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1 Microplastic contamination in tropical fishes: an assessment of different feeding habits

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9 **Abstract**

10 Marine ecosystems are reported to be contaminated by microplastics (MPs) (< 5 mm), however
11 the ecological mechanisms involved in the ingestion of debris by marine organisms are
12 relatively unknown. By developing and optimising an appropriate protocol of gut digestion for
13 fish species, this study explores a tropical estuarine environment to unriddle the processes
14 responsible for the different ingestion rates of plastic debris. A total of 82 fishes with different
15 feeding habits were analysed, *Centropomus undecimalis* (n = 30; Piscivore), *Bairdiella ronchus*
16 (n = 21; Zoobenthivore) and *Gobionellus stomatus* (n = 31; Detritivore). The microplastic
17 ingestion varied with the feeding strategy; *C. undecimalis*, the predator, was the most
18 contaminated species. Overall, most MPs were fibres (47%), followed by pellets (40%) and
19 fragments (13%), although these proportions varied among species. A high level of
20 contamination was found in the Estuarine Complex of Santa Cruz Channel, Northeast of Brazil,
21 with many sources of MPs contaminants, as for many other tropical estuaries, which probably
22 accumulate in the sediment and water column, with unknown consequences for human health.

23 **Keywords:** Trophic transfer, Plastic extraction, Trophic guild, Plastic pollution, Tropical
24 Atlantic

25 **Introduction**

26 Estuaries are well known for providing ecosystem services by supplying essential goods
27 (raw materials and food) and offering an attractive environment for population growth and
28 cultural activities (Atkins et al., 2011). In addition, they carry out an important ecological
29 function since fishes use these environments for protection, feeding, reproduction, settlement,
30 and nursery (Krumme et al., 2008; Potter et al., 2013; Lima et al., 2016b; Ramos et al., 2016;

31 Ferreira et al., 2019). Worldwide, estuaries are usually surrounded by large metropolises, and
32 the expansion of the urban population is directly associated with impacts on the coastal
33 ecosystems (Freeman et al., 2019). Plastic pollution is one of the most significant environmental
34 problems of the 21st century since large quantities of these materials are being mismanaged
35 and/or illegally disposed of in marine ecosystems (Dauvergne, 2018; Ostle et al., 2019). Once
36 in the environment, these plastic materials are weakened by natural processes (*e.g.*,
37 hydrodynamic forces, solar radiation, and biological actions) (Thompson et al., 2004; Jambeck
38 et al., 2015) and fragmented into smaller parts known as microplastics (< 5 mm).

39 Plastic debris poses several risks to marine biota (Galloway et al., 2017). Ingestion can
40 be hazardous, causing digestive injuries, decreasing predatory efficiency, or inducing toxic
41 effects (de Sá et al., 2015; Moore, 2008; Teuten et al., 2007; Barboza et al., 2018). Microplastics
42 can adsorb pollutants available in the water column, such as persistent organic pollutants
43 (POPs) (Oehlmann et al., 2009; Frias et al., 2010; Rochman et al., 2013) or heavy metals
44 (Ashton et al., 2010; Holmes et al., 2012), which may be further bioaccumulated and
45 biomagnified in the food web (Batel et al., 2016; Teuten et al., 2009). Furthermore,
46 microplastics may be transferred in the trophic chain by predating contaminated prey (Ferreira
47 et al., 2016; 2019; Chagnon et al., 2018). The trophic transfer has already been pointed out as
48 a relevant contamination mechanism for estuarine species (Athey et al., 2020).

49 Although the long-term effects of microplastic contamination are still unknown, the
50 scientific community continually emphasises the importance of using reliable and replicable
51 methods of investigations (Hermsen et al., 2018; Markic et al., 2019). A useful approach to
52 obtain plastic in marine wildlife is based on chemical digestion protocols, which are efficient
53 and low cost to work with (Karami et al., 2017; Kühn et al., 2017). Digestion protocols are a
54 practical and secure way to extract and isolate microplastics in organisms, being widely used
55 in the investigation of fish contamination by plastics (Foekema et al., 2013; Tanaka & Takada
56 2016; Bellas et al., 2016; Hermsen et al., 2017; Bessa et al., 2018; Pellini et al., 2018; Herrera
57 et al., 2019; Su et al., 2019). However, the lack of security procedures (*e.g.*, cleaned workroom
58 and blanks procedures) during the implementation of digestion protocols may lead to the
59 overestimation of contaminants since samples are more prone to airborne and cross-
60 contamination (Torre et al., 2016; Hermsen et al., 2017; Hermsen et al., 2018). Moreover, the
61 procedure must ensure data reliability through an effective and careful extraction of the

62 microplastics and the implementation of a robust sample size with a minimum of 10 samples
63 (Markic et al., 2019).

64 Several studies conducted in South Atlantic estuaries evaluated the ingestion of plastics
65 by marine organisms, mammals (Attademo et al., 2015), mussels (Santana et al., 2016; Birnstiel
66 et al., 2019), turtles (Guebert-Bartholo et al., 2011), and microplastics interactions with
67 ichthyoplankton (Lima et al., 2016a), and fish assemblages (Vendel et al., 2017). However, few
68 studies investigated the ecological and biological dynamics associated with microplastic intakes
69 on wild fishes (Ferreira et al., 2016; 2018; Amorim et al., 2020; Dantas et al., 2020).
70 Furthermore, the bioaccumulation of microplastics in marine species is highly influenced by
71 feeding strategies (Miller et al., 2020). However, the possible correlation between feeding
72 habits and MPs ingestion is not yet well known.

73 Three estuarine species were chosen to test our hypothesis that microplastic ingestion
74 varies according to the feeding strategies: (1) *Centropomus undecimalis* (Bloch, 1792); (2)
75 *Bairdiella ronchus* (Cuvier, 1830); and (3) *Gobionellus stomatus* (Starks, 1913). *C. undecimalis*
76 is an essential economic living resource for the commercial and subsistence fisheries in South
77 America (Carpenter, 2002). While adults of *C. undecimalis* inhabit coastal areas and migrate
78 towards the estuary, their juvenile stage uses the estuarine areas as a nursery ground (Ferreira
79 et al., 2019). *C. undecimalis* is classified as opportunistic predator, feeding on a large variety
80 of available preys in the environment, with a piscivorous tendency (Lira et al., 2017; Ferreira
81 et al., 2019). *B. ronchus* is classified as zoobenthivore (Ferreira et al., 2019), which preys on
82 invertebrates associated with the sediment (Elliott et al., 2007). *G. stomatus* is a detritivore fish
83 (Ferreira et al., 2019) consuming detritus and microphytobenthos (Elliott et al., 2007). Although
84 *B. ronchus* and *G. stomatus* were not of economic importance, they play a significant ecological
85 role within the estuarine ecosystem and are the main energy source for the *C. undecimalis* (Lira
86 et al., 2018; Gonzalez et al., 2019).

87 Understanding the role of microplastics as a component of anthropic pollution in this
88 ecosystem is crucial to assess adverse impacts on regional biodiversity and the quality of
89 fisheries resources that are being traded and consumed. In this context, the present study aims
90 to (i) apply an adapted extraction protocol to assess MPs in the digestive tract of fishes, assuring
91 the integrated quality control and appropriate sampling size, (ii) describe microplastics

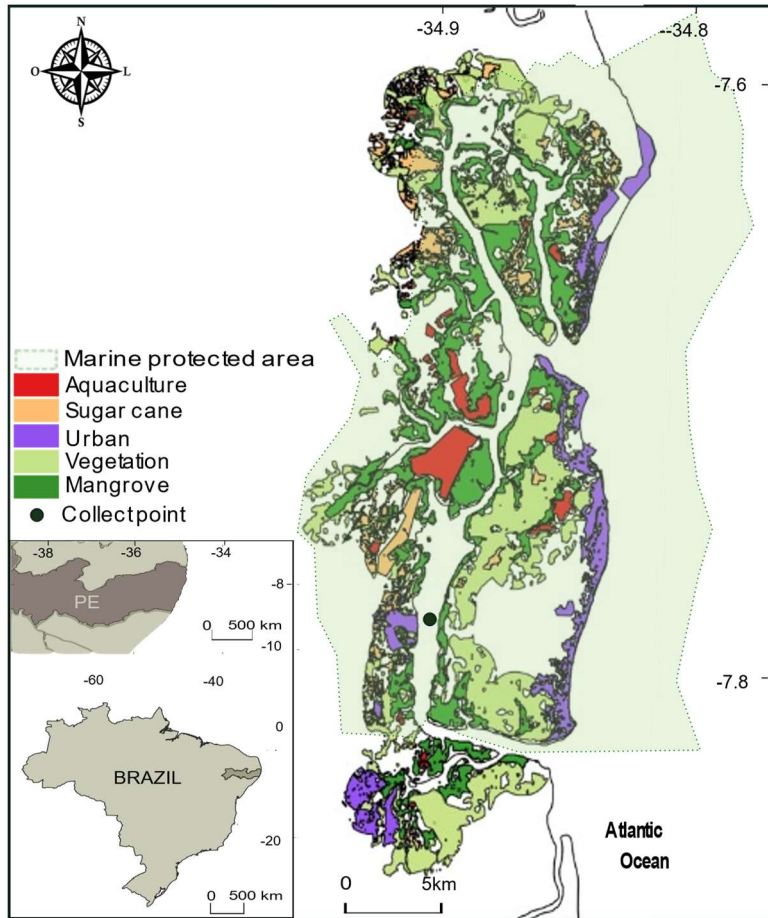
92 contamination in fishes in estuarine waters, and (iii) identify the main types of microplastics
93 considering the different feeding strategies.

94 **Materials and Methods**

95 **Study area**

96 The Estuarine Complex of the Santa Cruz Channel (ECSC) (Fig.1) is located along the
97 northeast Brazilian coast. The climate is classified as tropical, hot and humid, with an average
98 of 26°C ($\pm 2.8^\circ\text{C}$) annual air temperature and two seasons defined according to the level of
99 precipitation (rainy and dry seasons) (Medeiros et al., 2001).

100 The ECSC is a tidal channel that surrounds the Itamaracá Island, separating it from the
101 mainland, with a total area of 22 km, a maximum width of 1.5 km and a depth between 4 and 5
102 m (Lira et al., 2017). The main tributaries are formed by the Arataca, Botafogo and Igarassu
103 Rivers, and the predominant vegetation is the mangrove forest (Medeiros et al., 2001). The
104 ECSC is surrounded by two cities (Itapissuma and Itamaracá), which have their economies
105 mainly focused on the industrial and agricultural sectors (IBGE, 2011), whereas the local
106 economy is primarily supported by artisanal fisheries, aquaculture, and tourism (de Moura et
107 al., 2009).



108

109 **Figure 1.** Map of the Estuarine Complex of the Santa Cruz Channel (ECSC) adapted from Pelage et al., 2019.

110 **Sampling and Laboratory procedures**

111 Three demersal species were selected for this study, given their commercial and or
 112 subsistence importance for riverine populations and their ecological interactions in the trophic
 113 chain (Lira et al., 2017; 2018): *Gobionellus stomatus*, *Bairdiella ronchus*, and *Centropomus*
 114 *undecimalis*, classified as Detritivores, Zoobenthivores and Piscivores respectively, according
 115 to Ferreira et al. (2019) for the area. For the common Snook *C. undecimalis*, individuals were
 116 collected in the juvenile stage (Total Length < 26.3 cm) (Ferreira et al., 2019), ensuring that the
 117 obtained specimens had not left the estuarine ecosystem. This procedure was carried out to
 118 ensure that contaminants' intake occurred within the estuarine boundary since adults of *C.*
 119 *undecimalis* perform migrations toward coastal areas. As *B. ronchus* and *G. stomatus* are not
 120 caught outside the estuary of ECSC (Ferreira et al., 2019), we did not fix these species'
 121 ontogeny, and juveniles and adults were analysed. A total of 82 individuals were obtained by
 122 local fishermen. After each sampling, the specimens were labelled, and the individuals were

123 frozen. In the laboratory, individuals were identified (Menezes & Figueiredo, 2000), measured
124 and weighted. Each individual had their organs (stomach and intestine) carefully removed,
125 weighed and stored again for the digestion analysis. The minimum sample size for each species
126 was 20 individuals to avoid the bias of a low sample size, which is twice the number suggested
127 by Markic et al. (2019).

128 **Quality control and Extraction protocol**

129 Firstly, to guarantee quality control and avoid potential airborne contamination, several
130 steps were implemented. The entire process of digestion, filtration, identification and storage
131 of the microplastic samples was carried out in a cleaned and reserved room within the main
132 laboratory, reserved only for microplastic analysis. The flow of people was limited; cotton lab
133 coats and disposable latex gloves were worn during the entire process. Also, all used tools were
134 previously cleaned with alcohol 70% and rinsed with filtered distilled water. The solutions
135 utilised in the various procedures were filtered using a vacuum pump system (equipped with
136 laboratory glassware) through a 47 mm GF/F 0.7 µm glass fibre filter (Whatman).

137 The digestive tracts were rinsed with distilled water before being placed in a beaker,
138 submerged in NaOH (1 mol/L; PA 97%) solution, and covered by a glass lid (Fig. 2). The entire
139 digestive tracts were submitted to NaOH without further dissection of those organs to avoid
140 airborne contamination during handling. The proportion used was 1:100 w/v for 1 g of digestive
141 tract weight, 100 ml of NaOH (1 mol/L) solution. The mixture was oven-dried at 60° C for 24
142 h, mixed from time to time with a glass stick. The samples digested in the previous step, and
143 the procedural blanks were filtered using a vacuum pump system through a 47 mm glass fibre
144 filter (GF/F 0.7 µm Whatman). After filtration, filters were carefully set in a Petri dish (47 mm
145 diameter) and covered. These filters were oven-dried at 60° C for 24 h. The microplastics were
146 identified using a stereomicroscope (Zeiss Stemi 508) with 6.3 – 50 times magnification with a
147 detection limit of 20 µm, photographed (Axiocam 105 Color), measured (Zeiss Zen 3.2) from
148 the filter and stored in covered Petri dishes (Fig. 2). They were then categorised by type: (i)
149 fibres (filamentous shape), (ii) fragments (irregular shape) or (iii) pellets (spherical shape). The
150 digestion protocol is a useful tool to separate the organic materials and facilitates visual
151 identification, although it is not sufficient for identifying the polymers. Thereby, we also
152 applied the method described by Ferreira et al. (2019) to confirm plastic debris by drying the
153 samples in an oven to verify whether their physical characteristics changed or not.

154 Procedural blanks were made for each day of analysis before beginning the sample
155 digestion. For blanks, a beaker was filled with 50 ml of NaOH (1 mol/L) solution and covered
156 with a glass lid, and these blanks were exposed to the same protocol applied to the samples. A
157 total of 10 blanks were made; among them, four blanks were observed with eight tiny particles
158 (<100 µm) considered as paint fragments. Thereby, all particles further identified with any
159 resemblance to those observed in the blanks were excluded from posterior analyses.

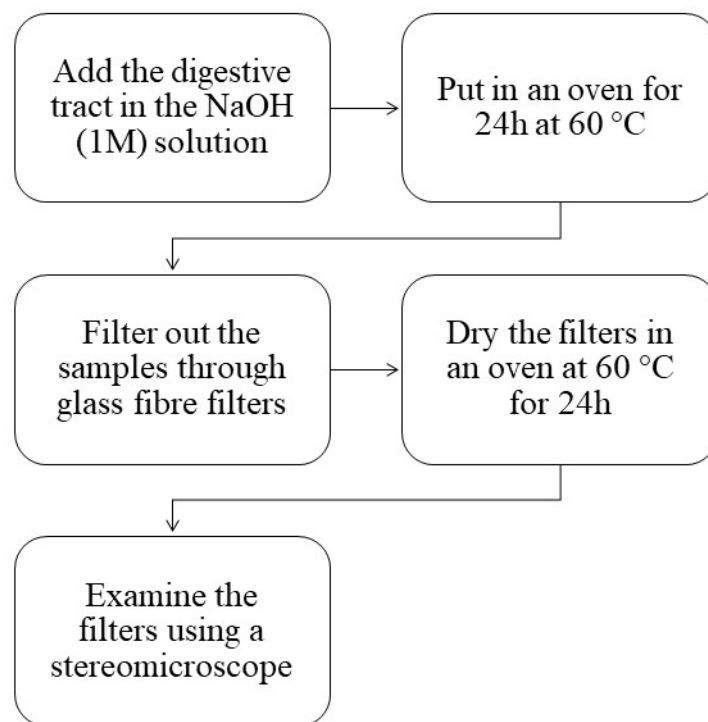


Figure 2. Flowchart with the stepwise of the extraction protocol.

160 **Data Analysis**

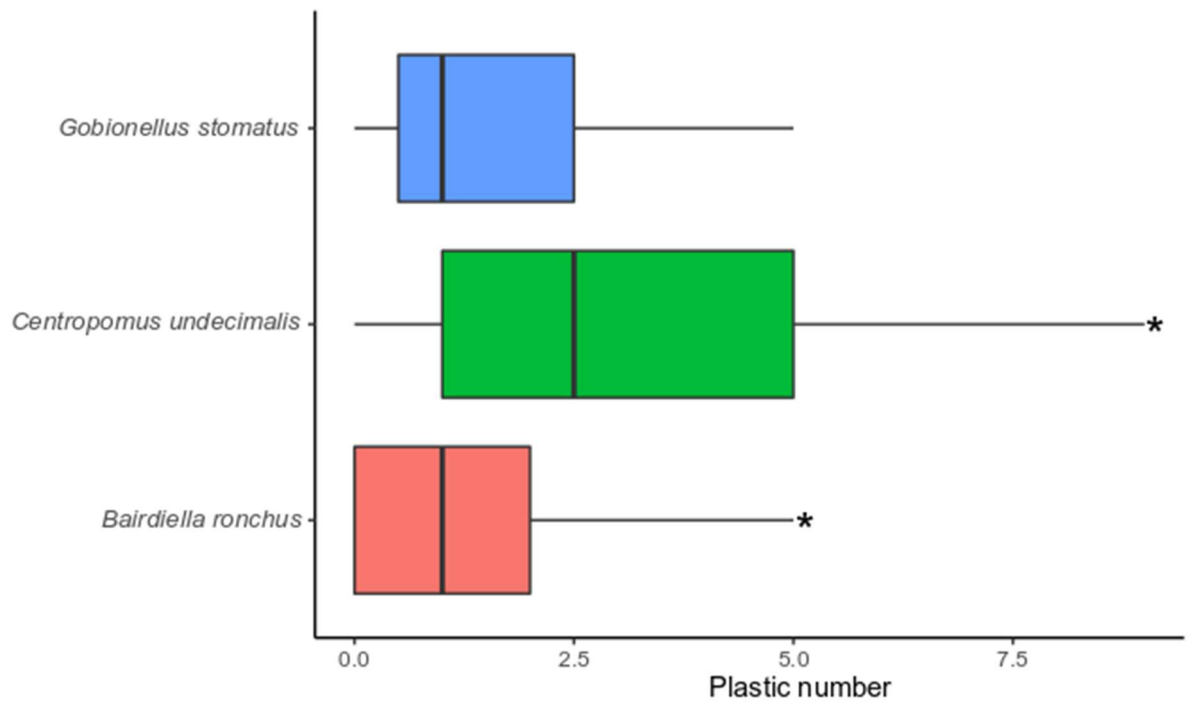
161 Kruskal-Wallis tests were used to verify if ingested microplastics (total MPs and
162 different types) presented significant differences in number and length in relation to species (*C.*
163 *undecimalis*, *B. ronchus* and *G. stomatus*). When the Kruskal-Wallis showed significant
164 differences, the *post hoc* pairwise comparisons Dunn's test was performed to investigate the
165 sources of variance (Dunn, 1964). All statistical analyses were carried out with the software R
166 version 3.6.3 (R Core Team, 2020) and were conducted considering a level of significance of
167 5%.

168 **Results**

169 A total of 30 individuals of *Centropomus undecimalis*, 31 *Gobionellus stomatus*, 21
170 *Bairdiella ronchus* were analysed. Microplastics were present in 77% of *C. undecimalis*, 74%
171 of *G. stomatus*, and 67% of *B. ronchus* individuals. A total of 176 particles of MPs were
172 recovered from 82 fishes (Table I).

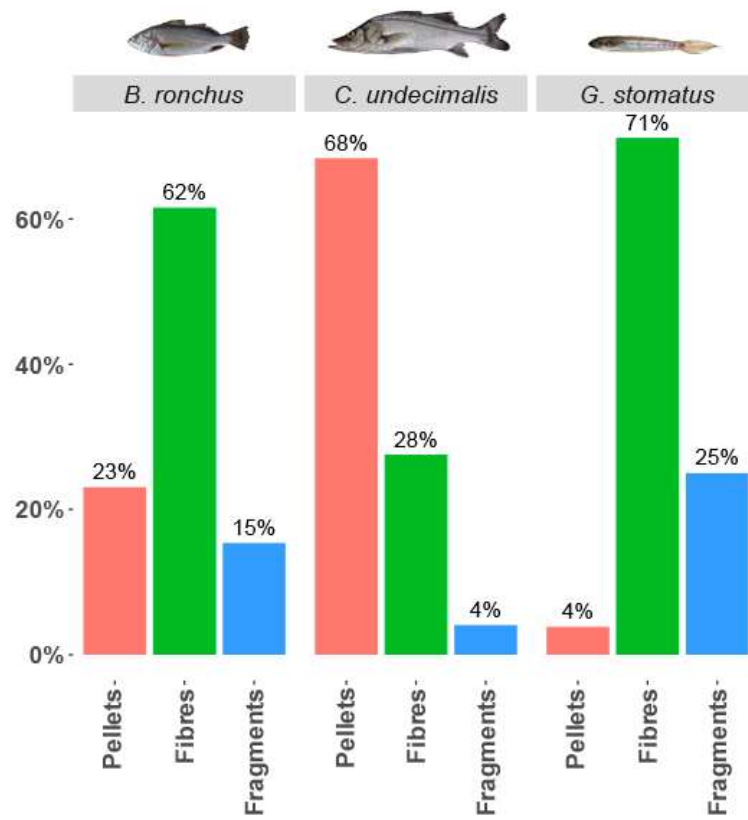
173 According to the number of MPs, ingestion significantly differed between species (*p*-
174 *value* ≤ 0.05), *C. undecimalis* being the most contaminated (3.3 ± 2.9 MPs fish⁻¹), followed by
175 *G. stomatus* (1.7 ± 1.5 MPs fish⁻¹) and *B. ronchus* (1.2 ± 1.3 MPs fish⁻¹) (Fig. 3). Significant
176 differences were recorded between *C. undecimalis* and *B. ronchus* (chi-squared = 7.873, df =
177 2, *p*-*value* ≤ 0.05). Concerning the length of ingested MPs, no significant differences among
178 the same type of MPs were observed when comparing MPs' size between species.

179 Regarding the types of MPs ingested by fishes, most were fibres (47%), followed by
180 pellets (40%) and fragments (13%), and proportions varied between the species. *C. undecimalis*
181 registered 68% of pellets, 28% of fibres, and 4% of fragments, *B. ronchus* and *G. stomatus*
182 registered 23% and 4% of pellets, 62% and 71% of fibres and 15% and 25% of fragments,
183 respectively (Fig. 4 and 5).



184

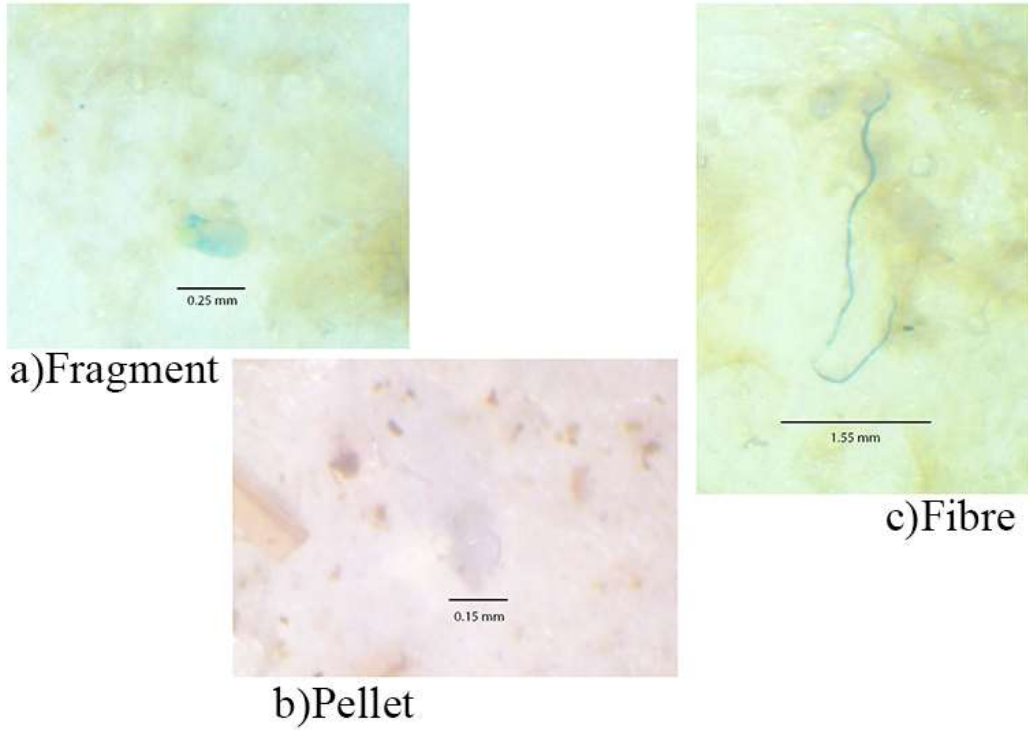
185 **Figure 3.** Mean number of microplastics ingested by fishes collected in the Estuarine Complex of the Santa Cruz Channel. The
 186 asterisks represent the statistical differences with a significance of 0.05.



187

188 **Figure 4.** Different types of microplastics (fibres, fragments, and pellets) ingested by fish species, expressed as a percentage.

Types of microplastics



189

190 **Figure 5.** Types of microplastics ingested by fishes of the estuarine complex of Santa Cruz Channel. a) fragment (irregular
191 shape); b) pellet (spherical shape); and c) fibre (filamentous shape).

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193

Family/Species	Ecological parameters			Biometry			Microplastics occurrence		
	N	Habitat use	Feeding habit	TL (cm) Min-Max	SL (cm) Min-Max	TW (g) Min-Max	MPs	FO%	TL (mm) Min-Max
Gobiidae									
<i>Gobionellus stomatus</i> (Starks, 1913)	31	Demersal	DV	9.3 - 11.9	7.0 - 9.0	3.78 - 7.89	52	67	0.02 - 5.00
Sciaenidae									
<i>Bairdiella ronchus</i> (Cuvier, 1830)	21	Demersal	ZB	12.5 - 16.5	10.0 - 14.0	22.2 - 62.4	26	74	0.11 - 3.35
Centropomidae									
<i>Centropomus undecimalis</i> (Bloch, 1792)	30	Demersal	PV	8.9 - 14.0	7.3 - 11.0	5.8 - 16.78	98	77	0.06 - 3.79

194

195 **Table I.** The ecological parameters and biological aspects of the analysed species. Feeding habit: DV (Detritivores), ZB (Zoobenthivores) and PV (Piscivores) obtained for the area by
196 Ferreira et al. (2019). TL (Total Length); SL (Standard Length); TW (Total Weight); FO% (Frequency of occurrence).

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206 **Discussion**

207 ***Quality Assurance and Quality Control***

208 In our study, we applied an analytical method to extract microplastics (MPs) in the
209 digestive tract of fishes, involving a careful procedure of quality control, using sodium
210 hydroxide (NaOH 1 M), and a sampling size with a minimum of 20 individuals for each species,
211 following the recent recommendations of Markic et al. (2019) and Hermsen et al. (2018).

212 The application of digestion protocols to extract microplastics in marine biota has been
213 growing worldwide (Lusher et al., 2017). Digestion protocol is a reliable method for isolating
214 microplastics in the biota, facilitating the observation of non-organic materials. For fish, the
215 most common method used is alkaline digestion. Indeed, for many authors, this is considered
216 the most suitable method to remove organic material and isolate plastic debris (Karami et al.,
217 2017; Kühn et al., 2017; Schirinzi et al., 2020). Although the efficiency of potassium hydroxide
218 (KOH 10%) has been more often tested, the use of sodium hydroxide (NaOH 1M) has also been
219 widely tested and demonstrated to be very useful (Bellas et al., 2016; Baalkhuyur et al., 2018;
220 Budimir et al., 2018; Morgana et al., 2018; Wiczorek et al., 2018; Su et al., 2019), and ensures
221 polymer integrity after chemical digestion (Budimir et al., 2018).

222 Although adaptations of digestion protocol have been carried out around the world
223 (Kühn et al., 2017; Karami et al., 2017), so far, only three studies in estuaries of South America
224 have used a chemical digestion protocol for microplastics extraction in the digestive tract of
225 fishes, with the implementation of adequate quality assurance and quality control (QA/QC)
226 (Arias et al., 2019; Ribeiro-Brasil et al., 2020; Garcés-Ordóñez, et al., 2020). Implementing
227 QA/QC procedures is essential to avoid cross-contamination of microplastic samples (Lusher
228 et al., 2017; Hermsen et al., 2017; O'Connor et al., 2019). This treatment is necessary to
229 minimise over/underestimation of microplastics due to airborne contamination and or loss of
230 particles during sample handling. Moreover, the sample size is also an essential factor in the
231 analysis. Among the studies in South America, only Arias et al. (2019) have chosen a sample
232 size $n > 10$. Such bias is unlikely to occur in our study, where the sample size was the highest
233 in South America. Therefore, our results are expected to adequately reflect the contamination
234 in the Estuarine Complex of Santa Cruz Channel (ECSC), and the protocol here used can safely
235 be replicated in other studies.

236 *Microplastic ingestion by fishes*

237 In our study, microplastic (MP) contamination rates were high (frequency of occurrence
238 of 73%) for the three analysed demersal estuarine species. Overall, demersal estuarine species
239 are frequently reported to have a high ingestion rate of MPs (Ferreira et al., 2016; 2019; Arias
240 et al., 2019). Demersal species inhabit and feed on the fauna associated with the substrate (Elliot
241 et al., 2017), which might be in direct contact with contaminated sediment. Indeed, the estuarine
242 sediment is a significant accumulation zone for MPs (Zhang, 2017). As estuaries are a
243 transitional ecosystem, often surrounded by urban areas and exposed to domestic sewage
244 discharge, they receive contaminants of both riverine and tidal inputs (Lebreton et al., 2017).
245 Thereby, these ecosystems are more prone to be contaminated by MPs, especially fibres
246 (Browne et al., 2011; Bessa et al., 2018). In addition to the sewage discharge, fibres can also be
247 originated by the use, maintenance, discarding and loss of fisheries gear (Lima et al., 2014).
248 MPs fibres occurred the most in the estuarine bottom, possibly due to the rapid sinking of these
249 types of MPs (Lima et al., 2014).

250 Overall, the most ingested MP type were fibres, as observed in most of the studies
251 worldwide (Wright et al., 2013; Foekema et al., 2013; Bellas et al., 2016; Herrera et al., 2019;
252 Bessa et al., 2018). Although all types of plastics were ingested by the species analysed in this
253 study, ingestion rates varied. Fibres were the most frequent type in *G. stomatus* (representing
254 71% of the ingested MPs) and *B. ronchus* (68%). *G. stomatus* and *B. ronchus* are detritivore
255 and zoobenthivore species, respectively, depending on the organisms associated with the
256 substrate or the organic matter available. Consequently, they are more vulnerable to the MPs
257 fibres contaminating estuarine sediments. However, pellets were the most frequent type in *C.*
258 *undecimalis* (68%), differently from the results observed by Ferreira et al. (2019) for the same
259 species, which registered mostly ingestion of filaments. Pellets also dominated the diet of fish
260 along the coast of Salvador (Brazil) (Miranda & Carvalho-Souza, 2016) and from the Amazon
261 estuary (Pegado et al., 2018). Different from the other types of MP ingested, pellets are primary
262 microplastics (Fendall et al., 2009), which are manufactured as MPs mainly for the cosmetics
263 industry (e.g., microbeads). This type of MP can be accidentally discharged into the
264 environment during the transport of this raw material (Ogata et al., 2009) or by the release of
265 domestic sewage (Tanaka & Takada 2016). The increase in urbanisation in the Santa Cruz
266 Channel (Pelage et al., 2019) surely amplified the sewage discharge in this area. Pellets can be

267 found floating in the water column, and they can even have fish eggs attached to them (Ivar do
268 Sul et al., 2013). Predatory fishes such as *C. undecimalis* can ingest the pellets directly by
269 confusing them with their natural prey. Moreover, when feeding, opportunistic predators ingest
270 a large amount of prey, which might increase the momentary build-up of MPs particles prior to
271 egestion.

272 Predator species are more vulnerable to microplastic contamination due to the trophic
273 transference, which occurs when they ingest contaminated prey (Eriksson and Burton, 2003;
274 Ferreira et al., 2016; Chagnon et al., 2018; Nelms et al., 2018; Ferreira et al., 2019).
275 Consequently, as we observed among the three analysed demersal fish species, the predator, *C.*
276 *undecimalis*, had the highest contamination rate (3.3 ± 2.9 MPs fish⁻¹) despite being in its early
277 life stage (juvenile), followed by *G. stomatus* (1.7 ± 1.5 MPs fish⁻¹) and *B. ronchus* (1.2 ± 1.3
278 MPs fish⁻¹). Differences in plastic ingestion might be associated with the feeding strategy of
279 species (Wright et al., 2013; Miller et al., 2020). Our study tested and confirmed the hypothesis
280 that microplastic ingestion varies with the different feeding strategies. However, regardless of
281 the diet preferences of the species, in our study area, it is reported many sources of MPs
282 contaminants, which probably accumulate in the sediment and water column, negatively
283 affecting the life-strategies of fish species and mostly the juvenile stages which utilise estuaries
284 as a nursery ground. Thus, the predators are more prone to be contaminated by microplastics
285 through two main exposure routes: (1) the highly contaminated estuarine habitats and (2) the
286 ingestion of contaminated prey.

287 Anthropogenic activities in the ECSC (urban areas, manufacturers, aquaculture plants,
288 and sugarcane fields) are found surrounding the whole floodplain, and these activities might be
289 an important source of microplastics and other contaminants (*e.g.*, pesticides and heavy metals).
290 Indeed, the ECSC has registered many impacts such as habitat loss and mercury releases
291 (Pelage et al., 2019; Araújo et al., 2019; Albuquerque et al., 2019), which likely affect the
292 estuarine community. Besides, our study has identified high contamination by microplastics in
293 fish species that are a relevant source of protein locally and regionally. Despite being a Marine
294 Protected Area, which provides essential ecosystems services, there is a lack of awareness and
295 public policies, highlighting the importance of monitoring and management policies to control
296 and mitigate social and health problems.

297 Further studies are needed regarding the microplastic impacts on marine fauna and
298 whether they could transfer adsorbed pollutants such as persistent organic pollutants (POPs),
299 heavy metals, and plastic additives to the food web are necessary, as microplastics particles can
300 be transferred along the trophic chain, the chances to accumulate other pollutants in the food
301 web increases. In fishery resources, this question is a public health matter because it is linked
302 to human uptake of these pollutants. Our study also emphasises the importance of implementing
303 protocols to extract microplastics in biological samples, which guarantee the quality of samples,
304 avoid under or overestimation and airborne contamination, and which can be easily replicable.

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317

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