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Microplastics in Mississippi River Fishes: A Watershed Approach

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MICROPLASTICS IN MISSISSIPPI RIVER FISHES: A WATERSHED APPROACH

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Table of Contents

List of Tables

List of Figures

Abstract

Microplastics are plastic particles less than 5mm that come from different sources, such as industrial products, cosmetics, and the breakdown of the macroplastics. Microplastics derive from terrestrial sources but concentrate in flowing freshwaters where they may enter aquatic food webs. The northern Gulf of Mexico has shown very high microplastic concentrations, which are assumed to be sourced from the Mississippi River and its watershed. This study seeks to study microplastics in fish in the Mississippi River and the Gulf of Mexico. Fish stomachs and intestines from fish underwent chemical digestion, filtration, and microplastics identification and enumeration through FT-IR spectrometry. Chapter one seeks to quantify microplastics in four different fish species (Shortnose Gar *Lepisosteus platostomus*, Largemouth Bass *Micropterus salmoides*, Bluegill *Lepomis macrochirus*, and Flathead Catfish *Pylodictis olivaris*) across the Mississippi River, and test the hypothesis that microplastic counts in fish will increase from the headwaters to the mouth. Fish were sampled from several sites along the main-stem Mississippi River (from Minnesota to Louisiana). All four fish species had microplastics in their stomachs and downstream locations had higher microplastics concentrations; however, location had no significant effect on microplastic counts among species except for Largemouth Bass. Chapter two sought to quantify microplastics across body size in two common estuarine fish species, the Hardhead Catfish and Southern Flounder. Hardhead Catfish are benthic omnivores, and among one of the most common estuarine fishes in the Gulf of Mexico (but with almost no fishery value). Southern Flounder are an ambushing flatfish that inhabit estuaries at younger ages before a move offshore as adults. I hypothesized that microplastic concentrations in stomachs (and intestines) as measured by the number of microplastic particles in fish would be higher in smaller-sized fishes than larger-sized fishes (according to total length), as smaller fish target

smaller prey and possibly directly ingest more microplastics. Fish were sampled in 2018 and 2019 within balanced size classes. Larger fish ingested more microplastics, which can be as a result of indirect consumption or accidental ingestion from the water column.

Chapter One. Microplastics in Fish in the Mississippi River Watershed

1.1. Introduction

Plastics include a wide variety of manmade products that are usually synthetic polymers blended with other chemicals (depending on their specific purpose). Plastics production has increased dramatically since the 1940s through mass production and consumption of plastics worldwide; global production of plastics was less than one million tons in 1941 (Thompson et al., 2009), and increased to 381 million tons by 2015 (Ritche & Roser, 2020). One result of the increase in plastic use is waste; plastics contribute to about 10% of the waste generated globally (Thompson et al., 2009) and an estimated 4.6 billion tons of plastic waste was discarded or landfilled between 1950 and 2015 (Ritche & Roser, 2020). Plastics are currently considered a serious environmental concern as they are responsible for the injury and death of many mammals, turtles, birds, and fish (Thompson et al., 2009). Further exacerbating the problem of plastic pollution is that people can see and observe plastic impacts on wildlife, creating aesthetic issues (Gregory, 2009). However, another problem associated with plastic pollution is the effect of microplastics in the environment, and this issue is particularly concerning because it is not observed with the naked eye, and therefore the scope and scale of the problem is not clear. Carpenter and Smith (1972) were among the first people to report microplastics when in the early 1970s they found small plastic pellets between 2.5 and 5 mm in the open ocean. Expansion of plastic production reached 299 million tons in 2013 (Gourmelon, 2015), and global concern about plastic pollution continues to increase. Thus, the initial problems detected by Carpenter and Smith (1972) are likely much more extensive today due to the plastic pollution accumulation for decades.

Microplastics

Microplastics are usually defined as plastics that are less than 5 mm in any dimension; however, there is no consensus lower size limit (Duis & Coors, 2016; Van Wezel et al., 2016). Microplastics are separated into two main categories. *Primary microplastics* are mass-produced in microscopic sizes typically for cosmetics, paint, and soaps (among other products). The other type is *secondary microplastics*, which include the breakdown of macroplastics such as tires and nurdles into microscopic pieces and fragments of microplastics. The process of defragmenting usually happens as a result of chemical and physical breakdown such, as photo-oxidation, mechanical stress, and wave-actions (Barnes et al., 2009; Cole et al., 2011; Van Wezel et al 2016).

Microplastics have been reported in oceans and coastal environments around the world (Cole et al., 2011), and as such there are likely different ways that microplastics enter and pass through aquatic environments. Microplastics can be directly introduced into the oceans through commercial and fishing vessels, aquaculture, oil rigs, lost or discarded fishing nets, and recreational activities such as tourism. However, in the marine environments, plastic pollution from land-based sources—including all the activities and plastic industries—contributes around 80% of what is found (Cole et al., 2011). The effluent from wastewater treatment plants is considered a major source of microplastics (Sanchez et al., 2014). For example, microplastics were detected in high concentrations (up to 24 microplastic particles per liter) in landfill leachate, which may runoff terrestrial systems and enter aquatic systems (He et al., 2019). Urban and agriculture areas usually discharge their wastewater into surface waters (e.g., rivers and streams), which makes surface waters a vector that transfers land pollutants to the ocean. Despite this simple pathway there is a lack of information about microplastics in rivers (and other

freshwaters) relative to the marine environments (Cole et al., 2011; Peng et al., 2018).

Microplastics have been found in fresh and marine water columns, sediments, primary producers, zooplankton, fishes, and even commercial salt. Because microplastics exist almost everywhere, many food webs are now potentially exposed to microplastic contamination (Cole et al., 2011; Peixoto et al., 2019; Scircle et al., 2020; Wang et al., 2019). Microplastics can have adverse impacts on microalgae growth and photosynthesis (Sjollema et al., 2016; Zhang et al., 2017). Microplastics can suspend on seaweeds, which make them available to the planktivores (Gutow et al., 2015). In a review, Botterell et al. (2018) reported that zooplankton ingest microplastics that results in adverse effects, such as the limitation of food intake and the possibility of damaging their alimentary canal. Microplastics can cause physical stress (especially to smaller organisms) such as clogging the digestive tracts and stress of ingestion, which can lead to physical deterioration, starvation, lower feeding activity (Anderson et al., 2016), reduced reproductive fitness and lower predator avoidance (Wright et al., 2013). Qiang & Cheng (2019) reported that after microplastics ingestion by larval zebrafish, they showed a significant decrease in their swimming speed and distance with implications to foraging and predator avoidance.

In addition to posing a physical threat to organisms, microplastics may also pose a chemical threat. Microplastics may become toxic to aquatic organisms through adsorbed pollutants such as endocrine-disrupting chemicals, waterborne pollutants, and harmful persistent organic pollutants (POPs). Many POPs are toxic and carcinogenic, currently controlled by Stockholm Convention (Gallo et al., 2018). POPs may be used in plastic manufacturing as plasticizers, heat retardants, and additives such as polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCDD), and polychlorinated biphenyls (PCBs), whereas other

POPs from other sources can also be attached or adsorbed on microplastics. These chemicals are usually found at the microlayer of the sea surface, where low-density microplastics are also most abundant. As lipophilic chemicals, POPs will adhere to the hydrophobic surface of microplastic. Microplastics coated with POPs can be transported over large distances or ingested by aquatic organisms. POPs can be toxic and cause serious problems such as mutagenesis, inducing endocrine disruptions, carcinogenesis, even biomagnified to higher trophic organisms (Cole et al., 2011). Batel et al. (2016) used microplastics with adsorbed benzopyrene to trace the movement of benzopyrene to brine shrimp and then to zebrafish, effectively establishing that microplastic pollutants can transfer throughout the food web.

There is no longer debate about whether or not microplastics are ingested by fish, as the existence of plastics in fishes digestive tracts has been extensively documented in marine (e.g. Davison & Asch, 2011; Güven et al., 2017; Wang et al., 2017) and freshwater environments (e.g. Collard et al., 2018; Silva-Cavalcanti et al., 2017). But specific information about the rate of plastic ingestion or comparing microplastic loads among different species remains unclear. For example, Murphy et al. (2017) reported that microplastic concentrations in fish is higher in coastal fishes compared to offshore fish species. However, they could not detect a difference in microplastics concentrations between demersal and pelagic fish due to sample size limitation. Lusher et al. (2013) reported that there was no significant difference of microplastics concentrations between demersal and pelagic species, while Güven et al. (2017) reported that demersal fish ingested less microplastic than pelagic fish. Andrade et al. (2019) reported that trophic guild did not make a difference for microplastic concentrations in fish they studied; however, they reported that the sizes of ingested mesoplastic particles (5.1 – 25mm) was different across guilds. Davison & Asch (2011) sampled 141 fishes from different 27 species,

and they only found microplastics in 9.2% of the fish stomachs. However, Lusher et al. (2013) studied microplastics in 504 fish and reported the average concentration of microplastics to be 1.9 microplastics particles per fish. McNeish et al. (2018) reported microplastics in 10 different fish species in Lake Michigan, the round goby *Neogobius melanostomus*, a zoobenthivore had the highest concentrations of microplastics in their digestive tract. From these studies and others, it is clear that plastic ingestion rates among fishes is variable and not well understood and could be inherently variable or the product of an emerging methodology.

The Mississippi River

The Mississippi River is the largest river in the North America and as it spans multiple latitudes, ecoregions, and biomes while encompassing 32 states, and discharging about 41% of the U.S freshwater to the Gulf of Mexico (Goolsby & Battaglin, 2001). It is directly affected by nearly all the anthropogenic activities in the United States. These activities include agriculture, urbanization, industry, and sewage discharges that affect the river's water quality with pollutants like DDT, fecal bacteria, and PCBS (National Research Council, 2008). More than sixty different organic compounds have been reported in the Mississippi River, including plasticizers, herbicides, polyaromatic hydrocarbons (PAHs), and other miscellaneous chemicals (DeLeon et al., 1986). Not only were POPs found in the Mississippi River, but microplastics have also been identified in different locations in the Mississippi River. Microplastics were measured from New Orleans, Louisiana to St. Louis, Missouri, and microplastics concentrations were found to increase downstream (Scircle et al., 2020). Also, in 2017, microplastics were reported in relatively high concentrations in the Gulf of Mexico off the coast of Louisiana (Di Mauro et al., 2017). Di Mauro et al. (2017) hypothesized that the high concentration of microplastics might be

influenced by the Mississippi River discharge.

Currently, there are no large-scale or system-wide studies about microplastics in the Mississippi River Fishes. However, there was a recent large-scale study in Mississippi River from St. Louis, Missouri to New Orleans, Louisiana (Scircle et al., 2020) that quantified microplastics in the water column in different locations in the Mississippi River. Also, multiple studies have reported microplastics in rivers waters and river sediment over a large scale: Ciwalengke River, Indonesia (Alam et al., 2019), Fall Creek and Six Mile Creek, New York (Watkins et al., 2019), Yangtze River, China (Xiong et al., 2019), and Pearl River, China (Yan et al., 2019). However, there are only a few large-scale studies about microplastics in river fishes. Horton et al. (2018) studied 203 km in the River Thames and Slootmaekers et al. (2019) studied the Flemish rivers in Belgium—yet they each investigated only a single fish species. To date, the only large-scale river study that examined multiple fish species was in the Xingu River tributary of the lower Amazon River—a scale of about 350 km (Andrade et al., 2019).

Conducting microplastic studies in fishes across large-scales, such as the Mississippi River, is important. Because 80% of plastic pollution in marine environments is estimated to come from terrestrial sources (Cole et al., 2011), understanding the characteristics of microplastics in large rivers may provide insight to the major source of marine microplastics. For example, plastic litter in the Gulf of Mexico is expected to come mainly from the Mississippi River. The Mississippi River's heterogeneous local habitats support different fish communities (National Research Council, 2008). Although for centuries, research has been focused on documenting the different fish species in the Mississippi River, virtually nothing is known about the relative consumption of microplastics by these fishes. Given the possible variability in microplastics consumption across fish species, one way to understand fish and microplastics

across large spatial scales is to focus on a set of widely distributed species. In theory, the same species should have (roughly) the same ontogeny, behavior, and feeding habits across space, and as such, differences in microplastic concentrations in the same species across a large area should be reflective of the microplastics in that location. Although the Mississippi River is home to an estimated 288 fish species (Ross & Brenneman, 2001), Bluegill *Lepomis macrochirus*, Largemouth Bass *Micropterus salmoides*, Shortnose Gar *Lepisosteus platostomus*, and Flathead Catfish *Pylodictis olivaris*, are widely distributed throughout the Mississippi River and also routinely sampled by fishery monitoring programs, making them good candidate species for large-scale studies.

The Species

After discussing the available options with the cooperating agencies regarding the species that are widely distributed throughout the Mississippi River, I ended up identifying four fish species (Table 1 and Figure 1) that are widely-distributed and typically common in the Mississippi River watershed, and that represent different foraging guilds. The purpose of different foraging guilds is to evaluate feeding habits and trophic guild as a possible factor in determining the number of microplastics in fish.

Table 1. Species of interest that are widely available in the Mississippi River and chosen for the microplastics sampling project

Species	Common Name	Trophic Guild
Pylodictis olivaris	Flathead Catfish	Benthic piscivore
Lepomis macrochirus	Bluegill	Insectivore / zooplanktivore
Micropterus salmoides	Largemouth Bass	Piscivore
Lepisosteus platostomus	Shortnose Gar	Piscivore

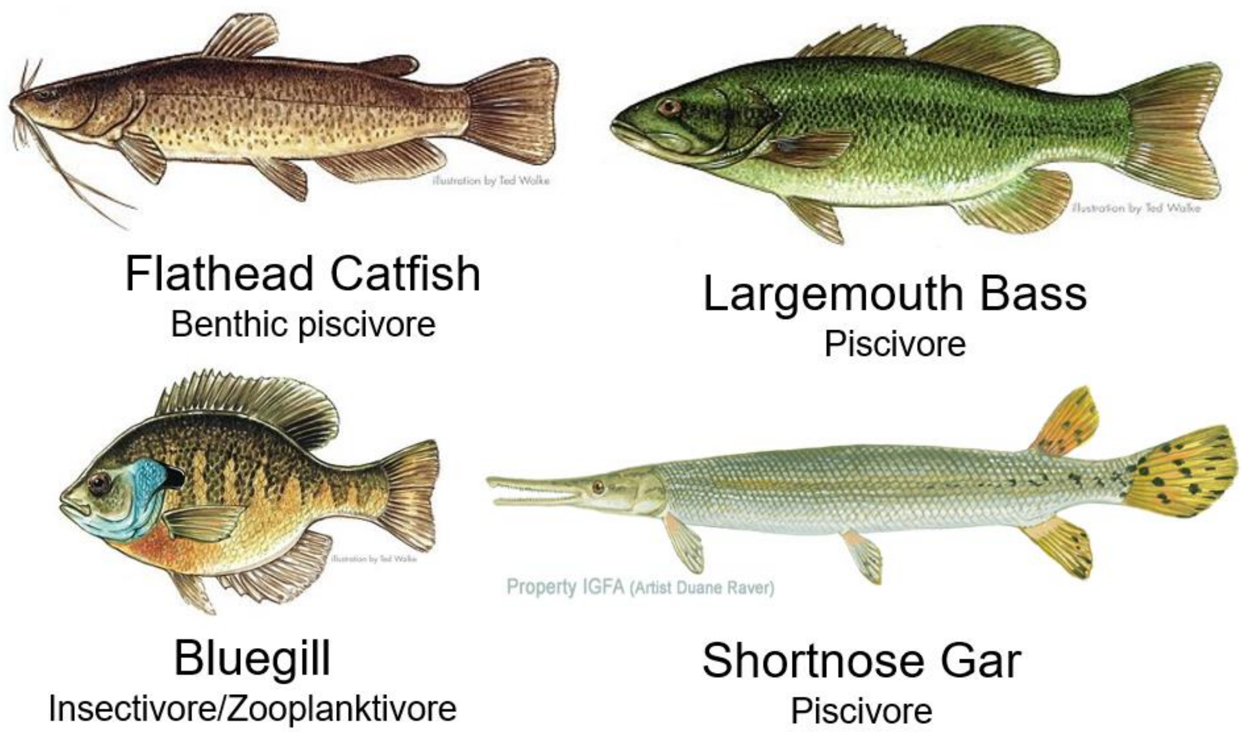

Figure 1. Species of interest that are widely available in the Mississippi River and chosen for the microplastics sampling project. Flathead Catfish, Largemouth Bass, Bluegill were illustrated by Ted Walke, while Shortnose Gar was illustrated by Duane Raver.

Bluegill *Lepomis macrochirus* are common warm water fish in the Mississippi River. They have a laterally-compressed body and live in waters that have dense rooted vegetation in which they live as they feed (Snow et al., 1960). Young Bluegill (total length [TL] < 150 mm) usually feed on plankton, such as copepods and small crustaceans, but as they grow (TL > 150 mm) their diets include insects and even small fish when their total length is larger than 200 mm, although they are not considered piscivores (Harris et al., 1999). Phillips & Bonner (2015) found microplastics in Bluegill stomachs in Texas, but they only examined 12 specimens. However, Peters & Bratton (2016) also examined microplastics in Bluegill in the Brazos River Basin in Texas, and 144 out of 318 Bluegill had microplastics in their stomachs.

Largemouth Bass *Micropterus salmoides* range from Canada to the southern U.S. The species haves fusiform body shape that compresses as they age. Much like Bluegill, Largemouth Bass usually inhabits river backwaters and shallow weedy lakes; however, Largemouth Bass are piscivorous and feed on fish and invertebrates, especially crayfish (Mraz et al., 1961). Microplastics were also reported in Largemouth Bass stomachs; however, the study only included 12 specimens (Phillips & Bonner, 2015). Hurt et al. (2020) studied microplastics in Largemouth Bass in Lake Bloomington and Evergreen Lake in Illinois, and microplastics were present in 100% of the specimens (n = 24) with high concentrations of microplastics (approximately 25 microplastic particles per fish).

Shortnose Gar *Lepisosteus platostomus* are abundant in freshwaters in the U.S. as it is common in the eastern two-thirds of the U.S. Unlike Bluegill and Largemouth Bass, much less is known about Shortnose Gar, due to its low commercial and recreational interest. Shortnose Gar are usually found in eddies and along the banks where the flow is slower than in mid-channel (Sutton et al., 2009). Shortnose Gar are considered opportunistic piscivores, yet and they

also feed on invertebrates. Their sagittiform body is adapted to stalking and ambushing preys (Sutton et al., 2009) rather than chasing. There were no reported studies of microplastics in Shortnose Gar, perhaps due to their overall lack of commercial and recreational importance.

Flathead Catfish *Pylodictis olivaris* are found throughout the Mississippi River from the Laurentian Great Lakes basin to the lower Mississippi River. Flathead Catfish are a relatively sedentary benthic species inhabiting substrates that are associated with other structures such as woody debris and riprap that provide them with cover (Daugherty & Sutton, 2005). Flathead Catfish are piscivores—they feed primarily on crayfish and zooplankton when they are small (TL < 250 mm), and their diet focuses more on fish as they grow (250–500 mm TL), until their diets become dominated by fish when they are larger than 500 mm TL (Jolley & Irwin, 2003). Also, there are no reported studies of microplastics in Flathead Catfish.

My goal was to understand microplastics pollution in the Mississippi River through quantifying microplastics in Mississippi River fishes. Mississippi River fishes were sampled for microplastic analysis throughout the river, and then comparisons were made to determine how microplastic concentrations in fishes differed between among fish species and locations in the river and basin.

1.2. Research Objectives and Hypotheses

Objective

The research in this chapter seeks to describe the relationships between consumed microplastics and fish *species* (i.e., foraging type) and *location* (i.e., site).

Hypotheses

Null Hypothesis for Species: There is no difference in microplastic concentrations among different fish species sampled across the Mississippi River.

Alternative Hypothesis for Species: Bluegill (feeding on small preys such as insects and zooplankton) will have higher microplastic concentrations than other foraging types due to their targeting of smaller prey items, which might mistakenly include microplastics or might include forage with incorporated microplastics following Batel et al. (2016).

Null Hypothesis for Location: There is no significant difference in microplastic concentrations in fishes sampled across different locations in the Mississippi River.

Alternative Hypothesis for Location (1): Microplastic concentrations will be higher at downstream sites (relative to upstream) in the mainstem Mississippi River, similar to water column results of Scircle et al. (2020).

1.3. Methodology

Study Area and Sampling

Fish were collected within existing fish sampling programs operated by state resource agencies: Louisiana Department Wildlife & Fisheries, Arkansas Game and Fish Commission, Missouri Department of Conservation, Wisconsin Department of Natural Resources, and Minnesota Department of Natural Resources. There were five sites on the mainstem of the Mississippi River (from Minnesota to Louisiana) and from two sites on major tributaries (Missouri River and Arkansas River) to represent 7 different sites (Minneapolis, Minnesota site, La Crosse, Wisconsin site, Missouri River site, Cape Girardeau, Missouri site, Caruthersville, Tennessee site, Arkansas River site, St. Francisville, Louisiana site) (Figure 1). Mainstem sites occasionally were sampled at more than one location, clustering was done to represent each state or city separately as follows: three locations around the Minneapolis, Minnesota site, one location in La Crosse, Wisconsin site, one location in Cape Girardeau, Missouri site, four location in Caruthersville, Tennessee site, one location in St. Francisville, Louisiana site, four locations in the Missouri River site, one location in the Arkansas River site). Fish were collected from the late summer and fall of 2018 and 2019. I did not get samples from Louisiana in 2019.

Figure 2. Map of Mississippi River Watershed with the sample collection sites on the mainstem and tributary. Red dot indicates study sites (7 = Minneapolis, Minnesota site, 6 = La Crosse, Wisconsin site, 5 = Missouri River site, 4 = Cape Girardeau, Missouri site, 3 = Caruthersville, Tennessee site, 2= Arkansas River site, 1 = St. Francisville, Louisiana site)

Fish Collection and Work-up

Fishes were collected from the Mississippi River, Missouri River, and Arkansas River primarily by electrofishing and gillnets by cooperating state agency partners. All fish were collected using electrofishing, except for: Flathead Catfish in Louisiana were collected using hoop nets (1.2m and 4.5m), and Shortnose Gar in Caruthersville, Tennessee were collected using 1.5m gillnets. I collected the fishes either by direct pickup from agencies (from nearby states), or through shipment (from distant locations) of freshly caught frozen samples with dry ice to Louisiana State University. All samples remained frozen and until processing. Fish samples were taken out of the freezer and kept in the fridge two days before processing to thaw. Fish samples were

weighed and total length (TL [mm]) was measured. Scissors were used to cut fish from the anus to the esophagus. The stomachs were removed and weighed (g) and preserved in glass jars.

There is currently no standard protocol to study microplastics in fish, as some researchers examine the whole digestive tract of fish (Güven et al., 2017; Karlsson et al., 2017; Lusher et al., 2013), while others examine only the stomachs (Andrade et al. 2019; Silva-Cavalcanti et al., 2017; Wagner et al., 2017). Researchers even use different identification techniques such as chemical digestions and Fourier transform infrared (FTIR) spectroscopy (Guven et al., 2017; Wang et al., 2017), chemical digestion and Raman spectrometry analysis (Collard et al., 2018; Karlsson et al., 2017;), and visual inspection of stomach contents under a dissecting microscope (Davison & Asch, 2011; Lusher et al. 2013; Silva-Cavalcanti et al., 2017). Using different techniques may also affect the result when comparing the concentrations of microplastics in fish—certain detection methods may have selectivity or biases toward different microplastic particle sizes or shapes.

Using a modified method (Foekema et al., 2013), stomachs were placed in in glass jars and fully submerged in a 10% filtered KOH (potassium hydroxide) solution, which dissolves organic matter leaving the plastics unharmed. The glass jars were kept in a water bath with a controlled temperature of 60ºC for 24 hours, manually stirring them every few hours until the organic matter was fully digested. After digestion, the slurry solution was vacuum filtered through a nylon net filter paper with 20 µm pore size. However, in case of the presence of a lot of organic matter and sediment left in the samples, a prior filtration step through 1000 µm sieve was done to separate the larger particles in a petri dish. Each filter paper was stored in a labeled covered petri dish and kept for 24 hours in an incubator at 50ºC to ensure that samples were dry before any further use. Macroscopic particles were removed with stainless-steel forceps and

processed using an FT-IR spectrometer (ThermoScientific iS5) to determine their identity and determine if they were plastics. The other smaller putative particles on filter papers were examined using an FT-IR microscope (ThermoScientific Nicolet iN10). Plastic polymer libraries were installed on both instruments, and I was able to identify whether each particle was plastic or not by matching the particle spectra with the libraries included in the Omnic Pecta software. Acceptable spectral match was defined as >70% match; however, spectra between 60–70% match could be confirmed or denied with a further visual examination. Lusher et al. (2013) relied on a 60–70% match with a visual examination. Samples with match percentage less than 60% were rejected. The plastic concentration was calculated as the number of plastic particles per fish.

To prevent contamination, nitrile gloves were used, I ran the gloves through the FT-IR and they were made of (Polybutadiene: *acrylonitrile 21% acrylonitrile*), and none of the microplastics in the samples had a complete match with this compound, although four samples were identified as polybutadiene acrylonitrile. All equipment and work surfaces were washed with distilled water prior to use, and stomachs and intestines were placed immediately in clean glass jars and new Petri dishes were closed immediately after putting the filter paper in each of them to prevent any aerial contamination. Control samples were processed regularly to test the possible contamination from the liquid potassium hydroxide, air, and equipment. No microplastics were identified in the control samples.

Data Analysis

The data analysis was conducted using R statistical software (Team. R, 20. Because the microplastic data that I collected was counts, and many fishes had zero counts, a Zero Inflated Poisson (ZIP) regression was used to test the relationship between the response of microplastics ingestion and the predictors of species and location. The use of a ZIP allowed for the accommodation of zero-inflation, while still performing the Poisson regression. The ZIP model is composed of two linked models. The first model is a logistic regression that models 0s and 1s using a Bernoulli distribution, which effectively estimates whether an observation is a value that should be included in the Poisson model for counts, or whether it is a zero that should be further excluded. The model is written as:

$$\omega_i \sim \text{Bernoulli}\ (\psi_i)$$

where ω_i is the logistic model estimate for fish i and ψ is the probability. The second model is a modified Poisson that models the counts,

$$C_i \sim \text{Poisson}(\omega_i \times \lambda_i)$$

Where C is the observed counts and λ_i is the Poisson parameter for counts. Note that the Poisson equation is very similar to a typical Poisson estimation, except the ω_i parameter is modifying what observations are included in the counts, and thereby reducing the number of zeros going into the Poisson regression. The above zero-inflated model structure was then used for two models that represented different questions. Below, for simplicity, I show the linear component of the regression:

$$\lambda = \alpha + \beta_1(species \times location) \text{ (model 1)}$$

1.4. Results

Sample Collections

A total of n = 229 fish samples was collected from five locations in the mainstem. n = 141 were collected in 2018, while n = 88 were collected in 2019. No samples were collected from Louisiana in 2019. The 229 fishes from the mainstem were collected from the five mainstem locations and the samples were found in all the locations, except for the absence of Flathead Catfish in Caruthersville (Figure 3).

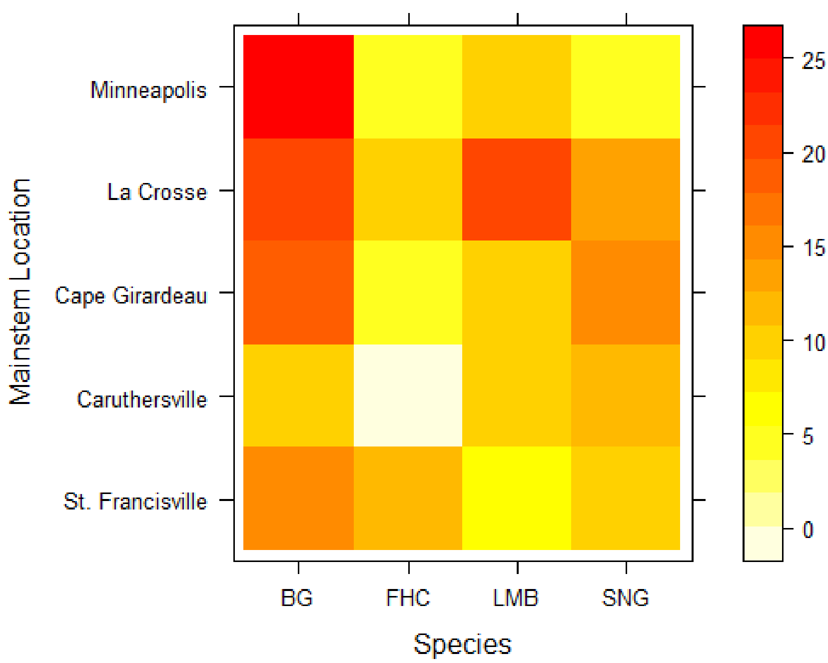

Figure 3. Heatmap of sample sizes of species split up by mainstem Mississippi River sampling locations. Species are listed on the bottom axis (BG = Bluegill, FHC = Flathead Catfish, LMB = Largemouth Bass, and SNG = Shortnose Gar) and the nearest city to the sampling location is listed on the left vertical axis. Darker colors (reds) indicate larger site-specific sample sizes while lighter colors (yellows) indicate lower site-specific sample sizes.

Microplastics Frequency

The most collected fish species were Bluegill, with 89 fish samples, while Flathead Catfish were the least collected with 31 samples. The percentage of overall fish with microplastics in their stomach contents was 12.22%, Bluegill showed the highest frequency of microplastic occurrence in their stomach with 13.48%, while Flathead Catfish had the lowest frequency of microplastic occurrence in their stomachs with 9.68% (Table 2). The percentage of fish that I found with microplastics in their stomachs were higher downstream locations compared to upstream (Figure 4).

Table 2. Fish species collected from the mainstem, and the frequency of microplastics occurrence in their stomachs

Species	**No. of fish**	**Average TL (mm) ± Standard deviation**	**No. of fish with microplastics**	**% of fish with microplastics**	**Average microplastics in fish**
BG	89	125.7 ± 41.8	12	13.48%	0.16
LMB	55	262.9 ± 93.4	7	12.73%	0.25
SNG	54	569.9 ± 77.4	6	11.11%	0.13
FHC	31	387.6 ± 92.7	3	9.68%	0.10
Total	229		29	12.66%	0.17

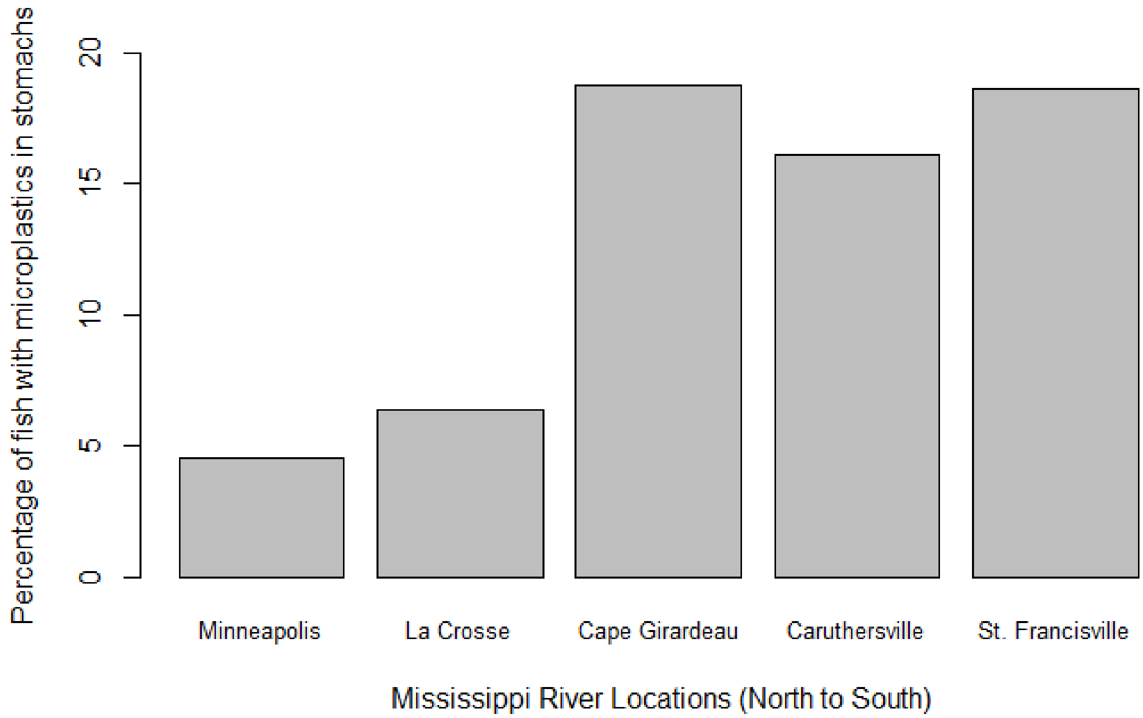

Figure 4. Barplot showing the relationship between different mainstem locations arranged from north to south (on the x-axis) and the percentage of fish that I found microplastics in their stomach (on the y-axis)

Zero inflated Poisson model

I used Zero Inflated Poisson model (model 1) to examine the relationship between the different locations and the microplastics counts ingested by fishes. The model reported a significant effect of zero-inflation model coefficients (binomial with logit link): Psi (ψ) estimate: 1.366, P value < 0.001, a statistically significant zero-inflation parameter estimate supports the choice of a zero-inflation model. A significant effect of the river kilometer was found for only one species, Largemouth Bass count model coefficients (Poisson with log link): Intercept estimate: 0.003, *P*

value < 0.01, while all the other species showed no effect of microplastic counts changing with river kilometer (Figure 5).

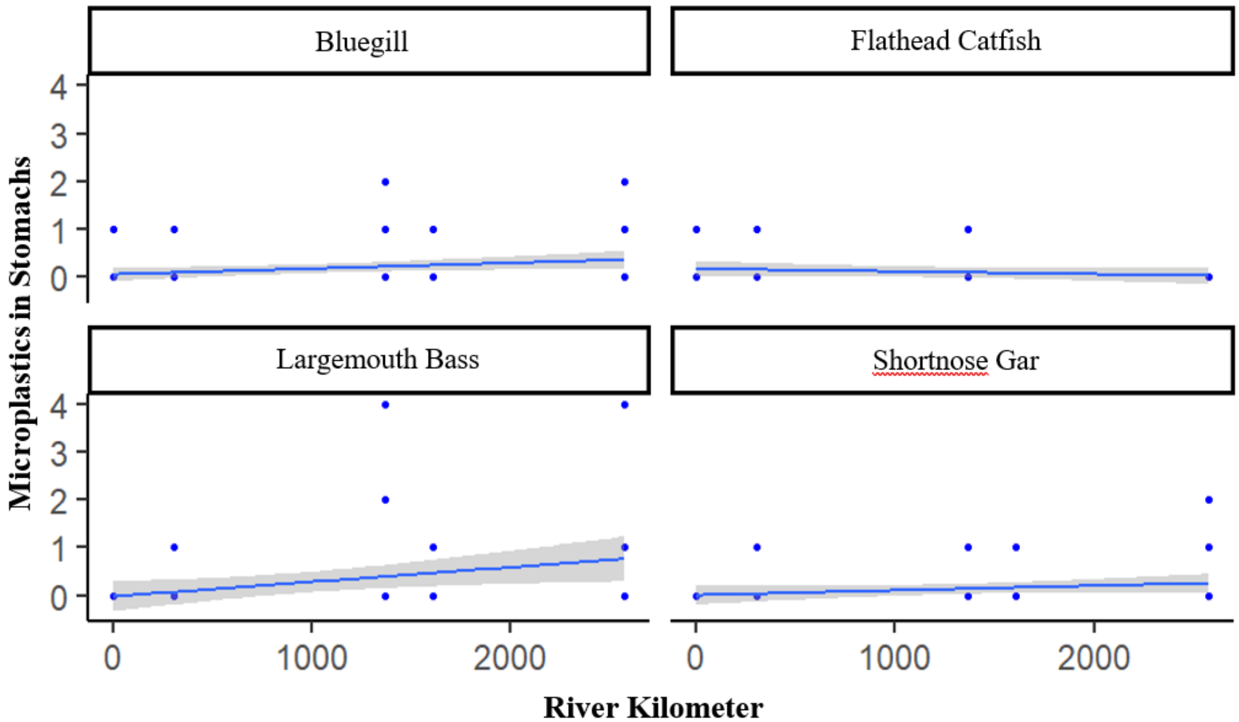

Figure 5. Different fish species with microplastics count in their stomachs in different locations in the mainstem from the north to the south (0 River Kilometer is the most northern location)

1.5. Discussion:

This study is the first macroscale study in the Mississippi River looking at microplastics in freshwater fishes. My results are largely in agreement with other studies that support the idea that freshwater fishes ingest microplastics (Collard et al., 2018; Silva-Cavalcanti et al., 2017). Plastics were found in the stomachs of all the four fish species—around 12% of the fish I sampled ingested microplastics. The number of fishes that ingested microplastics downstream locations was higher than the number of fish that ingested microplastics upstream (Figure 4). Only Largemouth Bass showed a significant effect of river kilometer on increasing microplastic counts in fish stomach. The number of microplastics found in Largemouth Bass stomachs was significantly higher downstream than upstream. Fishing gear and catch method can have an impact on the stomach emptiness and regurgitation differently between different fish species. I didn't have the control over the capture method as fish were provided by state agencies. However, most fishes were collected using electrofishing, which made the comparison easier between the microplastics concentrations in fish stomachs.

A recent study about microplastics in the Amazon freshwater fishes (Andrade et al., 2019) showed similar results—different fish species did not show significant differences in microplastic loads. Also, a recent study of microplastics in Mississippi River waters found that microplastics concentration in waters increase downstream, which matches my Largemouth Bass results. However, their study took place only from St. Louis, Missouri to New Orleans, Louisiana (Scircle et al., 2020).

Jabeen et al. (2017) reported that the amount of microplastics ingested are different between fish species, and they also found that demersal fish species ingested more microplastics. Rummel et al. (2016) found that pelagic species ingest more microplastics than demersal species.

I looked at microplastics in four different fish species, Bluegill and Largemouth Bass that inhabit shallow water zones (Trebitz et al., 1997) had more microplastics in their stomachs while Shortnose Gar usually found at surface of the water (Becker, 1983) and Flathead Catfish (demersal) had the lowest amount of microplastics. The microplastics counts in stomachs of the different fish species were close to each other that the results were not significantly different. My results show that microplastics ingestion does not differ in different fish species and habitats, which agrees with Lusher et al. (2013) as they did not find any significant difference between different species and habitats. Also, my study agrees with Andrade et al. (2019) and suggests that fish might ingest microplastics accidentally or through prey items as they are present in the water column. Benfield (1996) showed that turbidity and light intensity decreased the selectivity of prey items, which might contribute to the accidental ingestion of microplastics. Also, Abrahams (1997) mentioned that the reactive distance between the predator-prey interaction is reduced with increasing turbidity. I did not find a significant difference in microplastics loads in different species, but microplastics loads in fish stomachs did have a significant difference across different locations in the Mississippi River mainstem. The microplastic concentrations started to increase in fish stomach starting from Cape Girardeau, Missouri, this sudden increase in microplastics might be a result of the Illinois river discharge in the Mississippi River. That might imply that different fish species do not selectively ingest microplastics, but the more microplastics exists in water column, the higher the chance that fishes will ingest them.

The effects of microplastics are still unclear as most of the studies just focused on identifying microplastics in fish stomachs and intestines (Lusher et al., 2013; Cole et al., 2011; Andrade et al., 2019). However, some studies reported the potential impact of microplastics and all of them showed negative impacts on fish health (Anderson et al., 2016; Qiang & Cheng,

2019; Wright et al., 2013). Increasing microplastics in freshwater ecosystems can be dangerous due their direct impacts on fishes or their toxicity impacts which might affect fish populations, and furthermore public health through biomagnification of microplastics their associated POPs, as reported in Qiang & Cheng's (2019) laboratory experiment. Also, there is a need of developing a common protocol of identifying and quantifying microplastics in fish as different methodologies and protocols might affect the results and comparison between different studies and locations might be biased.

Chapter Two. Microplastics in Two estuarine Fishes in the Gulf of Mexico.

2.1. Introduction

Multiple studies have documented microplastics in estuarine ecosystems. For example, microplastics were detected in the sediments of Vitória Bay, Brazil (Neto et al., 2019). McEachern et al. (2019) reported microplastics in both waters and sediments of Tampa Bay, Florida. Microplastics were detected in three estuarine fish species, and 38% of 120 fish samples ingested microplastics in Mondego estuary in Portugal (Bessa et al., 2018). Kazour et al. (2018) reported microplastics in juvenile European Flounder *Platichthys flesus*; 58% of wild fish had microplastics in their digestive tract while 75% of caged fish had microplastics in their digestive tract. Microplastics have also been documented in benthic fish species in Texas Gulf coast (Peters et al., 2018).

Microplastics can be defined according to different characteristics. For example, microplastics can be categorized according to their shapes—they can be fibers, foams, fragments, beads, and films. Also, microplastics can be categorized according to their composition or what they are made, which usually refers to polymer type (Cole et al., 2011). Several recent studies that have looked at microplastics in estuarine fishes also consistently report that fibers are the most commonly detected (over 75% of the time) shape of microplastics (Bessa et al., 2018; McEachern et al., 2019; Neto et al., 2019; Peters et al., 2018). Some studies focused on microplastic polymers in estuarine systems. Peters et al. (2018) found that polyethylene terephthalate (PETE) and polyvinyl chloride (PVC) were the most detected polymers in estuarine fish, while Bessa et al. (2018) found that polypropylene, polyester, and rayon were the most detected polymers in estuarine fish.

The Mississippi River is an ecologically and economically important river in the North America. The Mississippi River has an average discharge of 16,791.89 m^3/s into the Gulf of Mexico (Kammerer, 1990). Microplastics were documented in the Mississippi River waters, and the microplastics concentrations were found in increase downstream (Scircle et al., 2020). At the receiving end of the Mississippi River is the Gulf of Mexico, a semi-enclosed water body that directly receiving discharges from the Mississippi River. Recent work has reported high loads of microplastics in the Gulf of Mexico waters, especially close to the mouth of the Mississippi River (Di Mauro et al., 2017). About 40% of the recreationally harvested fish and 25% of the U.S commercial fishery catches come from the Gulf of Mexico (Chen, 2017), which means that many people rely on coastal fish species from the Gulf of Mexico. Microplastics in the water column might affect fish species in the Gulf of Mexico and threaten the ecosystem and fish harvest. I looked for microplastics it two common coastal fish species by examining body size as a factor of interest and how it relates to the consumption of microplastics.

Two fish species were based on availability: Hardhead Catfish *Ariopsis felis* and Southern Flounder *Paralichthys lethostigma* (Figure 6). Hardhead Catfish is widely distributed in the Gulf of Mexico, southeastern U.S Atlantic, and parts of the Caribbean (Muncy & Wingo, 1983). Muncy & Wingo (1983) mentioned in their study that Hardhead Catfish constitute about 2 to 36% of bottom fishes by weight. Although Hardhead Catfish are not commercial species, this high abundance of Hardhead Catfish suggests an important role in the ecosystem, such that it might control, through predation, other fish species or provide prey for other predators. Hardhead Catfish live close to the mud and submerged sand flats and are an opportunistic feeder that has a varied diet including algae, crustaceans, seagrasses, worms, and fish (Muncy & Wingo, 1983). I could not locate any studies that examined the ingestion of microplastics by Hardhead Catfish.

Southern Flounder is a valuable recreational and commercial fish species. It is the most commonly harvested flatfish in the northern Gulf of Mexico (Corey et al., 2017). Southern Flounder have an estuarine-dependent life cycle as it spends its juvenile years in various parts of estuarine and coastal waters. Southern Flounder are an ambush predator—it camouflages on the bottom of the seafloor without motion and attacks prey when it comes within striking distance. Southern Flounder feed mostly on crustaceans in the juvenile stage, but their diet shifts more towards fish as they grow (Burke, 1995; Davenport, 2010). Philips & Bonner (2015) documented microplastics in stomach contents of Southern Flounder, but the sample size was only 8 fish and they did not mention how many fish ingested microplastics. However, they found microplastics in 10.4% of 116 fish samples of different marine fish species that they studied.

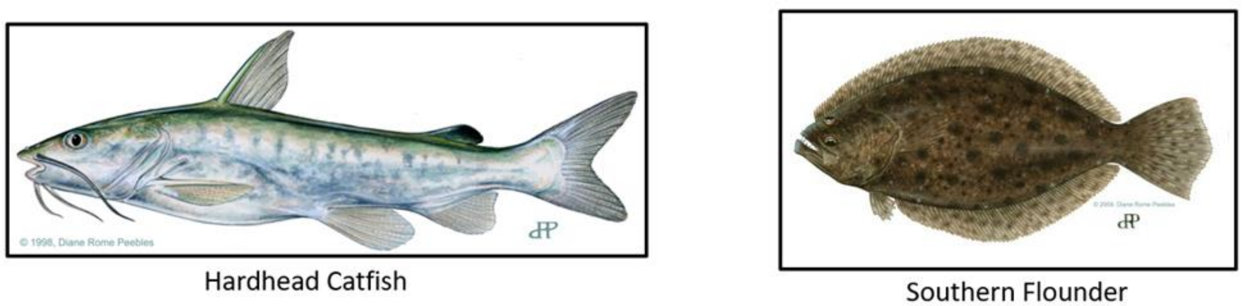

Figure 6. Fish species selected for the study in coastal Louisiana in the Gulf of Mexico. Hardhead Catfish *Ariopsis felis* on the right and Southern Flounder *Paralichthys lethostigma* on the left. Illustration by Diane Rome Peebles

Though it is now known that fish ingest microplastics, we do not always know the factors that contribute to individual fish microplastic loads. Therefore, to better understand microplastic pollution in estuarine ecosystems, I am attempted to quantify microplastic loads in two estuarine fishes and examine factors that might affect their ingestion such as body size, polymers types, shapes, and colors of microplastics. Studying those factors can give us an understanding of the mechanism of microplastics ingestion by fishes

2.2. Research Objective and Hypotheses

Objective

The research in this chapter seeks to help understand the relationship between consumed microplastics and fish size.

Hypotheses

Null Hypothesis for Fish Size: There will be no difference in microplastic concentrations across body size in two estuarine fish species.

Alternative Hypothesis for Fish Size: Microplastic concentrations will decrease with increasing fish size, as measured by the number of microplastic particles per fish, as smaller fish would eat more compared to their body size and their target preys are smaller that microplastics might be accidentally ingested.

2.3. Methodology

Study Area and Sampling

All fishes were collected from coastal Louisiana. All Hardhead Catfish were collected by the Louisiana Department of Wildlife and Fisheries (LDWF) as part of their fishery-independent sampling program. Sampling was conducted out of the Bourg and Lacombe LDWF field offices and primarily took place in Coastal Study Areas (CSA) I and V according to LDWF sampling procedures and gear SEDAR27 – RD – 06. Hardhead Catfish were collected in June and July 2018. The sample size for Hardhead Catfish was n = 40, which was equally balanced among four different size bins (0–99 mm TL, 100–199 mm TL, 200–299 mm TL, and >300 mm TL) in order to analyze the effect of body length on microplastics.

Southern Flounder were also collected by LDWF from both fishery independent and fishery dependent sampling programs. For fishery independent samples, Southern Flounder were collected from all five Coastal Study Areas (CSA) according to the marine fisheries section independent sampling activities manual of LDWF. For fishery dependent samples, fish were collected from two seafood dealers in Louisiana. Some Southern Flounder were collected from recreational fishing trips, and from recreational fishermen through Louisiana Sea Grant Extension Agents. Half of the Southern Flounder samples were collected with fish trawls (4.8m & 6m nets), while the rest of the fish were collected with different methods including hook line, electrofishing, seine and trammel nets. Southern Flounder collection took place from fall 2018 to summer 2019. The sample size for Southern Flounder was n = 50, which was equally balanced among 5 different size bins (0–99 mm TL, 100–199 mm TL, 200–299 mm TL, 300–399 mm TL, and >400mm TL) in order to analyze the effect of body length on microplastics.

Lab methods

Hardhead Catfish were measured for total length (mm TL), body weight (g), and gastrointestinal tract (GIT) weight (g). Hardhead Catfish were taken out of the freezer and kept in the fridge for one to two days to thaw before processing. After ensuring that fish were thawed, individual fish were dissected with scissors from the anus to the esophagus, the complete GIT was removed and weighed in grams, and stored in glass jars. Southern Flounder were measured for total length (mm TL), body weight (g), and stomach weight (g). Southern Flounder stomachs were dissected with scissors from the anus to the esophagus, the stomachs were removed and weighed in grams, in glass jars with appropriate size according to the digestive tract size. Digestive tracts of Hardhead Catfish and stomachs of Southern Flounder were digested in glass beakers using a modified method of Foekema et al. (2013). Samples were placed in glass jars and fully submerged in a 10% filtered KOH (potassium hydroxide) solution in order for the KOH to dissolve the organic matter and leaving the plastics unharmed. Glass jars with samples were kept in a water bath with a controlled temperature of 60ºC for 24 hours, manually stirring them every few hours until the organic tissues were fully digested. After digestion, the slurry solution was vacuum filtered through a nylon net filter paper with 20 µm pore size. Each filter paper was stored in a labeled, covered petri dish and kept for 24 hours in an incubator at 50ºC to ensure that samples were dry before any further use. Macroscopic particles were removed with stainless-steel forceps and processed using an FT-IR spectrometer (ThermoScientific iS5) to determine their identity and determine if they were plastics. The other smaller, putative particles on filter papers were examined using an FT-IR microscope (ThermoScientific Nicolet iN10). Plastic polymer libraries were installed on both instruments. I could identify if each particle was plastic or not by matching the particle spectra with the libraries using Omnic Pecta software. I adopted a

spectral match threshold of 70%; however, other spectra between 60–70% match could be confirmed or denied with a further visual examination. Lusher et al. (2013) relied on a 60–70% match with a visual examination. Samples with match percentage less than 60% were rejected. The plastic concentration was calculated as the number of plastic particles per fish or how many fish ingested microplastics.

To prevent contamination, nitrile gloves were used. I ran the gloves through the FT-IR, which reported that they were made of Polybutadiene (*acrylonitrile 21 percent acrylonitrile*), and none of the microplastics in the samples had a match with this compound. All equipment and work surfaces were washed with distilled water before use, and stomachs and GIT were placed immediately in the clean glass jars. New petri dishes were closed immediately after putting the filter paper in each of them to prevent any aerial contamination. Control samples were processed regularly to test the possible contamination from the liquid potassium hydroxide, air, and equipment. Glass jars with filtered KOH, but without stomachs were used as blanks, filtered, and scanned. No microplastics were identified in the control samples.

Data Analysis

Because the generated microplastic data were counts, and many fishes had zero counts, a Zero Inflated Poisson (ZIP) was used to test the relationship between body sizes (TL) and microplastics ingestion. The use of a ZIP allowed for the accommodation of zero-inflation, while still performing the Poisson regression (even if not zero-inflated). The ZIP model is composed of two linked models. The first model is a logistic regression that models suitability using a Bernoulli distribution, which effectively estimates whether an observation is a value (i.e., a zero) that should be included in the Poisson model, or whether it is a zero that should be further excluded. The model is written as:

$$\omega_i \sim \text{Bernoulli}\ (\psi_i)$$

where ω is the logistic model output, $_i$ is the observational units, ψ is the probability. The second model is a modified Poisson dealing with the counts

$$C_i \sim \text{Poisson}\ (\omega_i \times \lambda_i)$$

The above zero-inflated model structure was then used to represent our question.

$$\lambda = \alpha + \beta_1 \times (\text{Total Length in mm})\ (\text{model 1})$$

Where C is the observed counts, and λ is the Poisson parameter for counts where α is the intercept and β_1 is the slope. Model 1 was used to test microplastics in estuarine fish in the Gulf of Mexico to check if microplastics were increasing with increasing body size (TL mm). The model was used separately for each fish species as I was not comparing them.

Furthermore, I examined the frequency of microplastic occurrence in fish, the size of microplastics ingested relative to fish size (TL mm), and microplastics polymers, types (shapes) and colors that were found in fish stomachs and GIT. The data analysis was conducted using R statistical software (Team. R, 2020).

2.4. Results

Microplastics Frequency

Out of 40 Hardhead Catfish *Ariopsis felis*, a total of eight microplastics particles were identified in six individual fish GIT (Figure 7); four fish ingested only one microplastic particle except for two fish that each ingested two microplastics particles. Out of 50 Southern Flounder *Paralichthys lethostigma*, a total number of 16 microplastics particles were identified in 13 fish stomachs (Figure 7). Ten fish ingested only one microplastic particles, and 3 fish each ingested two microplastics particles.

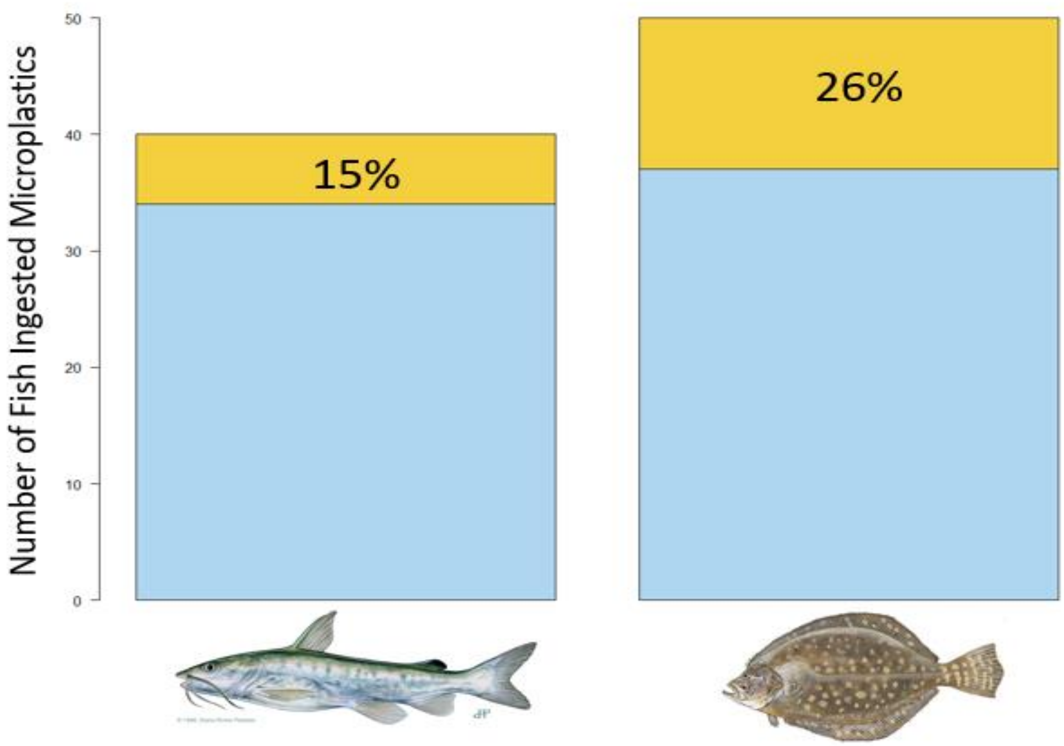

Figure 7. Barplot showing the relationship between fish species Hardhead Catfish and Southern Flounder (on the x-axis) and the percentage of fish that ingested microplastics (on the y-axis), blue color = fish didn't ingest microplastics, yellow color = fish ingested microplastics

The spectra of the nitrile gloves that were used during fish processing did not match any of the microplastics polymers spectra that were found in the fishes. Polypropylene and polystyrene polymers represented around 58% of microplastics polymers that were found (Figure 8). Seventy-five percent of the microplastics that Hardhead Catfish ingested were fragments shaped microplastics while 93% of the microplastics that Southern Flounder were fibers shaped microplastics (Figure 9). Regarding microplastics colors, 75% of microplastics ingested by Hardhead Catfish ingested were blue colored microplastics and 25% were white ones, while Southern Flounder ingested an equal portion of blue and black colored microplastics (Figure 10).

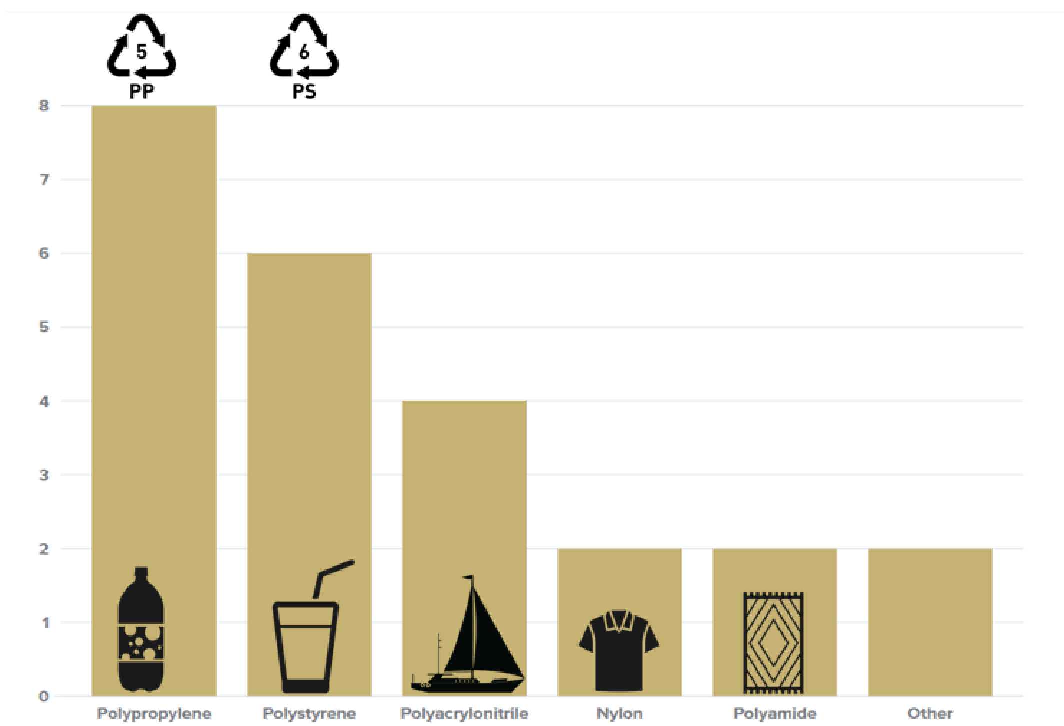

Figure 8. Barplot showing the type of microplastics polymers and some of their uses that were found in both fish species Hardhead Catfish and Southern Flounder (on the x-axis) and the number of the microplastics polymers (on the y-axis)

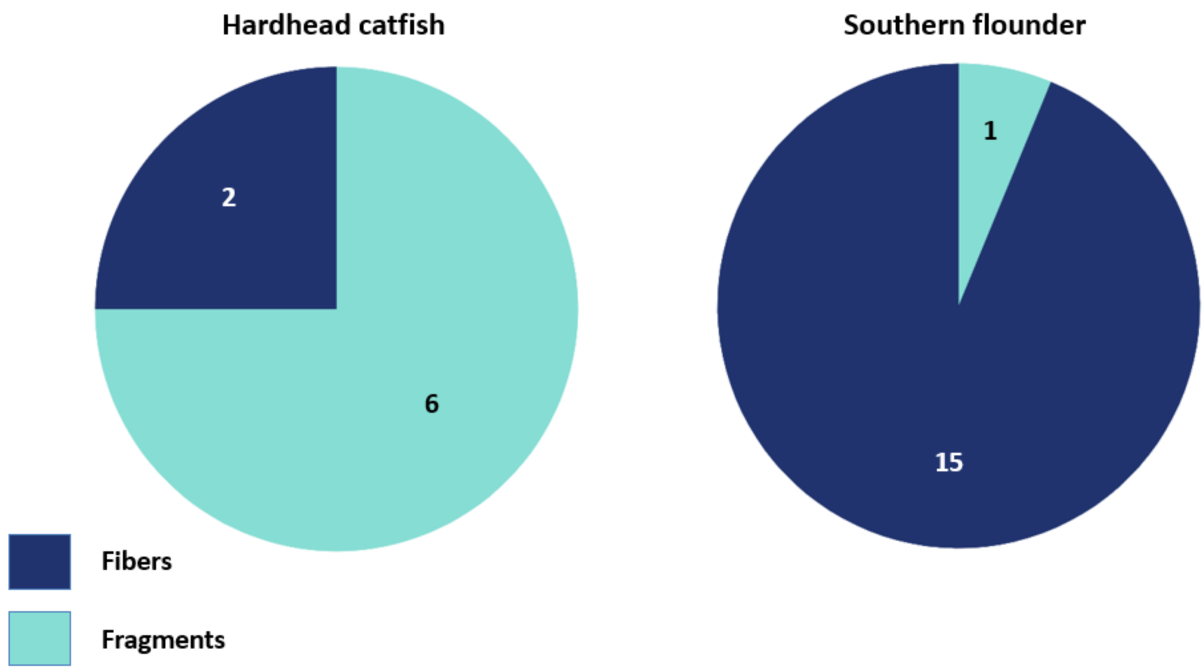

Figure 9. Pie chart showing the numbers of microplastics types (shapes) that were found in Hardhead Catfish and Southern Flounder

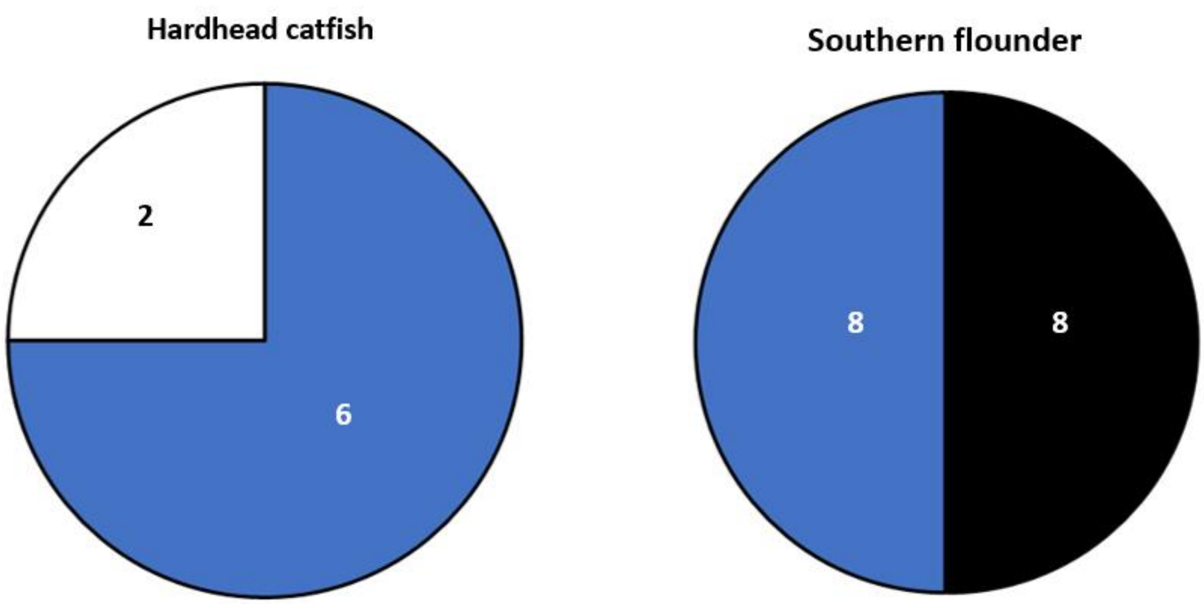

Figure 10. Pie chart showing the colors of microplastics that were found in Hardhead Catfish (blue and white) and Southern Flounder (blue and black)

Microplastics loads vs body size (TL)

The model estimated zero inflation in approximately 46% of the zeros. A significant positive effect was found with Count model coefficients (Poisson with log link): TL estimate: 0.009, P value < 0.03 of total length on the number of microplastics ingested was found for Hardhead Catfish (Figure 11, A). For Southern Flounder, the same effect was positive, but not significant ($P > 0.05$) (Figure 11, B). There was no obvious relationship between fishes' body sizes (TL mm) and the sizes of the microplastics ingested (Figure 12).

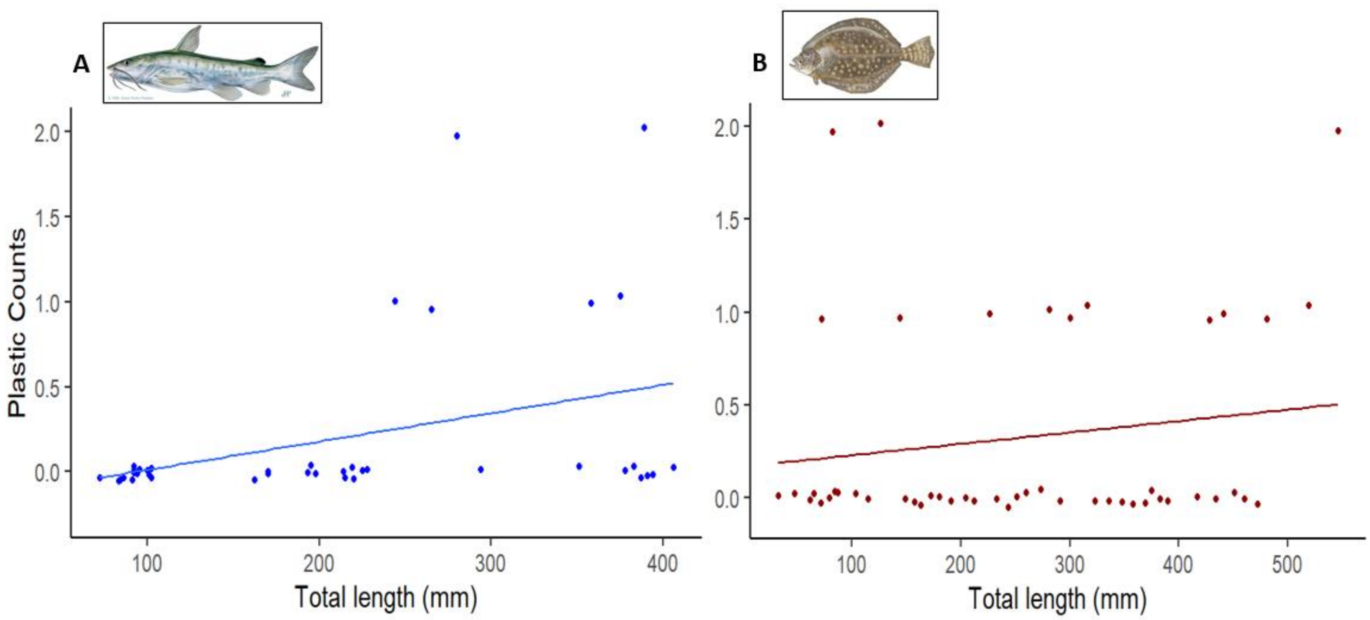

Figure 11. Hardhead Catfish total length (on the x-axis) and the microplastics count in their GIT (on the y-axis), B) Southern Flounder total length (on the x-axis) and the microplastics count in their stomachs (on the y-axis)

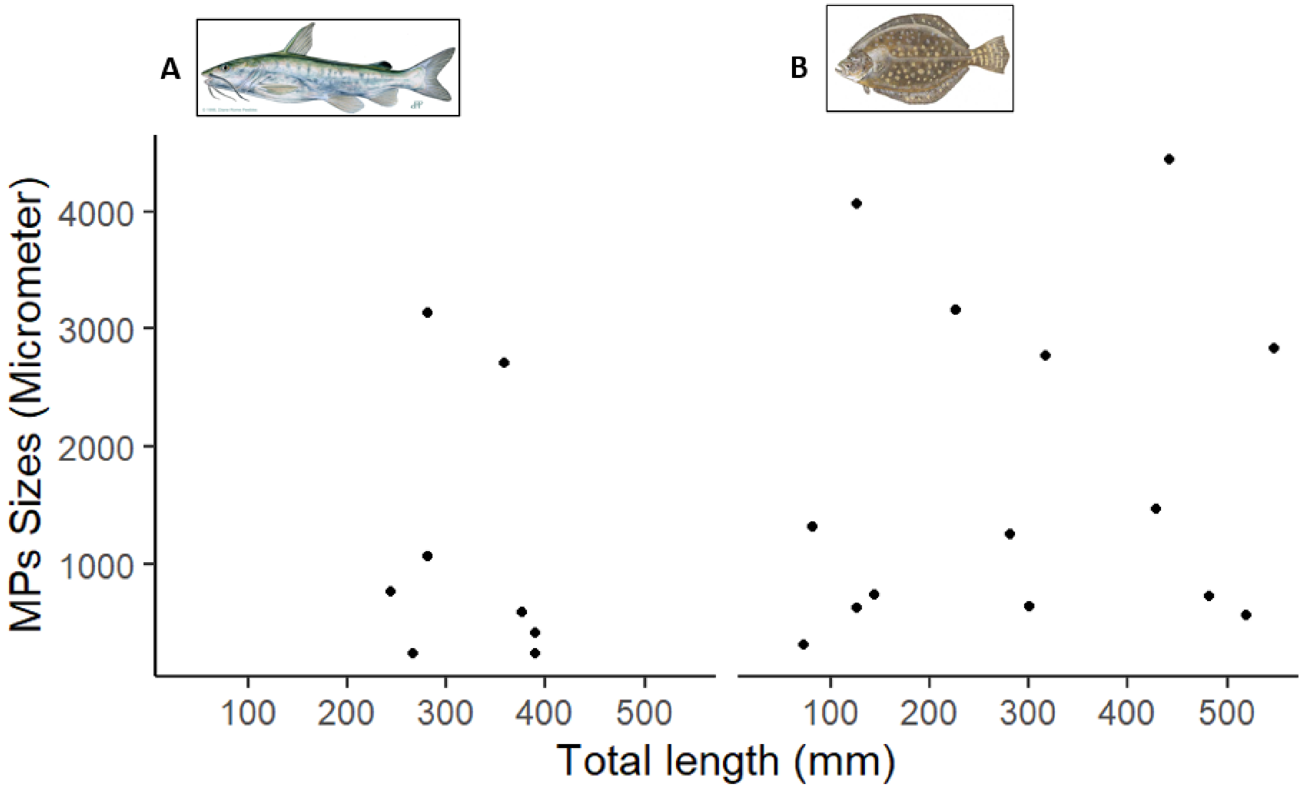

Figure 12. Hardhead Catfish total length (on the x-axis) and the microplastics sizes ingested in their GIT (on the y-axis), B) Southern Flounder total length (on the x-axis) and the microplastics sizes ingested in their stomachs (on the y-axis)

2.5. Discussion

This study is the first record of microplastics in Hardhead Catfish. I found that microplastics concentrations in Hardhead Catfish GIT significantly increased with increasing the body length. For Southern Flounder, I recorded higher concentrations of microplastics in Southern Flounder stomachs comparing to the only published study (Philips & Bonner, 2015) where the sample size was only 8 Southern Flounder and they did not mention how many fish ingested microplastics. However, they found microplastics in 10.4% of 116 fish samples of different marine fish species that they studied. Southern Flounder body length was not found to have any significant effect on microplastic loads in the stomach. Southern Flounder are ambush predators. This feeding behavior might contribute to the apparent random nature of microplastics in different sizes as microplastics might come indirectly from their prey or accidentally from the water column.

The goal of my study was to document microplastics in two estuarine fish species in the Gulf of Mexico and the understanding of microplastics concentrations across the different total lengths. Although I am not comparing the two fish species, I looked at microplastics in Hardhead Catfish in their whole digestive tract while only the stomachs in Southern Flounder. However, it was interesting to see that Southern Flounder had higher concentrations of microplastics, 26% of fish ingested microplastics, while only 15% of Hardhead Catfish ingested microplastics.

The main plastic polymers that were found were polypropylene and polystyrene, which represented >50% of the polymers found in fish species. Wessel et al. (2016) studied microplastics in the beach sediment of the Gulf of Mexico and they found that polypropylene and polyethylene are the most available microplastics polymers, followed by polystyrene, polyamide, polyamide, and polyester. Polypropylene and polystyrene are among the five most common plastic polymers produced globally in 2012 (Nerland et al., 2014). Polypropylene

represented 19% of global plastic production (54 million tons) while polystyrene represented 7% of the global plastic production (21 million tons).

Previous studies (Lusher et al., 2013; Boerger et al., 2010) reported that fibers represented the majority of microplastics shapes that were found in fish. Southern Flounder had the same representation as previous papers as they mostly ingested fibers. However, Hardhead Catfish mainly ingested fragments. Regarding the microplastics colors, Hardhead Catfish mostly ingested blue colored microplastics and some white ones, while Southern Flounder ingested an equal amount of blue and black colored microplastics. Alvarez-Zeferino et al. (2020) found microplastics in sediments in the Mexican beaches, and they found that the white colored microplastics were the most abundant followed by blue and green colors. It is still unclear if each fish non-randomly select shape or color of microplastics. More available reports and studies might contribute to useful information of microplastics ingestion by fish or it may be that fish accidentally ingest whatever microplastics they find.

Direct comparison of the two species is limited by the fact that for one species used the whole GIT and for the other species examined only the stomach. Another effect might be related to the specific location that the fish were collected from in coastal Louisiana. However, the sample size did not support separating.

The overall conclusion is that estuarine fishes do ingest microplastics, especially common polymers. Southern Flounder ingested more microplastics that Hardhead Catfish. And opportunistic species such as Hardhead Catfish, might have a trend of microplastics ingestion across their body size TL because they feed regularly on different prey and eat more as they grow and have larger total length. Finally, fishes might ingest microplastics accidentally from the water column or indirectly through their prey items, as fish size (total length) did not have an

effect on the sizes of the ingested microplastics.

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Vita

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