

Microplastic ingestion by two estuarine-associated mullet species,
Chelon richardsonii and *Chelon dumerili*
in the Breede Estuary, South Africa



Minor Dissertation

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Abstract

Microplastic (MP) ingestion in fish has the potential to cause a multitude of damaging effects to both organisms and the environment. Microplastics (MPs) are defined as solid plastic particles $\leq 5\text{mm}$ in size. The small size of MPs makes them extremely hazardous, as they imitate a range of food items on which organisms feed. Rivers and estuaries are links between terrestrial and marine ecosystems; thus, these systems hold key evidence of the magnitude and nature of MP pollution. Research particularly in the southern hemisphere is poorly documented for MP ingestion. This study shows MP ingestion by two mullet species (*Chelon richardsonii* & *Chelon dumerili*) in the Breede Estuary, South Africa. It was found that 98.4 % of the captured fish had ingested MPs and ingestion took place throughout the estuary by both species. The presence of brown/orange fragments and concentration of MPs found in the large ($\geq 200\text{mm}$) mullet, were consistent with what was found in sediment samples, indicating large mullet intake MPs via sediment ingestion. This was not the case for small ($< 200\text{mm}$) mullet, thus indicating small mullet selectively fed on particles and MPs in the water column. Small mullet ingested concentrations of MPs one order of magnitude greater than large mullet throughout the estuary, possibly reflecting the feeding transition from planktivorous juveniles to benthic adult detritivores. The MP conc. ($\text{MP}\cdot\text{g}^{-1}$) in small mullet was significantly ($p < 1.639\text{e}^{-12}$) higher compared to large mullet. A significant difference ($p < 0.008$) was found between MP conc. ($\text{MP}\cdot\text{g}^{-1}$) and species, this interspecific differentiation was driven by small ($< 200\text{ mm}$) fish, which selectively and visually feed. This study provides insights and new knowledge regarding MP pathways within estuarine food webs in South Africa. A better understanding of MP pathways is important to mitigate the resulting risks associated with fish, humans, and any other animal-related consumption.

Keywords: Breede River . Estuary . Microplastic concentrations . Mugilidae . Sediment pollution . South Africa

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Introduction

The first synthetic plastic was produced in 1907, but the plastic industry was only established in the late 1950s (Elizalde-Velázquez et al., 2020). From 1950 to 2015 the annual production of plastics increased by 200-fold, where global plastic production up until 2015 amounted to 7.8 billion tonnes (Roser and Ritchie, 2018). It is predicted that by 2050 plastic litter will be heavier than the fish in the ocean (Okunola et al., 2019). Today, plastic has become a part of human culture and is found even in the most remote places on earth (Woodall et al., 2014). A recent study showed that a regular grocery bag was deemed the deepest known piece of rubbish. It was in the Mariana Trench 10,898 meters deep (Chiba, 2018). Increased amounts of plastics in the environment have caused amplified interactions between plastics and organisms, therefore potential for ingestion and other adverse effects of plastics entering organisms are increasingly heightened. Two of the largest impacts of marine plastics on organisms include the ingestion of macro-plastics, meso-plastics, microplastics, and microfibrils by organisms and entanglement/collision due to lost fishing gear (Bakir et al., 2020). Various trophic levels within marine ecosystems are impacted by plastic ingestion. Species such as marine mammals (Nelms et al., 2019), sea birds (Provencher et al., 2018), fish (Wright et al., 2013; Naidoo et al., 2016), and invertebrates (Foley et al., 2018) have all been extensively studied, all resulting in harmful physiological effects via plastic interactions (Wright et al., 2013). Studies related to species effects have focused on flagship and economically important species. This bias has left other species and ecosystem implications poorly understood.

Plastics that end up in water systems can enter either through marine or land-based sources. Globally, 80 percent of plastics come from land-based sources, while the remaining 20 percent are marine (Li et al., 2016). Land-based sourced plastics involve the direct discard of plastics directly onto the shoreline, through river inputs where water flows through urbanised & industrial regions. Rivers and estuaries serve as plastic transporters into oceans. Plastics in the ocean are pervasive with particles being detected in the most remote marine atmospheres (Trianic et al. 2020). Plastic distribution and quantification have been studied well globally, but ecological and biological effects are still growing fields of research.

MPs are solid particles of plastic that are ≤ 5 mm in size (Wright, et al., 2013; Tirkey and Upadhyay, 2021; Naidoo and Glassom 2019), however, MP size classification is greatly debated. For this study, MPs are defined as ranging between 1 μ m and 5mm. Scientific literature categorises MPs into different shapes which include films, foams, fragments, fibres, and microbeads or pellets (Tirkey and Upadhyay, 2021). MPs are subdivided based on their source

of origin, namely primary and secondary MPs. Primary MPs are specifically manufactured to be a certain size and are used for a variety of purposes. An example of this would be microbeads or pellets that are used in many cleaning, household, and cosmetic products (Cheung et al., 2018). Plastics are extremely durable; they do not break down fully in treatment plants and eventually end up in some sort of aquatic system. Secondary MPs refer to the breaking down or fragmenting of plastic, mostly by photolytic and mechanical degradation (Chapman, 2007). Photolysis is the process where sunlight oxidises the chemical structure causing the plastic to become brittle and fragment into smaller pieces. In the marine environment, secondary plastics are predominantly fragmented by mechanical degradation because of wave action and sediment abrasion (Chapman, 2007). The composition of plastics allows them to persist in the natural environment for hundreds, even thousands of years.

Research has shown that the small size of MPs imitates food items on which many marine species feed (Lusher et al., 2013). Once ingested, MPs can have negative physical effects such as abrasions of internal organs and disrupting or blocking of organ function. MPs within a species can mislead the species into not feeding enough and effectively cause malnutrition. Additionally, MPs have chemical effects, where the leaching of certain chemicals from plastic additives and monomers can affect the endocrine system and be carcinogenic (Wright et al., 2013). Toxins and other harmful chemicals can also be absorbed by certain plastics, and depending on their constituents they can have detrimental effects (Cannon et al., 2016). MPs provide a transport medium for bacteria and pathogens to attach (Zhai et al., 2023). Studies documenting plastic consumption by organisms within Africa are scarce and even more so for estuarine systems, thus leading to a massive demand for scientists and other related experts to fill this knowledge gap (Pereao et al., 2020; Dahms et al., 2020; Aragaw, 2021).

Although research in MP ecology is growing at a rapid rate, studies have predominantly focused on specific organisms and habitats (McNeish *et al.*, 2018), estuaries, and rivers have largely been excluded from such studies. The literature that does include MPs in estuaries or river systems is extremely focused on mostly surface MPs (Anderson et al., 2018; Han et al., 2020) Consequently, information on plastics within the water column and sediment is lacking. A recent study by Defontaine et al., 2020, found MPs were present in the entire water column, and peak concentrations were located close to the riverbed of the Adour Estuary, France. Studies focused on only surface MPs would have a large underestimation of MP levels.

Mugilidae are commercially abundant schooling fish, commonly referred to as mullet. Mullet plays an essential ecological role within estuary systems and coastal inshore regions worldwide. Serve as a common food source for mostly larger piscivorous fish, bird, and marine

mammal species. Mullet represents keystone species in both shallow marine and estuary food web dynamics. Mullet are benthic feeders which makes them ecologically important in the energy flows of estuarine ecosystems but also increases their susceptibility to ingesting MPs (Cheung et al., 2018). Juvenile mullets are predominantly planktivorous feeders, with a large dietary breadth. As they mature from juveniles to adolescents and adults, their feeding habits transition to them being primarily benthic detritivores, ingesting sand, detritus, and epiphytes (Whitfield 2019; Marais 1980). This change in feeding habits may have important implications in terms of the amount, type, and size of the plastics being ingested.

Biologically, a large mullet has a more muscularly developed gastric mill, which is the organ used to grind food and sand. A more powerful gastric mill might easily break MPs into smaller pieces, which could imply that smaller mullets would be more susceptible to physical internal damage or obstruction, as their gastric mill is less developed. Associated patterns of plastic ingestion by estuarine mullet are the core of this investigation, and although understanding the implications that this would have upon human ingestion is not investigated here, it is an important consideration for future studies. Mullet might represent great indicator species as they feed throughout the water column and sediment during different life stages. To our knowledge, this is the first study to take place in the Western Cape where MPs in estuarine fish are investigated. In the last 10 years, only two research papers on microplastics in estuarine-associated fish have been conducted in South Africa, both were done in the province of KwaZulu Natal (Naidoo et al., 2016; Naidoo and Glassom, 2019).



Figure 1: On the left - *Chelon dumerili*, identified by: the presence of a yellow spot on the operculum, has groovy scales that displace easily, and its pectoral fins extend past the eye when bent. On the right - *Chelon richardsonii*, identified by: the presence of a yellow spot on the operculum, small scales, and its pectoral fins don't extend past the eye when bent.

In this study, I measured the concentration of MPs in sediment and in two mullet species (**Figure 1**) from the upper, middle, and lower reaches of the Breede Estuary, South Africa. As there was no previous information on MP concentrations in this system I developed five working hypotheses to structure this research: I expected (H1) that the mean MP conc. ($\text{MP}\cdot\text{g}^{-1}$) in fish would be higher in the lower reaches of the estuary, due to MPs there being the sum of that coming from the catchment and sea. (H2) MP concentration would increase positively as body size increases within species, because bigger fish would be able to ingest a greater amount of plastic particle sizes. In addition, larger fish with a wider gape were hypothesised (H3) to be

able to ingest bigger food/prey and plastic particles. (H4) Sediment MP conc. ($\text{MP}\cdot\text{g}^{-1}$) would be similar to MP conc. ($\text{MP}\cdot\text{g}^{-1}$) found in large ($\geq 200\text{mm}$) fish, based on evidence of larger fish having a benthic feeding behaviour. Finally, (H5) I predicted that there would be minimal difference between the MP conc. ($\text{MP}\cdot\text{g}^{-1}$) in the two species investigated, as juveniles across all species feed mostly on zooplankton.

Materials and Methods

Study site

The Breede Estuary is in the Western Cape of South Africa. The estuary mouth is situated at $34^{\circ}24.483'S/20^{\circ}50.833'E$ and is permanently open to the sea. The Breede River is 322 km in length from its source in Ceres to where it enters the Indian Ocean in Sebastian Bay (Taljaard et al. 2001, Flemming & Martin 2021). It's estimated that the Breede River catchment area is approximately 12 600 km^2 (Taljaard et al. 2001, Flemming and Martin, 2021). The estuarine section of the Breede, which in its definition corresponds to the distance upstream where tidal wave propagation occurs, is roughly 50 km with an open water area of 11,71 km^2 (Taljaard et al. 2001, Van Niekerk et al. 2019). The 50 km estuarine stretch was subdivided into 3 reaches (upper, middle, and lower), which were approximately 16.5 km per reach. Human population densities along the estuary are very small, comprised of three settlements Malgas, Infanta, and Witsands. In the past, as with most South African estuaries, the Breede Estuary was fished by commercial and recreational fishers, but today only recreational fishing and limited illegal fishing take place. During the holiday periods boat traffic may be more than 100 on some days (Lamberth et al. 2008, Lower Breede River Conservancy unpublished data), with boat-based angling being the most popular activity. The Breede Catchment falls within both the winter and bimodal rainfall regions with dominant rainfall and high flow in the winter months. Thus, the estuary is characterised as having a high flow variability and is subject to regular winter and spring flooding, where up to 50% of the annual discharge may be received over a few days (James et al. 2017), these freshwater flows of $20\text{--}95 \text{ m}^3\text{s}^{-1}$ can completely reset the system (Lamberth et al. 2008). In the present day, the estuary receives a Mean Annual Runoff (MAR) of $1\,034 \text{ m}^3 \times 10^6$ which is a 43 % reduction of that under reference, more pristine conditions (Van Niekerk *et al.* 2022). Discharge levels are an important factor in determining MP abundance hotspots; lower discharge rates, coupled with flood tides, will directly result in higher MP concentrations remaining in the estuary system (Defontaine et al., 2020). Heavier particles are more susceptible to being trapped in estuarine systems as their extra weight reduces the chance of them being flushed out to sea. Both the flow from catchment areas and

the incoming tides in the estuary cause a large river estuarine interface (REI) to be formed. The REI zone is a dynamic area where the mixing and settlement of particles take place, this could potentially serve as a MP accumulation zone or hotspot.

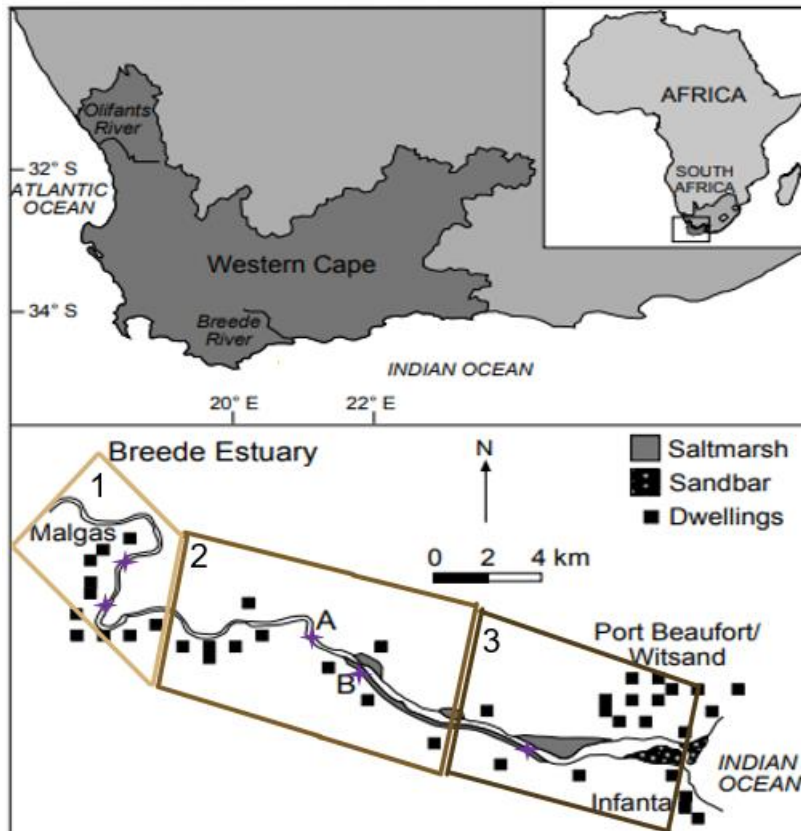


Figure 2: Top image - The Breede river within the Western Cape. Bottom image (Breede estuary) - 1 (light brown) The upper reach of the Breede Estuary; 2 - (medium brown) the middle reach of the Breede Estuary; 3 - (dark brown) the lower reach of the Breede Estuary. The purple stars indicate the approximate locations of the sampling sites (n=5). Image taken from Lamberth et al., 2008.

Quantification of microplastic contamination

To limit the contamination of samples I followed a protocol, Examinations and Standards, (2016), Edited by Amarein J Fourie and Danica Marlin - 2022 Edition. All equipment was acid washed (HCl) and rinsed with Reverse osmosis (RO) water (3 times) before being used, nitrile gloves and a 100% cotton lab coat were always worn in the laboratory. Equipment such as beakers and filtration apparatus were covered with foil when not in use, limiting airborne contaminants. All lab work took place under a laminar flow to prevent any MP contamination. Additionally, all surfaces were cleaned with acetone or HCl before and after procedures. Testing for contaminants was still executed despite the previously mentioned precautions, blank laboratory control samples (n = 10) were prepared using an open petri dish and a wet (RO water) 47 mm diameter with a 2.7 µm porosity GF/D filter (Whatman). Control samples were processed with the same protocol used for fish and sediment samples. Controls were

analysed under a microscope, resulting in 0.1 fiber particles per filter, resulting in contamination being dismissed.

Fish and sediment collection

Mullet (*C. richardsonii* and *C. dumerili*) were collected during a routine DFFE fish survey in the Breede Estuary, from the 8th to 10th of November 2022. Out of 20 routine sampling station sites, six stations were selected to get coverage of the upper, middle, and lower reaches of the estuary (**Figure 2**). Twenty fish per species within each area was the target sample size. In the upper reaches, two stations were sampled that were approximately 2 km apart, with the middle reaches being the same. However, only one station was sampled in the lower reaches as more than enough fish had been retained at one sampling site. All fish were captured with a seine and a gill net. The seine net (30 m × 2 m × 15 mm bar mesh with a 5 mm bar mesh in the middle 10 m including the cod-end) was used to sample small juvenile fish. It was set from a rowboat in a semi-circle and hauled to shore by four people. The gill nets comprised three panels of 30 m × 2 m monofilament nylon nets of 48, 51, and 54 mm stretched mesh.



Figure 3: Stephen Lamberth and Ricardo Williamson hauling in the gill net on the Breede Estuary, South Africa.

Fish were held in containers of water, sorted to the lowest taxonomic level, measured in millimetres for total length (mm TL), and returned alive to the system where possible. At each selected station, a subsample of dead mullet that were retained by the Department's personnel [MLRF research exemption permit Res 2022/72] was placed in labelled prewashed bags within an ice-filled cooler box. Once on land, fish were sorted by species per reach and placed in a chest freezer for analysis in this thesis. There was little to no risk of MP contamination as fish digestive organs were only extracted once in the laboratory.

Sediment samples within ca.10 m of each other were collected at the 5 fish collection sites, with three replicates per site (n=15). Using a 500 ml prewashed polypropylene container, sediment was collected by manually scraping the estuary bottom at the netting sites to ca. 5 cm deep. Sediment samples were immediately sealed, put directly onto ice, and then stored in a chest freezer until being transported to the laboratory. Using prewashed polypropylene containers and immediately sealing the container ensured no MP contamination could take place.

Microplastic quantification and characterisation

In the laboratory, the body length (mm TL) of each fish was measured, the gonad stage was determined (**Appendix I**), and the digestive tract (gastric mill, stomach, and intestines) was removed and placed into a precleaned polypropylene container. Under a laminar flow containing a UV light, digestive tracts were weighed separately using a balance and transferred into a prewashed (HCL & RO water, repeated three times per pot) 120 ml pot. Dissolving the whole digestive tract was preferred over dissecting and emptying the digestive material as I wanted to limit the potential of contamination as much as possible. A potassium hydroxide (KOH) stock solution of 1120 g/L was made and took two days to fully saturate. The digestive solution was then made using 150 ml of saturated KOH, 160 ml of NaClO, and 690 ml of RO water. KOH and NaClO were separately filtered (Regenerated cellulose 0.2 µm pore size filter disk) before the digestive solution (30%NaClO: KOH) was made. For each gram of extracted material, 5 ml of 30% NaClO:KOH solution (alkali digestion) was added to a pot, larger pots (200 ml) were occasionally used for heavier digestive tracts. Pots were then placed in sonic baths for 15 minutes, once complete, they were transferred to the incubator for 3 days at 42°C whilst shaking at 120 rpm. Since there was a high fat content observed in some samples after the incubation period, 20 ml of filtered detergent (NACLO or bleach) was added to all fatty samples, and these were left for a further two hours.

To make sure that no MPs were trapped between small amounts of excess sediment and digestive organs, samples were centrifuged. Samples were transferred into centrifuge tubes with 25 ml of filtered NaCl solution added to each tube. The NaCl solution displaces MPs to the top of the tube, while centrifuging displaces the excess material to the bottom of the tube. Tubes were centrifuged at 3 900 x G for five minutes. Samples were then filtered onto a 47 mm diameter with a 2.7 µm porosity GF/D filter (Whatman). Filters were examined using a stereo trinocular microscope with a camera adapter attached, and images on the computer were generated through the Zen Blue software (version 3.3). Staining of samples was avoided (pers comm, AJ Fourie), as properties of some plastics do not absorb, but rather adsorb liquids.

Protective eyewear was worn, and warning signs were placed around the room when using a crime-lite 2 torch (420-470mm, Blue). The crime light torch illuminated the MPs and was a fast way to get a rough count. These MPs were marked on the filter paper with a prewashed stainless-steel pen and then counted a second time with a P-LED incident double-arm white light through a stereo trinocular microscope. Using the microscope, MPs on the filter paper had to be counted in quarter sections and added together. About 90% of the crime light torch counts corresponded to the microscope counts, except for some small fibres that could only be seen on the microscope. The number of MPs per sample was recorded and photos of clearly visible MPs were captured. MP shapes were identified using six categories: foams, fibres, fragments, pellets, films, and nylon (not included in the ID guide) (**Appendix II**). MPs that I was able to identify were simply tallied.

In the laboratory, sediment samples were placed on foil-layered stainless-steel trays and spread evenly (approximately 4cm thick) using a prewashed stainless-steel spatula. The trays were placed into the freeze dryer at -40 °C for 14 hours. Samples that had dried completely were removed while others that still had moisture were placed back in the freeze dryer for a further 3 hours. The sediment samples were then placed into prewashed (RO water) and pre-weighed glass containers, dry weight was measured and recorded. Using a prewashed (RO water) teaspoon, 10 g of mixed sediment was taken as a subsample, weighed, and placed into a clearly labelled centrifuge tube that contained 40 ml of saturated NaCl solution (n=15). The samples were then centrifuged, filtered, and analysed with the same procedures used for fish samples.

Statistical analysis

The data in this study were not normally distributed, therefore non-parametric tests were carried out. All tests were carried out to a 95% ($\alpha = 0.05$) significance level. MP concentrations within the sediment were compared among reaches and with the ingested quantities in each reach. Further, MP concentrations within mullet alimentary canals were compared among reaches, size categories, and species. Kruskal-Wallis H tests (`kruskal.test()`) were used to model the relationship between both sediment and mullet MP concentrations MP conc. ($\text{MP}\cdot\text{g}^{-1}$) against the three reaches of the estuary. A post-hoc Dunn's test was run in order to interpret pairwise comparisons, this test required installation of the `descTools` package, and then `DunnTest()` was run. Mann-Whitney U tests (`wilcox.test()`) were executed for pairwise comparisons between MP conc. ($\text{MP}\cdot\text{g}^{-1}$) and species, and MP conc. ($\text{MP}\cdot\text{g}^{-1}$) against size category (large ≥ 200 mm TL and small < 200 mm TL). General or Generalised Linear Modelling incorporating multiple response variables to predict MP concentration was considered, but abandoned due to

the unequal sample sizes among the different strata, incomplete strata, and confounding of variables (i.e. sex and size). All tests were performed in RStudio (2022.12.0+353 "Elsbeth Geranium" Release).

Results

A total of 152 mullet samples were initially collected but due to issues pertaining to the filtering process, samples had some sort of oily or fatty constituent that prevented the sample from passing through the filter. A total of 124 mullet samples were analysed for MPs within their digestive tracts. Of the 124 mullet samples, plastic particles were found in 122 of them (98.4 %). A total of 1 877 particles were found, an average of 15.1 particles per fish. *C. richardsonii* caught in the middle reaches had the highest mean concentration of MPs. MPs particles were found in 62 of the 63 *C. dumerili* and in 60 of the 61 *C. richardsonii* that were kept. Of the two species, *C. dumerili* had the highest percentage (53.8%) of individual MPs whilst *C. richardsonii* had slightly fewer (46.2%). MPs were ingested by both species and in all three reaches of the estuary. The highest abundance of MP particles in an individual was 97, this was a *C. dumerili* located in the lower reach with a total length of 272 mm (Large size category). Although abundances of MPs were insightful, concentration ($\text{MP}\cdot\text{g}^{-1}$) of MPs was far more useful as it allowed for comparisons among fish of different sizes and between fish and sediment. MP concentration here was represented by the number of ingested MP particles divided by the digestive tract wet weight.

Table 1. Mann-Whitney and Kruskal-Wallis statistical analysis for MP concentration within fish and sediment between reach, size category, and species. Bold values indicate significance ($\alpha = 0.05$).

Sample type	df	Mann-Whitney		Kruskal-Wallis	
		W-value	P-value	X ²	P-value
MP concentration in weight of digestive tract against three reaches	2	-	-	8.08	0.018
MP concentration in particle number per gram of sediment against three reaches	2	-	-	3.08	0.158
MP concentration in weight of digestive tract against size category	1	418	1.639 e⁻¹²	-	-
MP concentration in weight of digestive tract against species	1	1392.5	0.008	-	-

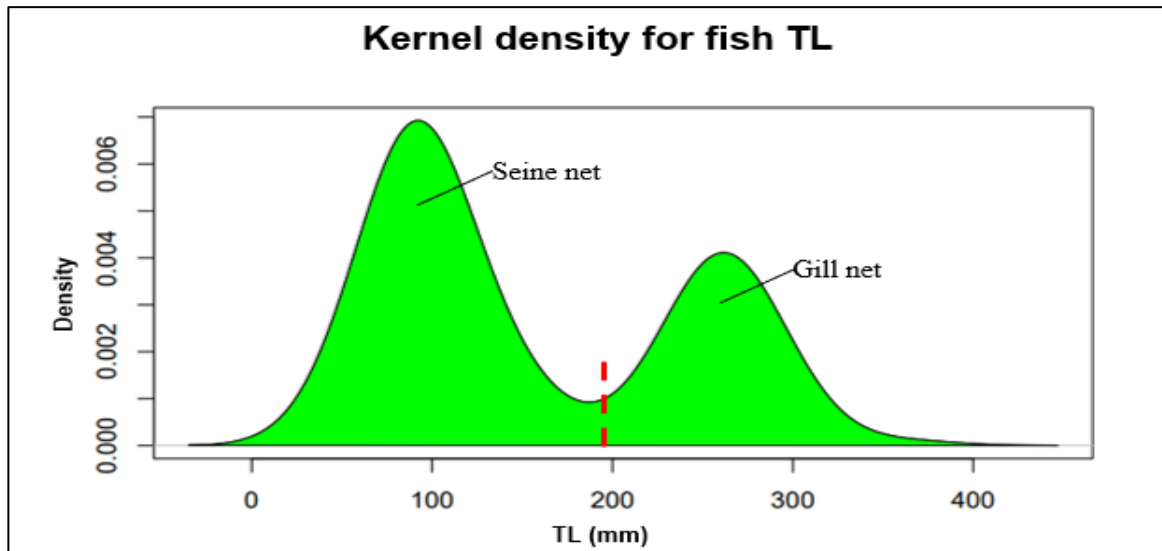


Figure 4. Kernel density of total length frequency [mm TL] of mullet in the Breede River estuary. The two sampling techniques yielded juvenile and adult fishes, respectively. The red dotted line indicates size at maturation(n=124).

In Order to sample a wide size range of fish, both small-meshed seine and larger meshed monofilament gill nets were used. The two netting types caught two distinct size ranges, resulting in a bimodal distribution (**figure 4**). Gillnet selectivity means that only a specific size range (+/- 200mm) can be captured by this technique, small fish will simply pass through, and very large fish have head diameters too wide or body depths too deep, to be caught. The seine net predominantly captured smaller fish. All fish that were greater than 200 mm (total length) were sexed as either male or female and were further characterised into the “large” size category (**figure 5**). Additionally, all fish less than 200 mm (total length) including four outliers that were greater than 200 mm, could not be sexed and were therefore deemed immature.

Table 2. MPs in the two mullet species analysed were separated by each reach; n–number of individual samples, size range (mm) TL measurement, Number of fish that ingested 1 or more MPs, Mean MP concentration is the average amount of particles per gram of wet digestive tract.

Species	Common name	Reaches	n	TL range (mm)	No. of fish with MP's	Mean MP concentration (MP.g ⁻¹)	SD MP concentration (MP.g ⁻¹)
<i>C. richardsonii</i>	Southern Mullet	Upper	8	80-128	8	19.45	16.50
		Middle	26	52-360	26	44.01	64.87
		Lower	27	85-292	26	3.37	4.87
Total	-	-	61	-	60	22.28	-
<i>C. dumerili</i>	Grooved Mullet	Upper	10	75-254	10	16.01	20.63
		Middle	34	66-286	33	1.71	1.58
		Lower	19	67-280	19	5.77	6.41
Total	-	-	63	-	62	7.83	-
Overall Total	-	-	124	52-360	122	15.05	-

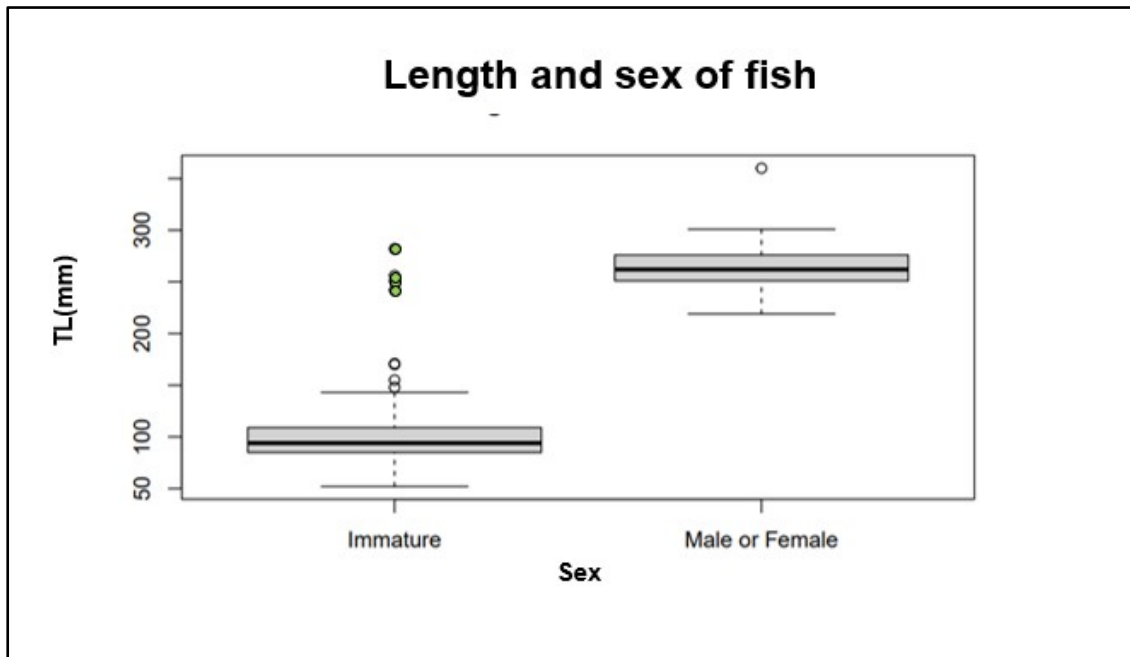


Figure 5. Mullet TL in relation to sex. Four outliers (highlighted in green) exist in the immature category, where TL is greater than 200 mm.

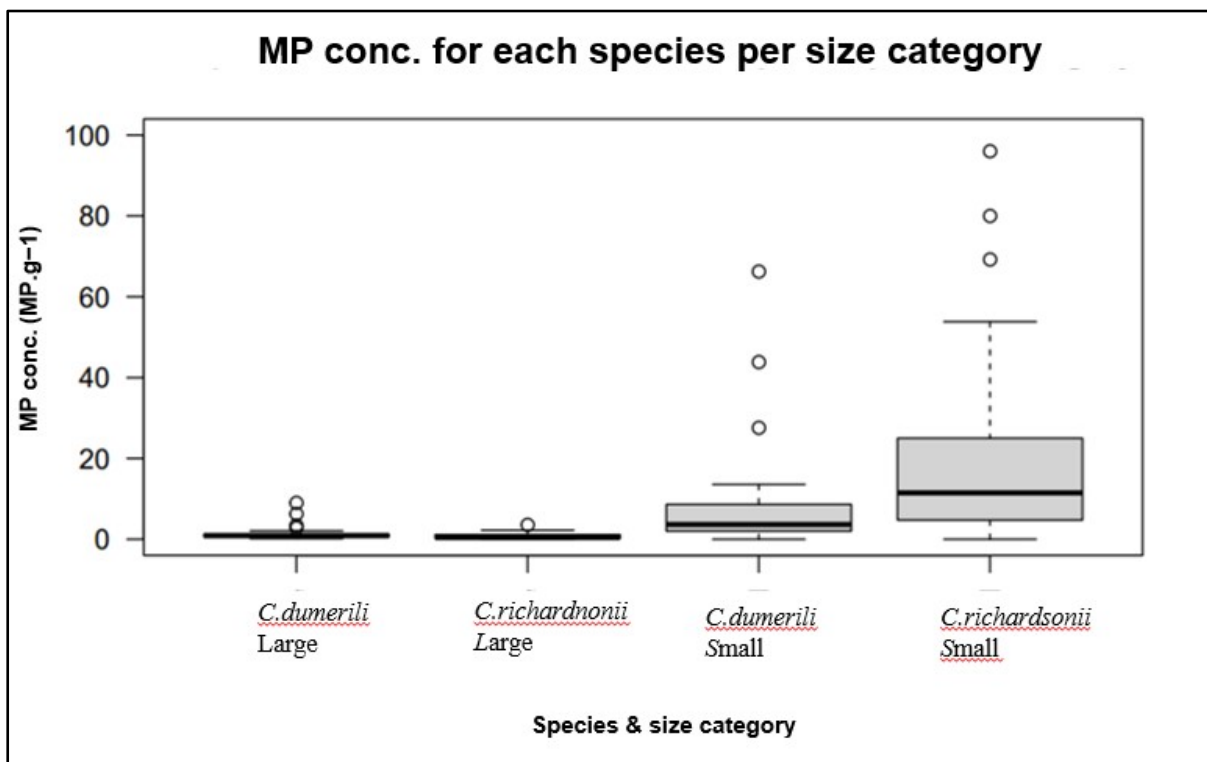


Figure 6. Mullet MP conc. in the alimentary canal differed among species and size categories. Small individuals of both species have more variable and significantly higher MP concentrations ($p < 1.639^{12}$) (Table 1). Small *C. richardsonii* have a higher MP concentration.

Immature fish were placed into the “small” size category (**figure 5**). MP conc. (MP.g⁻¹) by reach was found to be statistically significant as $p < 0.018$ was attained. When comparing MP concentration (MP.g⁻¹) and size category (large & small), there was a significant effect $p < 1.639 \times 10^{-12}$, with small mullet having a significantly higher MP conc. (MP.g⁻¹) (**Figure 6, Table 1**). Moreover, there was a much larger variation in the MP concentrations of small fish, the opposite was found for large fish. For individuals of both species that are in the small size category, MP concentrations are higher within each reach when compared to both species in the large size category. A significant difference ($p < 0.008$) was also identified between species as *C. richardsonii* had a higher mean MP conc. (MP.g⁻¹) than *C. dumerili* (**Table 1, Table 2, Figure 6**).

Table 3. MPs in sediment samples separated by each of the reaches; n—number of individual samples, 10 grams was taken from each sample as a sub-sample (sub-sample amount(g)), the total amount of individual MPs found (MP abundance), the average amount of MP per gram of sediment (Mean MP concentration (MP.g⁻¹)).

Region	n	MP abundance	Mean MP concentration (MP.g ⁻¹)	SD MP concentration (MP.g ⁻¹)
Upper	6	43	0.72	0.13
Middle	6	78	1.3	0.44
Lower	3	39	1.3	1.06
Total	15	160	1.1	-

MP particles were found in all 15 sediment samples. Within sediments, the upper reaches had a lower (0.72 (MP.g⁻¹)) MP mean concentration compared to the middle (1.3 (MP.g⁻¹)) and lower (1.3 (MP.g⁻¹)) reaches (**Table 3**). When comparing sediment MP conc. (MP.g⁻¹) Among the different reaches, a p-value of 0.158 results in non-significance (**Table 1**).

MPs were visually categorised with the aid of a MPs ID guide (Kunz 2020) (**Appendix II**) into 6 categories: Fragments, Fibers, Pellets, Foams, Films, and Nylon-like thread (not included in ID guide), hereafter referred to as nylon. In mullet, fragments were the most dominant MP across all reaches, species, and size categories within species. Films and fibers were also found in fish across all reaches, species, and size categories but to a lesser extent when compared to fragment abundances. Pellets, foams, and nylon were the lowest in terms of MP abundances in mullet, notably only small fish ingested foams. In terms of nylon ingestion, species in the upper reaches and of the small size category did not ingest nylon. Fibre abundances were equally abundant for both species located in the middle and upper reaches but both species in the lower reaches had very low MP fiber ingestion. Foams were only ingested by fish that were in the small size category. Although the dominant colour of MPs found was transparent or white, the

MPs that were identified in the sediment based on visual comparison were very similar in colour (brown & red) and shape to those found in larger mullet. Some fragments found in the sediment samples were almost identical to fragments extracted from large fish. 8 abnormally large (> 1mm) MPs were ingested, 6 of these were inside small-sized fish. The presence of biofouling was seen on MPs specifically ingested by some large fish and in sediment MPs. Upon visual examination, it was determined that fragments were the most abundant MP within the sediment samples across all reaches, while films and fibers were also abundant but not to the same extent as fragments. Fragments and films were the only two MPs present in all reaches. Nylon was present only in the lower and middle reaches, while fibers were present in the upper and middle reaches. Only one pellet or microbead was found in the sediment samples and it was in the middle reaches, while no foams were identified.

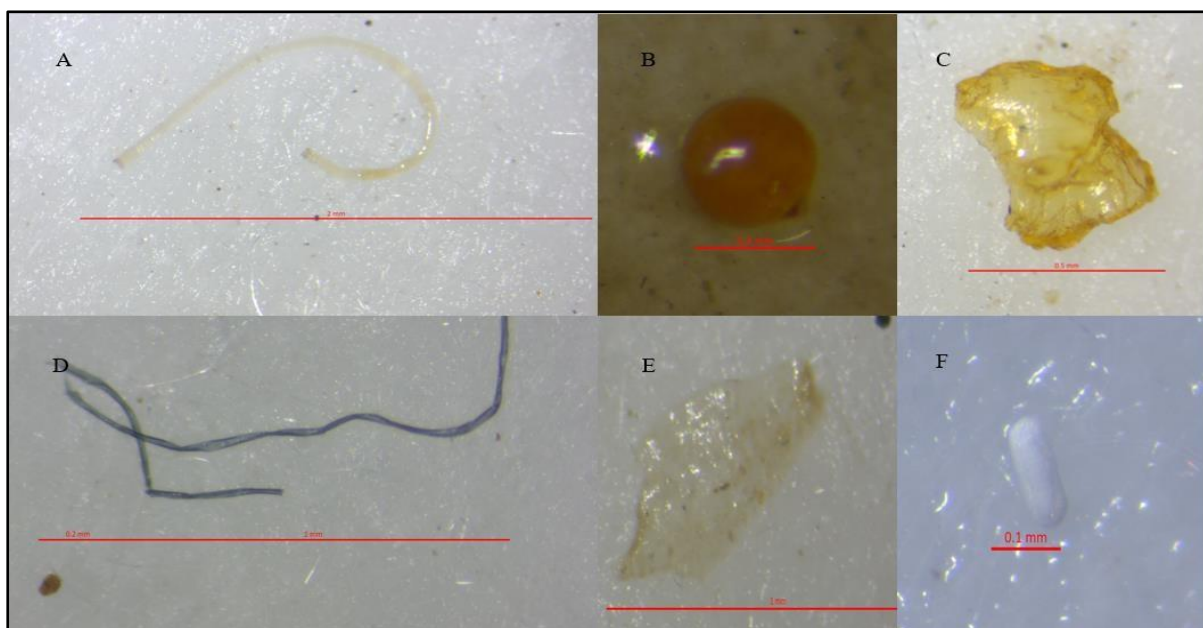


Figure 7. The 6 different types of MPs visually characterised. **(A)** - Nylon found in a large *C. dumerili*; **(B)** - A pellet of microbead found in a large *C. dumerili*; **(C)** - Fragment which had been ingested by a small *C. richardsonii*; **(D)** - A fiber found in a large *C. dumerili*; **(E)** - Film found inside a small *C. richardsonii*; **(F)** - Foam that was ingested by a small *C. dumerili*.

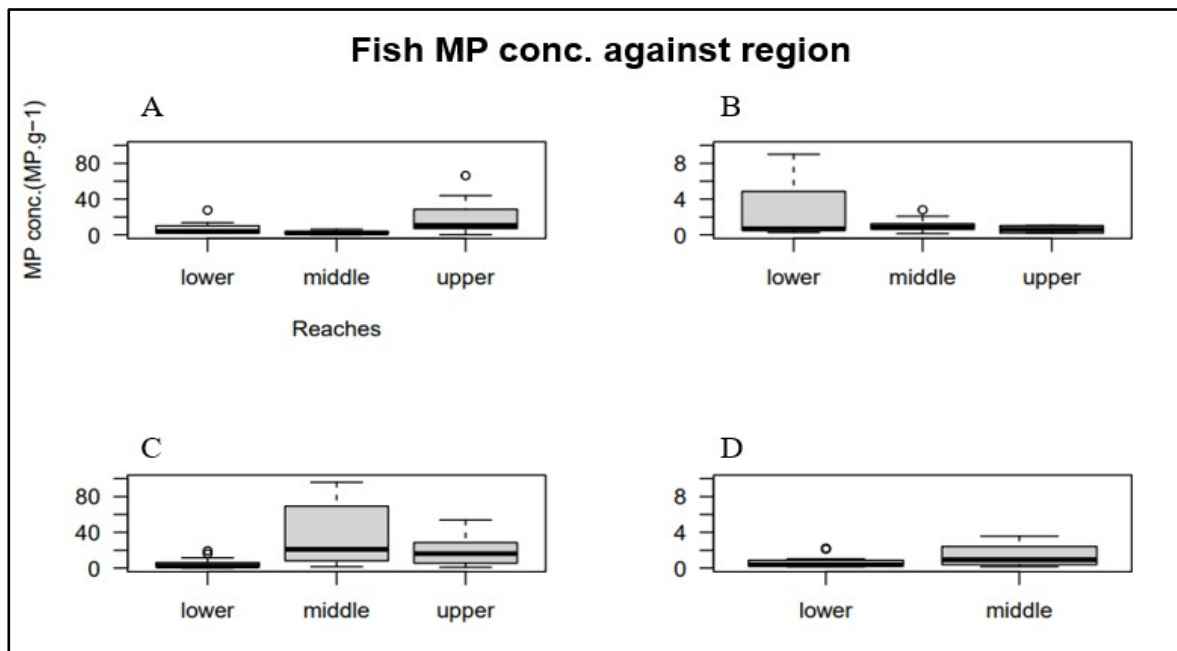


Figure 8. Mullet MP concentration among reaches for each species within a specific size category. **(A)** - Small *C. dumerili* with a y-axis up to 100; **(B)** - Large *C. dumerili* with a y-axis up to 10; **(C)** - Small *C. richardsonii* with a y-axis up to 100; **(D)** - Large *C. richardsonii* with the y-axis up to 10. No large *C. richardsonii* in the catch of the upper reaches. MP con. for fish that are in the small size category is significantly higher ($p < 1.639 \cdot 10^{-12}$) compared to large fish (**Table 1**).

Discussion

This study aimed to investigate the ecological impacts that are associated with plastic ingestion in two Mugilid species *C. dumerili* and *C. richardsonii* in the Breede Estuary, South Africa. This is the first study to report on MP ingestion in *C. dumerili* and the second for *C. richardsonii*. The study by McGregor and Strydom, (2020), examined MP loads in *C. richardsonii*, from the surf zone, this study sampled from an estuarine environment. McGregor and Strydom, (2020) found that surf-zone mullet were predominantly in a filter-feeding mode preying on surf-zone diatoms *Anaulus australis*, whereas in this study the estuarine mullet were selectively feeding or grazing detritus. MP prevalence levels were >95% in this study compared to 45% by McGregor and Strydom (2020), MP concentrations were higher, with an average of 15.1 MP.fish⁻¹ in this study, compared to the surf zone, ranging from 1.2 to 3.3 MP.fish⁻¹ in McGregor and Strydom (2020). Garcia et al. (2018) compared stomach contents and stable isotope signatures of two juvenile mullet species from an estuarine and a surf zone in Brazil and found that the diets of the two species differed considerably in the estuarine zone but were more similar in the surf zone. Both these Mugilid species are important prey for estuary and coastal predators, they are also economically significant as they represent the main target of the commercial net fisheries in the Cape region of South Africa. Given their position in food

web dynamics or trophic status, large volumes of MPs ingested by these species could have substantial implications for all species further up the food chain (Athey et al. 2020).

MPs in sediment & *C. dumerili* and *C. richardsonii*

Studies pertaining to MPs in estuarine systems are only recently starting to emerge and given that MP pollution has been reported from estuary environments globally (Pequeno et al., 2021; Perea et al., 2020; Pozo et al., 2019; Xu et al., 2020; Yonkos et al., 2014), I expected some degree of MP pollution in this study.

Results in this study showed that 98,4 % of individual fish analysed contained MPs in their digestive tract. These findings coincide with Pazos et al., (2017), who reported that 100 % of fish gut contents analysed, exhibited MPs and suggested that ingestion of MPs may be in relation to the extent of exposition. One outstanding observation in this study was that the main type of MPs found were fragments, this goes against most of the studies done in this type of research, where fibers have been the most dominant MP ingested by fish (Naidoo et al., 2016; Calderon and Hansen, 2018; McGregor and Strydom, 2020). High fragment abundances can result from numerous factors, which include sampling site selection and the timing of sampling. The human population density along the Breede Estuary is relatively low. Further upstream the river flows through towns which includes Ceres, Worcester, Robertson, Ashton, and Swellendam which could be a source of MP fragments, much of this may also be from farm produce packaging. Future studies could test this by using sediment traps to collect MPs (with sediment) in different parts of the catchment. The catchment area of the Breede River is 12600 km² and is 322 km in length which is large in relation to other rivers in South Africa, thus creating a high potential for MP to enter the system. Overall, much of the abrasion and breakdown of MPs may happen before it reaches the estuary. Fewer microfibres may be indicative of less wastewater treatment and urban runoff in this study compared to other catchments. Another possible reason for the few microfibres may be that invertebrates might have ingested them before they reached the estuary, this would have to be studied further as it is hypothetical. Population density is a significant factor when considering MP ingestion in fish digestive tracts (Lusher *et al.*, 2013; Yonkos *et al.*, 2014), both these studies found MP fibers as the dominant plastic. Sampling in this study was done in the summer (8th – 10th of November 2022) when discharge is characteristically low. However, the Breede River is known to experience high flow variability throughout the year compounded by winter and spring flooding. In summer, the estuary experiences relatively stable hydrological conditions, low freshwater inflow, and incoming tides generating turbulence and sediment deposition in the middle reach of the Breede Estuary. In addition, increased salinity results in finer sediments

(and finer MP) flocculating out. It seems that this behaviour inhibits the transport of MP between the upper and lower reaches in both directions, source to lower reach and ocean to upper reach. This indicates that the dominant MPs found in the upper and lower reaches at this time of the year (dry season) may originate from separate sources, while the middle reach is influenced by both. MPs found in the upper reaches of the estuary are associated with river basin contamination, while MPs in the lower reaches have local and/or marine origin associated with fishing activities and coastal villages (Lima et al., 2014).

Five different categories of MPs were found in the sediment of the Breede estuary, this was similar to MPs sediment shapes found in Xu *et al.*, 2020. Fragments were the highest across all samples (n=15), followed by film and fibers. Seven pieces of nylon were also found while only one pellet or microbead was identified. This MP shape distribution was not consistent with Alves and Figueiredo, (2019), where in their study fibers were the dominant MP detected in the sediment. However, certain aspects of my results correlate with Xu *et al.*, 2020, in that films and fragments dominated their sediment samples. Most plastic items initially float but biofouling and particle attachment cause them to sink (Pereao *et al.*, 2020). Evidence of this was found in this study as both sediment MPs and MPs in larger fish had visible biofouling. The timing of my sampling suggests that tidal influence and MPs associated with it may have been more influential compared to the source flow from the river. MPs from the ocean and within the estuary seem to be the source of pollution found in these fish, since fibres in fish were significantly reduced in the lower reach.

The upper reach had a low variation and abundance of MPs in sediment samples when compared to the lower and middle reaches. Additionally, this may just be due to most sediment deposition occurring in the middle & lower reaches, whereas sediment and MP's get transported past the upper reaches. The highest mean MP concentration in fish was 44.01 MP.g⁻¹, this was from the middle reach for *C. richardsonii* (**Table 2**). The hypothesis (H1) that the highest mean conc. (MP.g⁻¹) would be in fish located within the lower reach was rejected. Fragments contributed a large proportion to MP conc. (MP.g⁻¹) amounts, the fragments found in the Breede Estuary are most likely to be attributed to both current and historical fisheries activities that prevail in the system; these findings are similar to that recorded by Lima et al., 2014, working in the Goiana Estuary, Brazil. Furthermore, Lima et al., 2014 concluded that there was a direct relationship between the number of boats and environmental contamination. Predominantly in the warmer months, fishing activity in the estuary is very high, particularly during holiday periods. Boats on the estuary may be in excess of 100 on some days (Lamberth et al. 2008, Lower Breede River Conservancy unpublished data). Nylon was only identified in

fish and sediment samples taken from the lower and middle reaches of the estuary. This indicates an influence of fishing activities for predominantly the lower and middle reaches, other signs of fishing-related MPs include polystyrene MPs which could be traced back to cheap cooler boxes, transparent films in which all types of bait are packaged in, and foams that are attached to jetties and boats.

Brownish/orange fragments were consistently found in my sediment samples, and in some large fish (>200 mm). It is possible that the brownish colour is directly from brown plastic bottles such as ginger beer, but chemicals from rising humic and tannic acids might be another possible reason. The latter has been found in a study by (Li et al., 2022) where humic and tannic acids resulted in the discoloration of polystyrene MPs. Personally, I've observed numerous plastic floats and buoys stained brown by water that flows through Table Mountain sandstone and Fynbos. Leaf litter from riparian vegetation also produces humic acid. Both sediment and large fish equally had low MP conc. (MP. g⁻¹) (**Table 3, Figure 6**), and the presence of brownish /orange fragments shows a strong indication that large fish are ingesting MPs through the sediment in the Breede Estuary. Based on these results, (H4) sediment MP conc. (MP.g⁻¹) would be similar to MP conc. (MP.g⁻¹) found in large (≥200mm) fish was accepted. Various studies have indicated MP ingestion based on feeding ecology, to my knowledge, no studies in South Africa have compared sediment MPs to fish-ingested MPs.

Size and Feeding strategies.

Initially, I hypothesised (H3) that larger fish would ingest larger-sized plastics, however, my results showed this not to be the case and therefore this hypothesis was rejected. In smaller fish, there was certainly a trend of very large MPs being ingested. The results infer that smaller fish actively selected large zooplankton-like MPs from the water column or benthos whereas large fish ingested sediment. This study identified eight abnormally large (according to my sample) MPs (>1mm) that had been ingested, 6 of these belonged to fish of the small size category, with 5 of the 6 being fragments. This may allude to the biology of smaller fish in which their morphological adaptive structures have yet to become fully developed. Mugilidae can exploit specific trophic niches based on certain morphological adaptations that develop through certain life stages (McGregor and Strydom, 2020). Biologically, a larger mullet has a much more muscularly developed gastric mill, which is the organ used to grind food and sand. A more powerful gastric mill might easily break MPs into smaller pieces, which implies that smaller mullets would be more susceptible to physical internal damage or obstruction as their gastric mills are less developed.

Studies have been able to identify a relationship where the average amount of particles ingested increases with body length (Boerger *et al.*, 2010; Naidoo *et al.*, 2016), while others have shown the opposite, stating the type of food sources within a system, life stage and associated feeding behaviour to be of more significance regarding MP ingestion (Pazos *et al.*, 2017; McGregor and Strydom, 2020). This study concurred with the latter, therefore (H2) stating that MP conc. (MP. g⁻¹) would increase positively as body size increases within species was rejected. Comparing MP particle abundance is not a suitable method because Mugilidae feeding ecology changes so drastically through their life stages. Additionally, measuring MP conc. (MP. g⁻¹) was far more informative in understanding the relationships that *C. dumerili* and *C. richardsonii* have with MPs.

In both species, studies pertaining to their specific dietary shifts are limited. However, in the Mugilidae family juvenile mullet are predominantly planktivorous feeders (Whitfield 2019). As they mature from juveniles to adolescents and adults, their feeding habits transition into them being primarily benthic detritivores, where they ingest sand, detritus, and epiphytes (Whitfield 2019). Furthermore, McGregor and Strydom, (2020) found that when *C. richardsonii* is in its developmental stages they obtain a larger niche breadth. As a result, fish ingest more MPs during their younger developmental stages. The larger mullet (>200mm) are mostly deposit feeders; therefore, they sift through the sediments and can serve as a bioindicator of sediment and the associated MPs within it. Omnivorous fish ingest much larger quantities of MPs when compared to carnivorous and herbivorous fishes, the reasoning being that omnivorous fish have a wider diet source (Mizraji *et al.*, 2017). My findings were similar, in that small (<200 mm) fish contained a significantly ($p < 1.639 \cdot 10^{-12}$) higher MP conc. (MP. g⁻¹) in comparison to large (>200 mm) fish (**figure 6, Table 1**). This is strong evidence that coincides with previous research, showing that for both species in their early developmental stages (wider diet source), they are far more susceptible to high MP conc. (MP. g⁻¹) and the associated negative impacts.

Even though both species showed a significant difference between MP conc. (MP. g⁻¹) and size, a significant difference ($p < 0.008$) was apparent between MP conc. (MP.g⁻¹) and species (**Table 1**). These results show that there is interspecific differentiation and is driven by small (<200 mm) fish, which selectively and visually feed (**figure 6, Table 1**). H5 which states, that there would be minimal difference between the MP conc. (MP. g⁻¹) in the two species investigated was rejected. The interspecific difference in MP concentrations for this study most likely ascribes to different diets and/or feeding strategies. There is some evidence for resource partitioning in co-occurring mullet species both globally and locally. Blay (1995) assessed the

food and feeding habits of four species of juvenile mullet (Mugilidae) in a tidal lagoon and highlighted that “interspecific competition for food was possibly limited by species preferences for substrate particles of particular size range and differences in feeding chronology”. Blaber (1977) suggested that sympatric mullet species with parallel diets most likely evade competition via the selection of different-sized food particles. *C. dumerili* has been documented to ingest larger particles compared to other mullet species within its habitat (Masson & Marais, 1975; Payne, 1976). Marais (1980) conducted a study in the Swartkops Estuary (South Africa), where it was found that *C. dumerili* and *C. richardsonii* showed different feeding periodicities. Although the exact reason for interspecific differentiation in this study cannot be pinpointed, the previously mentioned factors show strong evidence for resource partitioning and reduced interspecific competition among the different mullet species. The significant difference ($p < 0.008$) found between the two species within the Breede can be attributed to one or many of these mentioned factors. It was found that low-density (positively buoyant) foam MPs were only ingested by small fish which is another indicator of their planktivorous feeding behaviour.

The paper by Naidoo and Glassom, (2019), conducted a pilot study on the glassfish *Ambassis dussumieri* which highlighted the significant effect that MP ingestion has on reducing the fish length and body depth. The negative growth effect that MPs have, was also shown in a bioaccumulation experiment, they concluded that fish ingesting zooplankton already containing MPs, experienced impeded growth rates (Uy and Johnson, 2022). Growth rates of affected *C. dumerili* and *C. richardsonii* would most certainly be susceptible to such adverse growth effects, as results in this paper show smaller fish ingesting higher MP conc. due to either actively ingesting MPs directly, ingestion via bioaccumulation of prey items, and/or potentially a mixture of the two. There have been no recent studies comparing MP ingestion or differences in feeding ecology between *C. dumerili* and *C. richardsonii*. Research is limited in terms of species-specific differentiation in feeding ecology amongst the Mugilids, but the few existing studies suggest that species in South Africa have overlapping habitat use and diets (McGregor and Strydom, 2020; Whitfield 2019). *C. richardsonii* had a higher MP conc. ($\text{MP} \cdot \text{g}^{-1}$) than *C. dumerili* for fish in the small size category, while MP concentrations for large species were equally low (**Figure 6**). However, MP conc. ($\text{MP} \cdot \text{g}^{-1}$) for small fish had a large variation while the opposite was found for large fish. This suggests that small fish are ingesting MPs incidentally and sporadically, meanwhile large fish are ingesting equal amounts of MPs. Between species, differentiation was further broken down by assessing MP conc. ($\text{MP} \cdot \text{g}^{-1}$) for each size category per each reach of the estuary.

C. richardsonii predominantly ingested MPs in the middle reach whilst *C. dumerilii* ingestion was evenly spread out across all three reaches, the highest being in the lower reach (**Table 2**). This further confirms that either MPs are passing through the upper reach from source flow or marine-associated MPs are not able to move as far up the estuary as the upper reach.

Accumulation and egestion

MP ingestion has been well documented globally, in various organisms. Although it was not part of this study, accumulation and/or egestion of MPs holds key information in understanding the degree of effects that MPs can have on a particular species. There are many factors involved in determining whether a species can egest MPs. Studies have documented accumulation in fish, but this has been mostly executed in laboratory environments (Naidoo and Glassom, 2019), and information about this in wild species is lacking. The physical size and shape of a plastic particle about an organism's digestive organs is highly influential in its ability to egest a particular particle. In lobster, fiber particles are retained for months, due to fibers intertwining or catching onto internal body organs (Naidoo and Glassom, 2019). If MPs are to accumulate in an organism, the most likely effect would be suppression of feeding (Wright *et al.*, 2013). Chemical components of the digestive tracts in animals can cause the leaching of certain harmful chemicals (Hirai *et al.*, 2011). This means that if MPs are egested, absorption of harmful chemicals is still possible. Future studies are needed to demonstrate the MP effects on wild species, as contaminants in the water are extremely complex.

This study found that the larger dietary breadth of smaller fish resulted in higher MP conc. (MP.g⁻¹) ingestion, suggesting that if large fish have low MPs conc. (MP.g⁻¹) in their gut, then *C. dumerili* and *C. richardsonii* do not accumulate MPs. Consequently, the number of MPs found in the fish digestive tracts may be regarded as an indirect instantaneous measure of the number of MPs in the estuary (Pazos *et al.*, 2017). However, without knowing the retention times of MPs within *C. dumerili* and *C. richardsonii*, one can only propose this on a theoretical basis and a more detailed analysis of the retention time of MPs in the digestive tracts of these species would be required to gain a concrete understanding of MPs egestion rate. The data gathered in this study suggest that trophic transfer may be a crucial route of exposure to other marine and estuarine species, given the trophic status of the two species investigated. Although tissue samples were not examined in this study for MPs, this is another pertinent problem in the context of trophic transfer of MPs. McIlwraith *et al.* (2021) demonstrate the presence of MPs in the gastrointestinal tract, fillet, and livers of seven species of sportfish from Lake Simcoe, Ontario, Canada. However, further work is required to better comprehend the mechanism of translocation.

In terms of highly impacted habitats, estuaries are one of the most affected by plastic pollution (Athey et al., 2020). Estuarine systems also provide nursery habitats for various species, where many are at their most vulnerable life stages. Further understanding of MPs within estuary food webs is vital in reducing plastic litter and would lead to more knowledge in terms of the human-associated impacts.

Post hoc considerations

Even though small (<200 mm) fish ingested higher MP conc. (MP. g⁻¹) compared to large (>200 mm) fish, having an increased size range of fish and more sub-sized categories would enable a comprehensive understanding of MP ingestion relative to size. The results shown in this study regarding the size and ingestion of MPs are still substantial. As shown in the paper by Lima et al., 2014, seasonal fluctuations of MPs in estuaries are high because of varying discharges throughout the year. In mind of this, future studies should sample seasonally or even monthly and compare MP ingestion in terms of river and estuary discharge rates. The use of a Fourier Transform Infrared (FTIR) was beyond the scope of this study, but filters have been retained for further analysis in the future. The most obvious issue and limiting factor in all MP analysis studies is the need for a globally standardised approach. This standardised approach should focus on the analysis aspect and revise a standard extraction method. In doing so, comparing the results of other studies will be far more accurate and would enable a much clearer picture of MP pollution. The possibility of breaking MPs when processing samples cannot be ruled out, however, it is unlikely to introduce bias or affect the outcome as all samples were treated the same and a standard method was followed.

Conclusions

This is the first study of MP ingestion by fish in estuaries in the Western Cape, South Africa. Overall, 122 of the 124 *C. dumerili* and *C. richardsonii* from the Breede Estuary ingested MPs (98.4 %), with fragments being the dominant MP across all aspects. A pattern in which smaller (<200 mm) fish that fed in the water column were found to have ingested higher concentrations of MPs compared to larger (≥ 200 mm) benthic-feeding fish. This may have negative implications for other juvenile fish that feed similarly. Furthermore, species feeding ecology and dietary breath were found to be of great importance in regard to MP conc. (MP.g-1) and could be applied to other organisms. Gaining information on such traits which make organisms more susceptible to MPs can lead to more targeted mitigation efforts. It is imperative that a standardised approach for MPs is found, this will enable stronger MP comparisons that can be applied to various scales. Research must identify MP sources of pollution and trophic transfer

relationships that exist. The Breede Estuary is relatively remote and unpolluted in comparison to other estuaries in South Africa (Van Niekerk et al. 2022). These combined findings emphasize a much larger issue may exist in more compromised estuaries within the country. Future research efforts need to quantify MPs entering estuaries through both marine and land-based sources of pollution.

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Appendices

I

Chelon richardsonii and *Chelon dumerili* gonad maturity stages and the criteria used to allocate gonads to the various stages. GL = gonad length, GW = gonad width, TL = total fish length, GM = gonad mass, OM = ovary mass, TM = testis mass, BM = fish body mass. (adapted from Bennett, 1993).



	State	Males	Females
1	Immature	Testicular portion of testis thin and threadlike, usually off-white (cream) or greyish. TM = 65-75% GM, GM < 0.5% BM, GL = 7-12% TL, GW = < 2% TL	Ovarian portion of gonad clear, pinkish, elongate and slightly rounded. OM = 65-75% GM, GM < 1% BM, GL = 8-14% TL, GW = 1-3% TL
2	Early development or recovery	GM = < 0.1% BM, GL = 7-14% TL, Developing testes elongate, flattened, and whitish, TM = 65 – 75% GM. GW = 1-4% TL. Recovering testis flattened and brownish. TM = > 90% GM, GW = 2.5 – 5.5% TL.	Developing ovary translucent, pale orange or yellow and rounded in cross-section. OM = 65-75% GM, GM < 1% BM, GL = 8-16% TL, GW = 2-5% TL. Recovering ovary translucent, orange or pinkish, slightly flaccid but rounded in cross-section. OM + > 90% GM, GM = 0.7-2% BM, GL = 10-20% TL, GW = 3-7% TL
3	Late development or recovery	Gonads more rounded in cross-section, some milt may be present in <i>vas deferens</i> . GM = 0.6-1.8% BM, GL = 10-17% TL. Developing testes whitish or cream. TM = 70-80% GM, GW = 3-5.5% TL. Recovering testes pale brown or cream. TM = > 90% GM, GW = 5-7% TL	Ovaries opaque and yellow, eggs very small, hardly visible to the naked eye, OM = > 80% GM, GM = 1-2.5% BM, GL = 10-22% TL, GW = 3-8% TL
4	Ripe	Testis cream, white more rounded in cross-section, varying quantities of milt in <i>vas deferens</i> . GM = 1.25-4% BM, GL = 15-20% TL, TM = > 85% GM, GW = 5-8% TL	Ovaries yellow and turgid, eggs visible but not easily teased from matrix. OM = > 85% GM, GM = 1-4% BM, GL = 14-22% TL, GW = 4-8% TL
5	Pre-spawning or ripe and running	Testis turgid and white, milt may be extruded from cloaca under slight pressure or flow freely when handled. GM = 2-8% BM, GL = 15-28% TL, GW = 4.5-11% TL, TM = > 90% GM	Ovaries similar in appearance to stage 4 but larger, eggs may be squeezed from the cloaca or flow freely. Hydrated eggs occasionally present, OM = > 90% GM, GM = 2.5-7.5 BM, GL = 19-28% TL, GW = 5.5-11% TL
6	Partially spawned	As in stage 5 but white colour replaced by obvious signs of varying degrees of haemorrhaging.	Gonad size and proportions similar to stage 5 but fairly extensive haemorrhaging evident. Hydrated eggs frequently present
7	Spent	Testis flaccid and reddish due to extensive haemorrhaging. GM = 1-3.5% BM, GL = 15-26% TL, GW = 3-10% TL, TM = > 90% GM	Ovaries flaccid and bloodshot, eggs occasionally visible, OM = > 90% GM, GM = 1-3.5% BM, GL = 16-26% L, GW = 3-10% TL.

II

MP identification guide assisted in determining the shapes of microplastics ingested (Kunz, 2020).

