geomean (95% CI) sum PRC PCB concentration was 5.77 ng/g wet weight (3.88-8.58) ng/g wet weight. Linear regression analysis

indicated a highly significant ($p<0.001$; ANOVA) relationship between sum-PRC PCB concentration in fish and the microplastic content of the food consistent with the difference in PRC-PCB concentrations across food treatments. However, sum PRC-PCBs in fish from the microplastic amended treatments were significantly lower ($F_{1,48}=22.149$; $p<0.001$; ANOVA) than sum Aroclor PCBs despite generally higher PRC contents in food. However, these differences between PCB groups were dependent on the treatment as indicated by a highly significant interaction term ($F_{4,48}=8.678$; ANOVA) for the general linear model.

PCB Assimilation efficiencies

Among PRC-PCBs, 5/275 (1.8%) congener/treatment AE combinations were missing due to lack of detection. There were no PRC-PCB cases where the calculated AE exceeded 100%. For Aroclor PCBs, there was 1/480 cases with missing/non-detected concentrations and 31/480 cases (6.4%) where the calculated AE exceeded 100%. The back-transformed arcsine mean (95% CI; count) AE for PRC-PCBs was 13.36% (12.27 – 14.49%; n = 270). The arsine mean (95%CI; count) AE for the selected Aroclor-PCBs was 51.64% (48.97 – 54.32; n = 448%), or 3.9 fold higher than measured for microplastic sorbed PCBs. PCA on the combined AE data revealed 2 significant component axes (60.1% of the variation) identified by scree plot and its intersection with a broken stick model. PCA 1 was dominated by Aroclor PCBs with strong loadings (correlations >0.6) observed for PCBs 52, 44, 95, 101, 99, 87, 110, 149, 118, 153, 105/132, 177 and 180 and moderately strong loads (correlation >0.5) for PCBs 138 and 170/190. PCA 2 had strong loadings for all of the PRC-PCBs (correlations from 0.84-0.97). These results provide support to indicate that microplastic-sorbed PCBs exhibited different behavior compared to diet-matrix associated Aroclor-PCBs. Table 3.1 refers to the PCA and the variance explained

by each axis. Given the differences in behavior of PRC-PCB and Aroclor-PCBs identified by PCA, GLMs were subsequently explored for each PCB group separately.

For Aroclor-PCBs, the interaction term for the GLM model was not significant ($t_{1,444}$=1.377; p>0.05) and therefore the interaction term was removed. The simplified GLM explained 7.8% of the variation of the arcsine AE Aroclor data. The coefficients for log K_{OW} ($t_{1,445}$=2.583; p<0.05) and microplastic treatment group ($t_{1,445}$=-5.802; p<0.005; t-test) were significantly different from zero. The multiple regression fit GLM is provided below:

$$Arcsine\ (AE_{Aroclor-PCB}) = -0.009 \pm 0.002 \cdot \%Microplastic + 0.087 \pm 0.034 \cdot logK_{OW} + 0.36 \pm 0.22 \quad (5)$$

The GLM model ran on the PRC-PCB AEs also exhibited a non-significant interaction term. The simplified GLM for this PCB group explained 19.5% of the variation in AE data and both log K_{OW} and microplastic treatment coefficients were highly significantly different from zero ($t_{1,267}$=-4.507 and 6.836, respectively, p<0.001 in both cases; t-test). The fitted GLM for PRC-PCBs was given by:

$$Arcsine\ (AE_{PRC-PCB}) = 0.007 \pm 0.001 \cdot \%Microplastic - 0.056 \pm 0.012 \cdot logK_{OW} + 0.62 \pm 0.08 \quad (6)$$

Notably, Aroclor-PCBs and PRC-PCB AEs showed opposing trends with microplastic contents and congener K_{OW}. Aroclor-PCBs showed a decrease in AE with increasing microplastic content while PRC-PCBs increased in their AE with increasing plastic contents. Figure 3.1 contrasts the relationships between back-transformed arsine mean PRC-PCB and

Aroclor-PCB AEs as a function of microplastic content in diet. The two model lines are predicted to intersect at just above 39% microplastic content of food.

Similarly, Aroclor-PCB and PRC-PCB AEs exhibited opposing trends with chemical K_{OW}, with an expected decreasing trend apparent for PRC-PCB AEs and increasing trend for Aroclor-PCBs. Closer examination of the K_{OW} relationship for Aroclor-PCBs revealed slopes that approached zero for the 0, 5 and 15% treatments, non-significant positive slopes for the 10 and 20% groups and a significant positive slope for the 25% group. For PRC-PCBs, all microplastic treatments exhibited a negative slope with log K_{OW}. Figure 3.2 demonstrates the change in slope of the PCB AE vs log K_{OW} for each of the Aroclor and PRC-PCB treatment groups. There is a positive relationship between the slope of the AE vs log K_{OW} relationship for Aroclor PCBs and microplastic content but no apparent relationship for the PRC-PCBs. In order to make the pattern clearer, Figure 3.3 (bottom panel) presents PCB AEs as a function of log K_{OW} for selected Aroclor groups (0% and 25% microplastic treatments) along with the PRC-PCB AEs for the combined groups. From Figure 3.3 it is apparent that the significant positive slope for the high plastic treatment was due to a decrease in low K_{OW} Aroclor PCBs that approached the AEs observed for PRC PCBs, whereas higher-K_{OW} Aroclor-PCB congeners tended to exhibit similar AEs between the 25% and 0% groups.

3.4 Discussion

The magnitude of dietary assimilation efficiencies of Aroclor-PCBs in fish for the 0% control group from the present (62.4% [55.6-70.0%]) fell within the range of PCB-AEs from other studies performed on fish fed food without microplastics (Fadaei et al., 2017; Gobas et al., 1993; Kobayashi et al., 2010; Li et al., 2015; Liu et al., 2010). Gobas et al showed that low-fat pellets have higher assimilation efficiency than high-fat pellets and they hypothesized that this

was due to low-fat pellets being more easily digestible than high-fat pellets. However, Liu et al. (2010) failed to replicate these findings for PCB AEs in goldfish. Other studies have reported somewhat lower PCB AEs on the order of 12-24% (Buckman et al., 2004; Fisk et al., 1998; Granby et al., 2018; Stapleton et al., 2004). However, differences in AE values between studies often occur as a result of differences in the method of AE calculation. In the case of Buckman et al. (2004) and Fisk et al. (1998) AE's were estimated from the chemical bioaccumulation slope calculated during an uptake study and dividing the uptake slope by the average food concentration and average feeding rate. Such approaches are considered less precise than individual mass balance studies, as used in the present research, because they assume all fish held in communal tanks had equal access to the food provisioned to the tank and are further confounded by growth and/or chemical elimination processes occurring during the uptake period.

Studies investigating the assimilation of microplastic-associated POPs to aquatic organisms are much more limited. Microplastics have been shown to transfer POPs to lugworms, amphipods, fish, and seabirds (Besseling et al., 2013; Browne et al., 2013; Chua et al., 2014; Colabuono et al., 2010; Herzke et al., 2016; Rochman et al., 2013). Besseling et al. (2013) observed that the presence of polystyrene (0.074%) in sediments caused an increase of bioaccumulation of PCBs by lugworms by a factor of 1.1-3.6 compared to lugworms exposed to the same contaminated sediment without any microplastic particles present. However, the accumulation of PCBs was also shown to decrease as the plastic content increased beyond environmentally relevant levels which corroborates the findings from the present study for Aroclor-PCBs.

Granby et al. (2018) evaluated the toxicokinetics (both chemical assimilation and depuration) of PCBs and PBDEs in European seabass (*Dicentrarchus labrax*) exposed to diets

with and without microplastics. The uptake portion of the study lasted 80 days followed by a 51 d depuration study. Apart from negative controls, there were three feeding treatments that included i) clean food containing contaminated microplastic particles at 2% food weight, ii) contaminated food containing clean microplastic particles at 4% food weight and iii) contaminated food without microplastic particles. Notably, the authors did not find differences in PCB or PBDE AEs among diet treatments indicating that microplastic-associated POPs had the same bioavailablility as POPs associated with the normal diet. In their study, AEs ranged from 38-55% for PCBs and 12-107% for PBDEs from diet (i), 33-54% for PCBs and 12-111% for PBDEs from diet (ii) and 34-63% for PCBs and 9-121% for PBDEs from diet (iii; control). PCB AEs were broadly consistent with the magnitude of Aroclor-PCBs measured in the present study but were not consistent with the much lower AE values measured for microplastic-associated PRC-PCBs. However, there were differences in the approach used to create microplastic dosed food between this and Granby's study. Granby et al. (2018) created different diet treatments as large pools of food prior to the initiation of the study, an apparently necessary procedure to ensure constant diet concentrations over the pro-longed uptake period. This could have allowed time for PCBs and PBDEs to redistribute between the microplastics and the food matrix during food storage. In the present research, the contact time between microplastics and food pellets was controlled (<24 h) to minimize redistribution artifacts and may explain the discrepancy in observed results. Previous research demonstrated that gut retention of microbeads in fish is of similar duration as food digesta and on the order of 24-48 h post food/microplastic ingestion (Grigorakis et al. 2017). Therefore, the shorter micro-plastic/diet matrix contact time controlled for in the present study may be more realistic to what occurs in nature compared to the much longer (up to 80 day) contact time as applied in Granby's study.

The second study measuring POP AEs in microplastic was that of Wardrop et al. (2016) who generated microplastic-associated PBDE AE estimates of 0-12.5% and were similar in magnitude to the PRC-PCB AEs from the present study. Wardrop et al.'s feeding treatments were similar to Granby et al (2018) and the present study, but differed in that Wardrop and colleagues did not directly incorporate microplastic particles into fish food, but rather administered 70g of fish flakes that was mixed together with 10g of contaminated microplastic particles (i.e. ~14% microplastic content) to fish tanks. This necessitated the assumption that fish consumed the provisioned fish flakes and microplastics to completion which is difficult to verify. Nonetheless, their estimates of PBDE AEs were consistent with the present microplastic associated PCB AE estimates.

In the present study, diet matrix associated PCBs (Aroclor-PCBs) showed progressive decreases in chemical AE with increases in dietary microplastic content as well as increases in AE with increasing log K_{OW}. The observed hydrophobicity effect for Aroclor-PCB AE is somewhat unusual, with most studies reporting a neutral (Buckamn et al., 2014; Fisk et al., 1998; Stapleton et al., 2004) or negative relationship between PCB dietary AE and chemical K_{OW} (Fadaei et al., 2017; Liu et al., 2010). However, closer inspection revealed that the hydrophobicity effect was partially (although not statistically significant) dependent on the microplastic content of food. Treatments with no, or low microplastic contents of food (0-15% microplastic contents) exhibited non-significant relationships between Aroclor-PCB AE and log K_{OW}, whereas a pronounced significantly positive relationship was apparent for the 25% microplastic treatment. Furthermore, it was only the least hydrophobic Aroclor-congeners from the high microplastic treatment groups which behaved differently compared to other treatments, such that the low K_{OW} compounds in the 25% microplastic group assimilated in a similar way as

the PRC-PCBs did (Figure 3.3b). One hypothesis for these observed patterns is that lower K_{OW} Aroclor-PCBs became partially dissociated from the diet matrix and sorbed onto microplastics present in food. The apparent K_{OW} trend is consistent with expected kinetic limitations for PCB/lipid desorption that would favor the redistribution of less hydrophobic Aroclor-PCB congeners from food to plastic. However, this pattern was not mirrored for low-K_{OW} microplastic associated PCBs. In the case of PRC-PCBs, assimilation efficiencies increased with increasing microplastic content of food and this pattern remained consistent across different congener K_{OW} values.

One unrealistic aspect of the present research was the very high microplastic contents used among food treatments. The lowest microplastic content in food was 5% by weight which is several orders of magnitude higher than food microplastic contents under environmental conditions. Microplastic contents in the gut contents of fish have been reported to range from 0.18-4.3 microplastic particles/fish (Bessa et al., 2018; Boerger et al., 2010; Lusher et al., 2012; Cheung et al.,2018) compared with the 28-170 microbeads added to food pellets in the present research. Taking the highest microplastic content (4.3 particles) and average fish weight (765 g) of Cheung et al.'s (2018) study (and applying the mean microbead weight from the present research) would indicate a microplastic content of environmental fish on the order of $2x10^{-5}$ % by weight. Assuming a similar microplastic particle content in smaller forage sized fish (~2 g), would indicate a microplastic content of approximately 0.01% by weight. Applying Eqs. 3 and 4 to the above microplastic contents would indicate an AE of 7.2% and 61.7% for a microplastic-associated and diet matrix-associated chemical having a log K_{OW} value of 6.25. The diet associated chemical AEs would not be significantly impacted by the presence of such small microplastic contents of diet compared to a zero-microplastic content diet, whereas the

microplastic-associated POPs are predicted (Eq. 3) to be 36% less bioavailable than observed for the lowest microplastic treatment applied in the present study. Thus under realistic microplastic contents in present natural food web components, POP bioavailability from microplastics is likely to be lower than measured in the present research.

Overall, the data on microplastic associated POP AEs from the present research support the conclusions of other researchers, that POPs partitioned into microplastic particles are unlikely to significantly contribute to elevated POPs exposures in fish or aquatic food webs (Bakir et al., 2014; Herzke et al., 2016; Koelmans et al., 2013; Lohmann 2017). First, microplastics represent a very small mass of third-phase organic material in the natural environment compared to dissolved and particulate organic carbon (Gouin et al., 2011). Gouin et al. (2011) describes the ratio of organic carbon/polyethylene to be on the order of 10^7: 1 with respect to volume. Similarly, even though the partition capacity of plastics such as polyethylene approaches the partition capacity of biological organisms (Smedes et al., 2017; Koelmans et al., 2016; Rios and Jones, 2015), the total mass of microplastics and microplastic-associated POPs is far lower than the mass of ingested food and food-associated POPs. Third, the data from the present research implies lower chemical bioavailability of plastic-associated chemical compared to food-associated chemical. The latter is likely due to gastrointestinal magnification of POPs from the diet that occurs from the net assimilation of lipids, fatty acids and non-lipid organic matter during food digestion that generates a fugacity gradient in the GI-tract favoring elevated net chemical uptake beyond equilibrium partitioning predictions (Gobas et al. 1988, 1999). In contrast, microplastics are not assimilated within the GI-tract and therefore do not contribute to gastrointestinal magnification nor food web biomagnification but can contribute to enhanced clearance of chemicals via the fecal egestion pathway especially when microplastic-associated

POPs have a lower fugacity than diet-associated or GI-magnified POPs present in food items (Besseling et al., 2013; Granby et al. 2018).

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Table 3.1. Geomean (95% CI) of fish body weights, lipid contents and concentrations of sum PRC-PCB and sum Aroclor-PCBs in control and microplastic amended food.

Treatment	Fish Body Weight (g)	Fish Lipid Content (%)	Amount of Food consumed (g)	Food sum PRC-PCB Concentration (ug/g dry wt)	Food sum Aroclor-PCB Concentration (ug/g dry wt)
0%	16.9	0.98[a,b]	0.020	Not Detected	21.70[a,b]
	(9.1-31.5)	(0.91-1.06)	(0.017-0.023)		(15.32-30.74)
5%	14.5	0.95[a,b]	0.019	15.39[a]	13.21[b]
	(11.6-18.2)	(0.87-1.04)	(0.017-0.022)	(10.20-23.23)	9.60-18.17
10%	15.6	0.88[b]	0.021	24.50[b]	17.95 [b]
	(10.4-23.3)	(0.66-1.20)	(0.019-0.024)	(21.24-28.26)	(11.91-27.05)
15%	14.5	1.02[a,b]	0.021	43.81[c]	18.64 [b]
	(11.2-18.9)	(0.94-1.13)	(0.019-0.024)	(34.68-55.33)	(11.72-29.65)
20%	19.0	1.19[a,b]	0.021	66.57[d]	22.80 [a,b]
	(16.2-22.3)	(0.83-1.71)	(0.018-0.024)	(51.89-85.41)	(16.76-31.02)
25%	17.7	1.25[a]	0.020	70.42[d]	28.50 [a]
	(13.8-22.7)	(1.05-1.49)	(0.018-0.022)	(57.73-85.90)	(28.50-37.74)

[*]Geomeans with different superscripts across rows are significantly different ($p<0.05$; Tukeys HSD). Fish body weights and food consumed were not significantly different across treatments (>0.05; ANOVA).

Component Axis	Variance Explained (%)	PCB AEs with Strong Loadings in Bold, Loadings >0.5 in Regular Text
PCA1	44.42	**PCBs 52, 44, 95, 101, 99, 110, 149, 118, 153, 105,** 138, **177, 180,** 170/190
PCA2	15.68	PCBs 21, 43, 62, 68, 57, 89, 112, 125, 166, 204, 205
PCA3	8.00	PCBs 87, 110

Table 3.2. Principle Components Analysis (PCA) on PCB AEs in goldfish across treatments

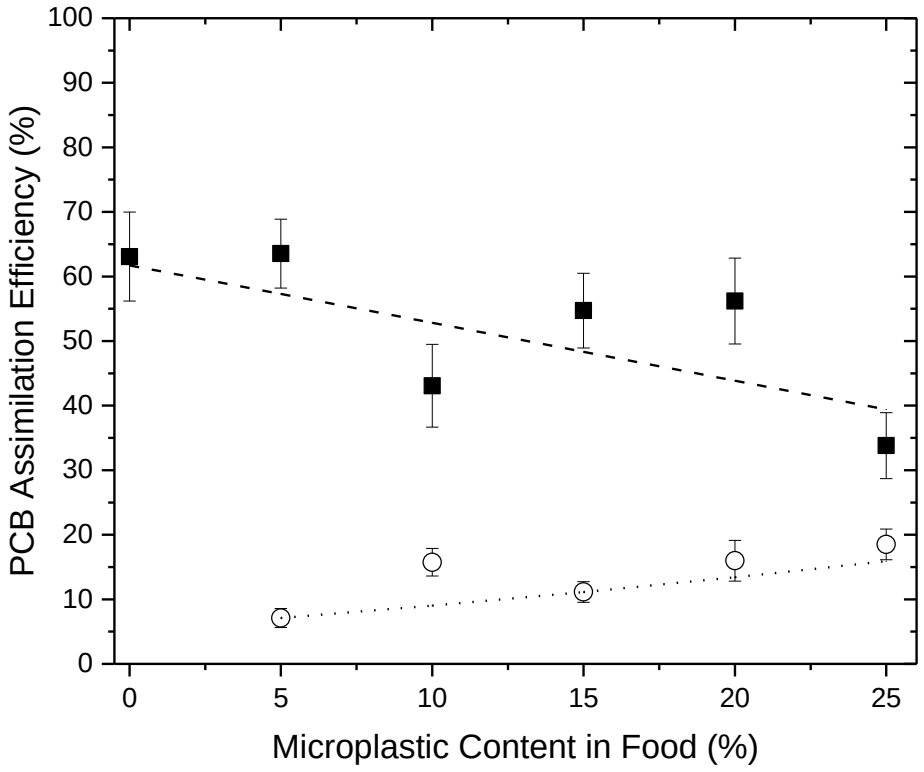

Figure 3.1. Squares – Diet-Matrix associated PCBs; circles PRC-PCBs sorbed to microplastics. Model lines fitted to Eq. 5 and 6 for a normalized log K_{OW} value of 6.25

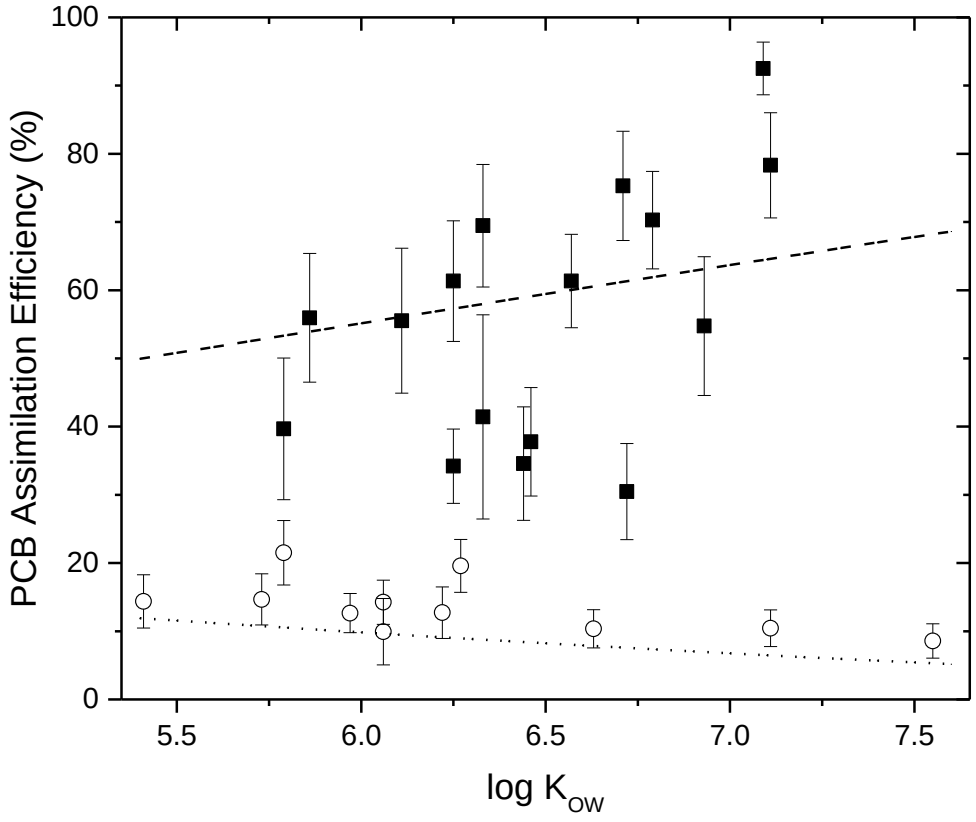

Figure 3.2. Squares – Squares- Diet-Matrix associated PCBs; open circles are PRC-PCBs sorbed to microplastics. Lines represent model fits to eq 5 and 6 under an assumed 5% microplastic content

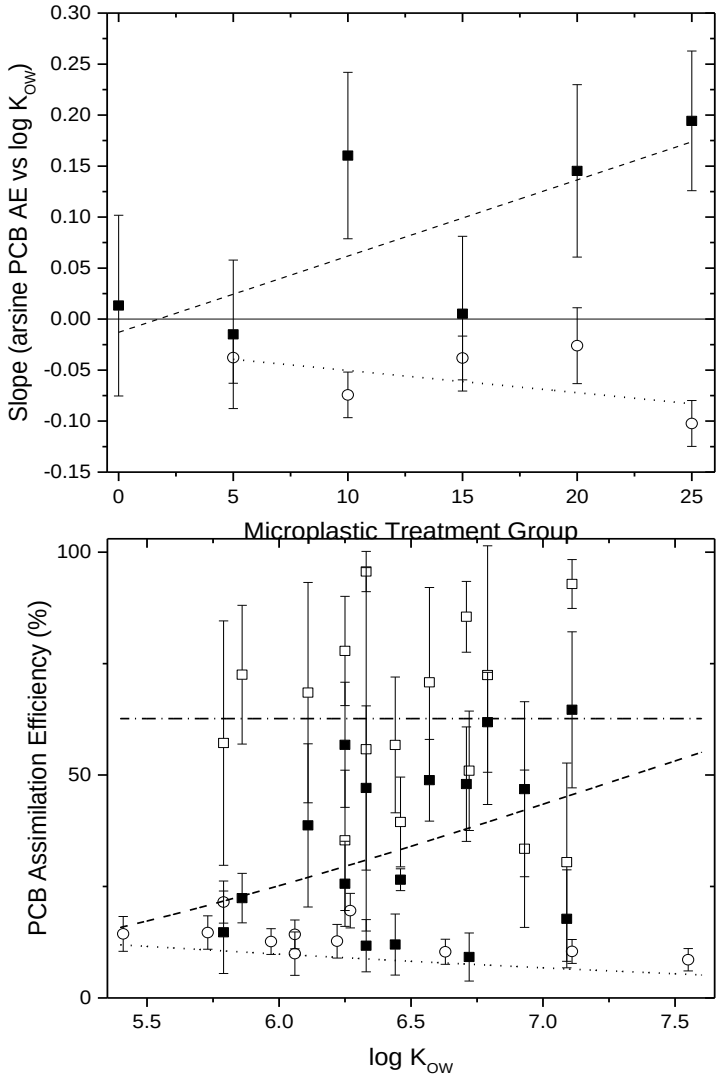

Figure 3.3. Top figure – AE vs K_{OW} slopes for Aroclor PCBs square and PRC-PCBs; circle. Bottom figure. PCB assimilation efficiency vs K_{OW} for the 0% Aroclor (open square), 25% arocclor (solid square) and PRC-PCBs (open circle).

CHAPTER 4

General Discussion

4.1 Thesis Objectives and Hypotheses Tested

The main objectives of this thesis were to investigate the gastrointestinal retention of microplastics in fish and to explore potential interactions between microplastics and hydrophobic persistent organic pollutants (POPs) affecting POPs bioaccumulation and toxicokinetics in fish. Chapter 2 of this thesis focused on microplastic retention in the gastrointestinal (GI) tract of fish while Chapter 3 characterized dietary assimilation efficiencies (AE) of polychlorinated biphenyls (PCBs) in fish in the presence and absence of microplastics in food.

In chapter 2, the gastrointestinal (GI)-tract retention of two microplastic types (microbeads and microfibers) was measured in gold fish after feeding them a food pellet containing a defined number of microplastic particles. Both microbeads and microfibers are common types of microplastics found in environmental samples (Browne et al., 2010; Eriksen et al., 2013). Microbeads are engineered microplastics used in commercial personal care products such as facial cleansers, toothpastes and other applications and enter the aquatic environment via waste water treatment plant discharge (Browne et al., 2011). Microfibers are secondary microplastics commonly generated from laundering synthetic clothing and textiles (e.g. nylon, polyester and fleece fabrics) that also enter the environment through waste water treatment plant discharges (Browne et al., 2011). Both types of microplastics were added to commercial fish pellets, fed to goldfish, and the retention of digesta and each microplastic type determined through time. Three hypotheses, as outlined in Chapter 1, were tested in Chapter 2 and their results are outlined below:

Hypothesis 1: microbeads, derived from personal care products, are retained and lost from the gastrointestinal tract of fish at the same rate as food/digesta

Hypothesis 1 was accepted, since ANCOVA revealed that the retention of microbeads was not significantly ($p>0.05$; ANCOVA) different than the retention of digesta.

Hypothesis 2: microfibers, derived from laundered textiles, are retained and lost from the gastrointestinal tract of fish at the same rate of food/digesta

Similar to Hypothesis 1, Hypothesis 2 was accepted since ANCOVA revealed that the retention of microfibers was not significantly ($p>0.5$; ANCOVA) different than the retention of digesta.

Hypothesis 3: microbeads have similar retention in the gastrointestinal tract of fish as microfibers

This hypothesis was accepted since both microfibers and microbeads were found to exhibit non-significant differences ($p>0.4$; ANCOVA) in their gut retention time to one another.

Overall the time to evacuate 50% and 90% of digesta, microbeads and microfibers from goldfish under the operating experimental temperatures was 10.0 and 33.4 h, respectively. These results indicate that plastic microbeads and microfibers are not likely to accumulate in the GI-tract of fish

The objective of chapter 3 was to determine dietary assimilation efficiencies of microplastic-associated PCBs and how it relates to dietary assimilation from regular food items consumed by fish. A novel duel tracer design was applied that allowed separate, but simultaneous,

determination of microplastic-associated PCB AEs and diet matrix-associated PCB AEs. The three hypotheses tested in Chapter 3 were as follows:

Hypothesis 1: microplastic-associated PCBs have similar AE values as diet matrix-associated PCBs

This hypothesis was rejected. The geomean (95% confidence interval) AE for microplastic-associated PCBs was 13.36% (12.27-14.49%) whereas the geomean AE for Aroclor-PCBs was 51.64% (48.97-54.32%) representing 3.9 fold difference in bioavailability between the two.

Hypothesis 2: diet matrix-associated PCB AEs are unaffected by the presence of microplastic particles in the diet across different treatments that vary microplastic contents (0 to 25% microplastic content in food by weight).

This hypothesis was rejected. Diet matrix associated PCB AEs were significantly related to the microplastic content of the diet and decreased as a function of increasing microplastic contents. However, significant differences in diet matrix-associated PCB AEs were only observed after microplastic concentrations exceeded 10% of the diet content by weight which reflects unrealistically high microplastic concentrations under environmental conditions. Applying Equation (5) of Chapter 3 and a more realistic prey microplastic concentration derived from the literature (estimated at 0.01 % microplastic content of food) indicates that diet matrix associated PCBs are unlikely to be appreciably different from a zero microplastic content diet under normal environmental conditions.

Hypothesis 3: PCB AEs from microplastics or the diet matrix demonstrate similar relationships with chemical hydrophobicity

This hypothesis was rejected. Multiple regression models predicting matrix-associated PCB AEs and microplastic-associated PCB AEs (Eqs. 5 and 6 of Chapter 3) demonstrated opposing hydrophobicity interactions to one another. Microplastic-associated PCB AEs were negatively related to PCB hydrophobicity whereas diet-matrix associated PCB AEs were positively related to PCB hydrophobicity. However, upon closer inspection of the patterns, the positive relationship between diet-matrix associated PCB AEs and log K_{OW} only occurred for the high microplastic treatment groups (20 and 25% microplastic contents in food by weight). The apparent positive relationship between diet-matrix associated PCB AEs with hydrophobicity was interpreted to be in part an artifact of redistribution of low K_{OW} PCBs in the high treatment groups from the diet matrix to the microplastic particles added to food. This interpretation was supported by the fact that low K_{OW} diet matrix PCB congeners demonstrated similar AE values as microplastic associated AEs. As in the case of hypothesis 2, the apparent microplastic-diet matrix PCB AE interaction only occurred at extremely high food microplastic contents of food that are unlikely to be found under environmental conditions. Thus, although hypothesis 3 was rejected, under normal environmental conditions of microplastic concentrations in water, sediment and food, it is anticipated that hypothesis 2 is indeed valid.

4.2 Implications of thesis findings

First, determining the gut retention of microbeads and microfibers led to the conclusion that microplastic particles do not bioaccumulate in fish; which further leads to a conclusion that the assimilation and elimination of POPs in fish is likely to be a result of individual meals/microplastic ingestion events. This implies that microplastics commonly characterized in

GI-tracts of natural fish populations (Bessa et al., 2018; Boerger et al., 2010; Lusher et al., 2012; Cheung et al., 2018) essentially represent the microplastic particle content of the 'last meal consumed' rather than a long term time integrated exposure over several successful meals consumed by the sampled organism. From the perspective of POPs/microplastic interactions, the results from Chapter 2 imply that there is a limited period of time in which microplastics and microplastic-associated POPs compounds present in potential food/prey items are in contact with one another placing some constraints on the capacity for microplastic-associated POPs to become assimilated by fish. A second important conclusion from Chapter 2 is that microplastics are unlikely to accumulate as a separate, non-lipid organic pool, in the gut tract of fish. This means that K_{BEX} (Equation 1 of Chapter 1) will be proportional to the microplastic content of ingested food and plastic partition capacity but does not require a separate microplastic bioaccumulation sub-model to be developed as part of a microplastic-POPs interacting toxicokinetic model. In other words, microplastics can simply be treated as a proximate component of the diet of the organism in a modified microplastic bioaccumulation model rather than being treated as its own compartment with separate gut tract uptake and evacuation characteristics. Finally, the information generated by Chapter 2 was necessary because the results were used help develop the study design for chapter 3 where we attempted to control the microplastic-diet matrix contact time and reduce POP redistribution artifacts. Overall, microplastic particles sampled from the GI-tract of wild fish tend to corroborate with the overall thesis conclusion that microplastic particles are unlikely to bioaccumulate in fish or fish prey items (Bessa et al., 2018; Boerger et al., 2010; Lusher et al., 2012; Cheung et al., 2018).

In chapter 3, the assimilation efficiency of PCBs from microplastic particles, as well as its relative contribution to the bioavailability of POPs, in fish was investigated. The results of

Chapter 3's assimilation efficiency study imply that PCBs absorbed to microplastic particles are less bioavailable than food matrix-partitioned PCBs. Microplastics have been shown to transfer POPs to lugworms, amphipods, fish, and seabirds (Besseling et al., 2013; Browne et al., 2013; Chua et al., 2014; Colabuono et al., 2010; Herzke et al., 2016; Rochman et al., 2013), but, to the best of our knowledge, this is the only the third study to investigated how AEs of microplastic-associated POPs compares to the AE of food matrix-associated POPs. Further, their studies examining the AEs of microplastic-associated POPs have either allowed contaminated microplastics and contaminated food to be in contact with one another for extensive time periods (Granby et al., 2018) or they did not directly amend microplastics into the food matrix (Wardrop et al., 2016) confounding the interpretation of bioavailability differences. Based on the results of Chapter 3, even short microplastic-diet matrix contact times on the order of 24 hours can result in some degree of redistribution of PCB congeners between food components and microplastics present in food especially for low K_{OW} PCBs and treatments containing high microplastic concentrations. Thus, care needs to be taken to minimize redistribution artifacts by controlling the contact time between microplastics and food pellets (<24 h).

Another observation from Chapter 3 was that microplastic-associated PCBs increase in their AE with increasing microplastic contents such that microplastic-associated PCBs were predicted to have equivalent AEs as diet-matrix AEs at a hypothetical microplastic concentration in food of approximately 39% by weight. It is unknown if this intersection is a consequence of PCB redistribution between diet matrix and microplastic components of the food or other unknown factors related to the physiology of PCB assimilation. Regardless, microplastic concentrations in food need to be greater than 5-10% by weight in order for such interactions to become important. Given realistic microplastic concentrations in food on the order of <0.01%,

it is unlikely that the main conclusion from Chapter 3, that microplastic-associated PCBs are less bioavailable than diet matrix-associated PCBs, do not occur under typical environmental conditions. Indeed, extrapolation of Eq 6 from Chapter 3 to environmentally realistic microplastic concentrations in food items would imply AEs on the order of 7.2% or about 32% lower than measured in the lowest microplastic diet treatment and 88% lower than the geomean bioavailability of diet matrix-associated PCBs.

Combining the overall findings of chapter 3 with literature observations that microplastic particles represent an extremely small fraction of organic volume within the environment and in ingested food items (Gouin et al., 2011), it can be concluded that microplastic particles are unlikely to impact the accumulation of POPs by fish. These conclusions may change if microplastics continue to accumulation in the environment. However, microplastic concentrations in food items would need to become several orders of magnitude higher before such affects likely to occur.

4.3 Data gaps and future studies

Although the quantity of published studies regarding microplastics is rapidly growing, there are still many gaps of knowledge regarding microplastic-POPs interactions that can affect bioaccumulation of POPs by fish. Granby et al (2018) demonstrated that high microplastic content (uncontaminated with POPs) can increase the rate of elimination of POPs in fish. Future studies should investigate the net effect of microplastics on the uptake and elimination of POPs in fish and characterise this as a function of microplastic types. With such diversity among different plastic types (shape, physio-chemical properties) that are represented in marine and fresh water systems, it is important to gain an understanding of how different types of plastics cause different effects in organisms. For instance, polyethylene (PE) has a strong affinity for

POPs compared to polyvinyl chloride (PVC), so it stands to reason that studies replicating chapters 2 and 3 of this thesis could yield a different set of results had PVC been used instead of PE. Special interest should be paid to the more common plastic types and expand to less common plastic types as the depth of knowledge surrounding microplastic-POP interactions increases. Further, little is known about the food-web dynamics of microplastics in marine or fresh water systems aside from microplastics seemingly lacking the propensity to bioaccumulate in organisms. There are many questions about microplastic-organism interactions to be addressed. For instance, do some organisms ingest more microplastic particles than others? Are certain types of plastic more likely to be ingested by organisms? Fish and other organisms have shown that they ingest microplastics in small quantities but published studies only encompass a small percentage of aquatic organisms.

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