Method Article

Ecology of microplastics contamination within food webs of estuarine and coastal ecosystems

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A B S T R A C T

The aim was to describe a methodology developed to study the relationship among the spatio-temporal patterns of habitat utilization, feeding ecology and microplastics (MPs) contamination across the different ontogenetic phases of fishes belonging to different trophic levels and living along the riverine-estuarine-coastal food web. The Goiana Estuary's water column was examined for the seasonal and spatial variation of MPs and their quantification relative to zooplankton, demersal fish species contamination following the same sampling design. The density of MPs in the water column determines their bioavailability. Interest in studies on MPs distribution in relation to spatial and temporal variation of environmental factors and fauna are increasing in quantity and quality. If the ecological strategies presented in this study were replicated in other estuary, comparisons could be made in order to describe how ecosystems work. Standard protocols for sampling, extraction, enumeration and classification of MPs and others pollutant ingested by fishes have been developed and are presented here to encourage comparisons. Standardized and comparable sampling designs and laboratory procedures are an important strategy in order to devise and transfer managerial solutions among different sites and comparisons along time when studying the same environment.

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Method details

Estuaries are very productive ecosystems that offer a number of environmental services and resources. However, anthropogenic interference usually results in loses that affect directly the fauna

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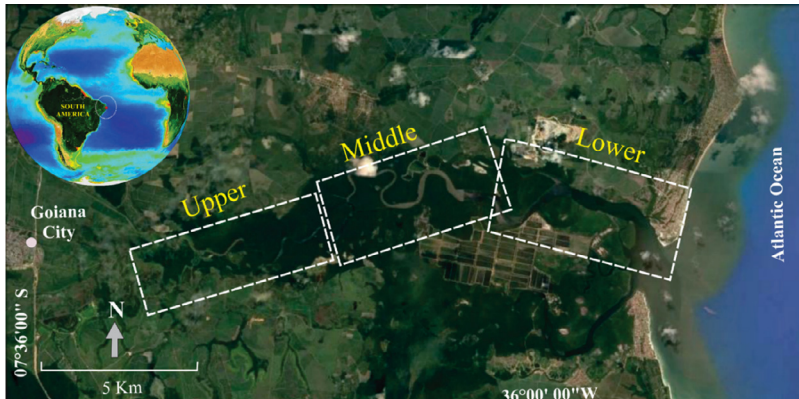
Fig. 1. Estuary of Goiana River. The main channel was divided in three areas (Upper, Middle and Lower) according to the gradient of salinity and geomorphology of the estuary.

across different trophic levels. One of the most conspicuous forms of interference is pollution by plastic wastes. Once in the environment, plastics fragment into smaller particles that eventually reach the size of MPs (< 5 mm). In addition, studies revealed that fisheries (including the wear and tear of gear) and domestic effluents (fibres) produce and dispose of a large amount of microfilaments that also reach estuaries. The main sources of MPs to estuaries are river basins, followed by autochthonous sources [1]. From there, they eventually reach the sea.

MPs present high densities in estuaries, where they become easily available for the biota [2]. Among these organisms are fish, which have different feeding habits and habitats uses, depending on species, ontogenetic phase and seasonal influences. If one of the phases within the life cycle of a fish species uses an estuarine habitat when it is contaminated by MPs, the chances of direct and indirect MPs ingestion increase. When feeding, fish may pray on dietary items previously contaminated [2]. Contamination usually increases towards the top of the food web, and higher trophic levels are more contaminated by MPs, especially developed phases that prey on fish [3]. The trophic transfer of MPs along food webs of the Goiana Estuary suggests that top predators show higher contamination levels in their later piscivorous phase [3–6].

The aim of this study was to describe a methodology developed to study the relationship among the spatio-temporal patterns of habitat utilization, feeding ecology and MPs contamination across the different ontogenetic phases of fishes in different trophic levels of a riverine-estuarine-coastal food web. For this purpose, Goiana Estuary was used as a case study.

Methods to develop studies on ecology and conservation of estuarine and coastal ecosystems

Study area

The Goiana River basin has a total drainage area of 2900 km^2 [7]. The network of small channels also serves as receptor of domestic and industrial effluents and wastes along its short and low-volume flow. The population served by this basin for water is of ~500,000 inhabitants. Average water flow is around 11 m^3 s^{-1} (0.5 to 25 m^3 s^{-1}). The last 17 km of its course is under the influence of tidal cycles, characterizing the estuary (Fig. 1).

Sampling strategies

To explain the correlation among the fauna, seasonal and spatial fluctuation of the environmental variables (gradient of salinity, dissolved oxygen and water temperature) the water column of Goiana Estuary (Fig. 1) was examined for the seasonal and spatial variation of MPs (< 5 mm) and their
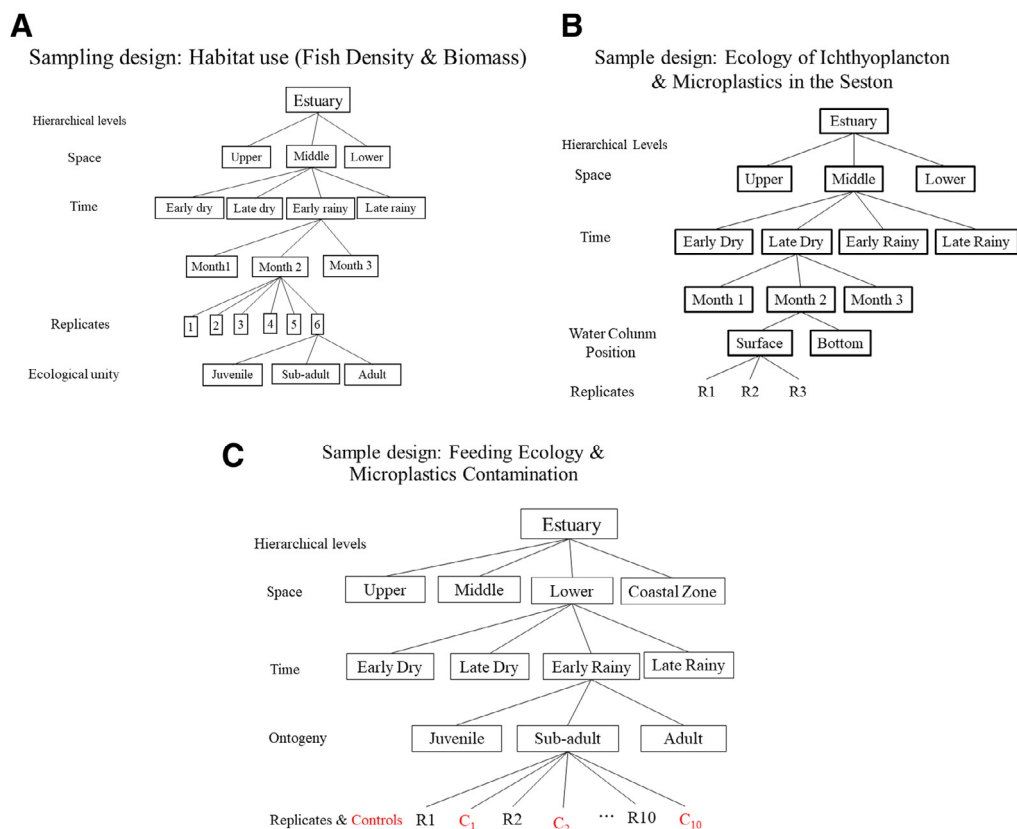
Fig. 2. Sampling design to test the hypothesis about seston (a: density of ichthyoplankton, zooplankton and microplastics), ontogenetic fish phases distribution (b: juvenile, sub adult and adult), and feeding ecology/MPs contamination (c) in relation to space (different area of the estuary: upper, middle and lower) and time (early and late dry season, and early and late rainy season). Controls (C_1... C_{10}) used to avoid MPs airborne contamination.

quantification relative to seston (*e.g.,* MPs and zooplankton) and demersal fish species contamination following a purpose-specific sampling design to test the following hypothesis [8]:

- the seasonal variability of environmental contaminants (metals, POPs and MPs) in water and sediment [1,8–10] (Fig. 2a);
- the seasonal patterns of estuarine use by different ontogenetic phases (larvae, juveniles, subadults and adults) of faunal communities, populations or species [2–6,10,13-15] (Fig. 2b) and;
- the seasonal patterns of faunal contamination (tissues or gut content) along the estuarine main channel (upper, middle and lower areas) [2,4,10,16–18] (Fig. 2c).

The information that will be produce following these sampling designs procedure the species of each trophic level will be studied take into consideration each ontogenetic phase (larvae, juvenile, sub-adult and adult). As each ontogenetic phase needs a habitat in a season period, it will be possible to analyze with precision the history of individual contamination in function of the variable time and space. Differences among South American and other estuaries can then be detected with greater precision and detail [8].

In this study, the methodological strategy was described for each estuarine habitat (main channel: environmental variables, seston, demersal fishes and their contamination when they prey). The novelty of this proposed methodology is linking the MPs contamination with the seasonality, fish
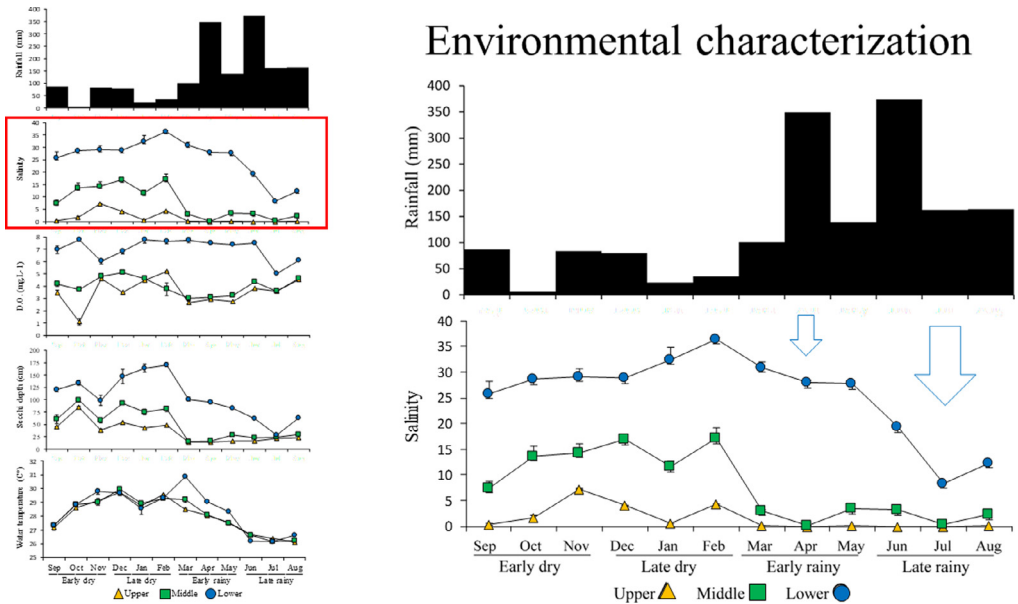
Fig. 3. Seasonal and spatial environmental fluctuation (Rainfall, Salinity, water temperature (°C), dissolved oxygen (mg.L), Secchi (cm) depth for Goiana Estuary main channel.

development phase and area of the estuarine ecosystem that the fish phase needs to complete their life cycle.

Environmental variables

Rainfall was measured by an automatic meteorological station located at the southern Estuary mouth (Fig. 1). Water temperature (°C) and salinity (WTW LF 197, Wissenschaftlich Technische Werkstätten, Wellhein, Germany) were recorded at the surface and bottom before fish and seston samples were taken (Fig. 3). This environmental information was correlated with biological and MPs densities to describe the seasonal and spatial estuarine environmental conditions during sapling period. (Fig. 4)
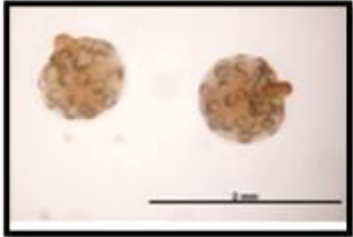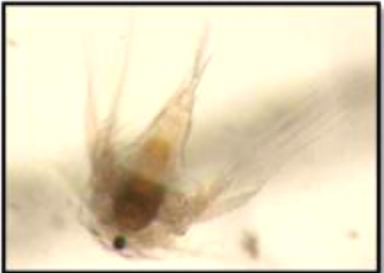
Seston sampling

The samples conducted to study the seston were 3 superficial (0 – 1 m) and 3 bottom (3 - 6 m) water sample replicates taken monthly (12 months) in each area of the estuary (Fig. 2a) by towing a conical plankton net (300 μm; Ø 0.6 m; 2 m long) for 15 min at average speed of 2.7 knots [1]. The volume filtered per tow was calculated using a flowmeter.

Laboratory procedures for seston analysis

Samples were divided into smaller aliquots (100 mL) to facilitate the separation of plankton and plastic debris from the organic matter, which was made with the aid of a stereomicroscope [ZEISS; STEMI 2000-C (× 5); www.zeis.com]. Once in the laboratory, fish larvae, fish eggs and MPs were totally separated from the bulk sample and their counts per unit were converted to a standard volume of 100 m^3 (Table 1). The ichthyoplankton taxonomic identification was based on a developmental series, working backwards from the adults and juveniles captured in the same region, from characteristics common to successively earlier ontogenetic stages [1,9,18]. Total length (L_T) was

Table 1
Density of seston componentes: microplastic, fish eggs and larvae, and zooplakton.

Itens	Total Density	
	(N 100 m^{-3})	%
Microplastics		
Soft plastic	10.7	0.08
Paint chips	7.58	0.06
Hard plastic	7.40	0.05
Microfilament	0.36	0.00
Sub-total (A)	**26.06**	**0.18**
Ichthyoplankton		
Larvae	51.03	0.37
Eggs	30.48	0.22
Sub-total (B)	**81.5**	**0.59**
Zooplankton		
Cirripedia Nauply	8223.4	60.15
Hidromedusa larvae	2762.1	20.20
Brachyura Zoe	848.6	6.21
Copepod Calanoid	754.8	5.52
Sub-total (C)	**13,564.1**	**99.2**
Total (A + B + C)	***13,671.7***	***100***

Microplastics

Fish Larvae

Fish Eggs
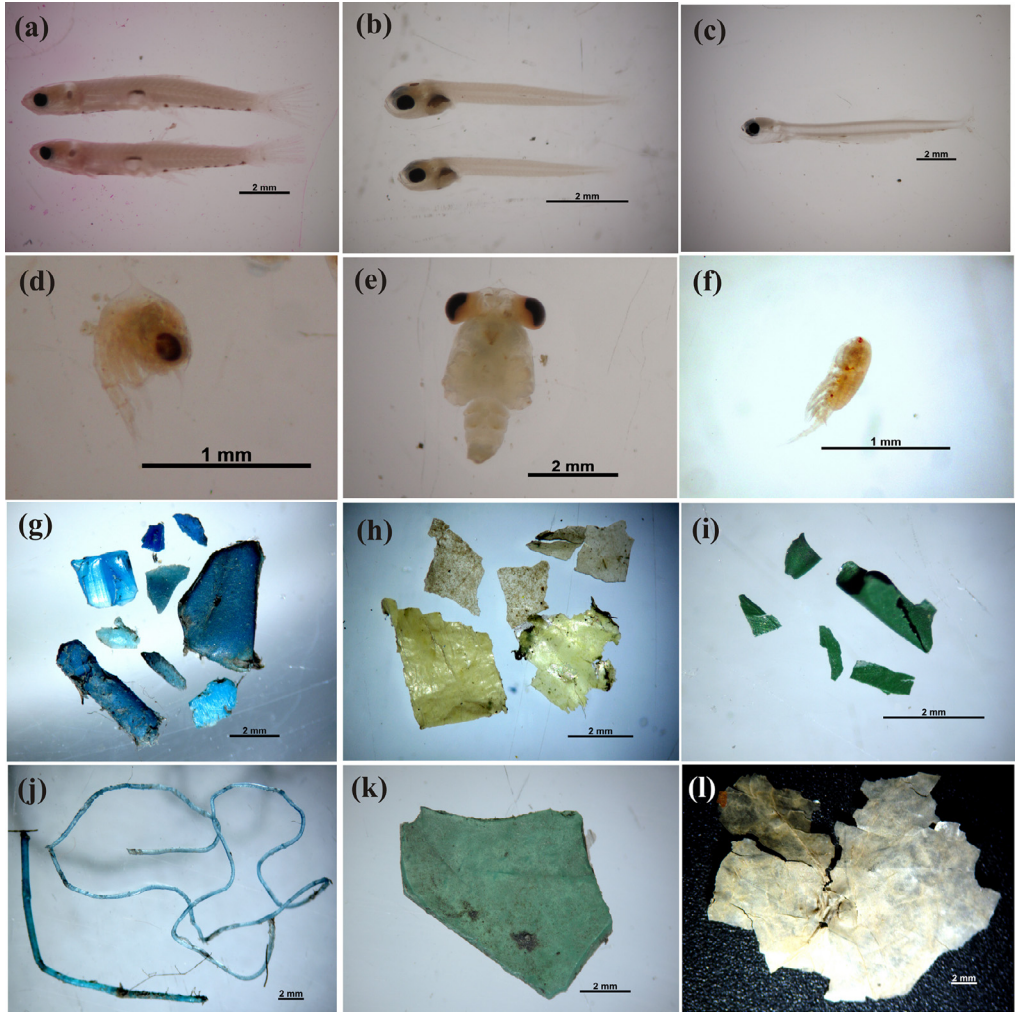
Zooplankton

Fig. 4. Examples of plankton and plastic debris found in the Goiana Estuary. Fish larvae: (a) *Gobionellus oceanicus*, (b) *Atherinella brasiliensis*, (c) *Anchovia clupeoides*; zooplankton: (d) zoea of *Ucides cordatus*, (e) Megalopa of *U. cordatus*, (f) copepod calanoida; microplastics: (g) blue hard plastics, (h) yellow soft plastics, (i) green paint chips; Macroplastics: (j) blue threads, (k) green hard plastic, (l) white soft plastic. Images captured with a digital camera Canon PowerShot G10 coupled to a stereomicroscope - ZEISS; STEMI 2000-C.

measured to establish the development stage sizes of the most important fish larvae species. For counting the zooplankton, three sub-samples of 10 mL were removed from a diluted 700 mL sample for each creek, using a Stempel pipette, with subsequent reposition. Each zooplankton taxon from the three aliquots was counted separately to calculate a mean count. Mean counts were then extrapolated to 700 ml and, as ichthyoplankton and microplastics, corrected to a standard volume of 100m^3.

Demersal fish ecology studies

Distribution pattern

River and estuary, samples were taken with an otter trawl net. The net was 8.72 m long with a mesh size of 35 mm in the body and 22 mm in the cod-end. The length of ground rope was 8.5 m,

and the head rope was 7.1 m long. To obtain a representative sample of all the fish sizes, a cover with a smaller mesh size (5 mm) was used over the cod-end [11]. The position of the boat before and after sampling was recorded by means of a GPS (Garmin – GPSMAP 421 s - USA) and was used for the calculation of the swept area. For each sample, the swept area (A) was estimated from Eq. (1):

$$A = DxhxX_2 \tag{1}$$

where, D is the length of the path, h is the length of the head-rope and X_2 is that fraction of the head-rope (h x X_2) which is equal to the width of the path swept by the trawl, the wing spread [11]. The otter trawl width was measured at the level of the otter boards at different velocities of the trawl when trawling. The ideal velocity was recorded between 3.7(2.0) ($h = 3.4$ m; X_2= 0.48) and 6 0.5 kmh^{-1} (3.5 knots) ($h = 3.8$ m; $X_2 = 0.53$). Above and below this optimal velocity range the otter trawl did not work well. The fraction of the head-rope which was close to the width of the swept area by the net during a haul was assumed to be $X_2 = 0.5$. The catch per unit area (CPUA) was used for the estimation of density (D) and biomass (B). It was calculated according Eq. (2) by dividing the catch by swept area (ha):

$$D = C_N x A^{-1} \left(ind.xm^{-2} \right) \tag{2}$$

And

$$B = C_M x A^{-1} \left(g.xm^{-2} \right) \tag{3}$$

where, C_N is the catch in number and C_M is the catch in mass of fish. The total mean density (D_T) and biomass (B_T) was estimated from Eq. (4) and Eq. (5), respectively:

$$D_T = D_a x X_1 \tag{4}$$

And

$$B_T = B_a x X_1 \tag{5}$$

Where, D_T and B_T are the mean total catch, in number and in mass respectively, per unit area of all hauls, "*a*" is the total sampled area and X_1 is the catchability coefficient. The catchability coefficient was set at 1 [19]. Six replicate trawls were made per month in each estuarine habitat of the main channel (upper, middle and lower) with an otter trawl net (Fig. 2b).

Ontogenetic phase definition

The fish species were assigned to different size classes (juvenile, sub-adult and adult) based on 2 criteria. The first criterion was the inflection point of the length−weight curve according Eq. (6), which was used to distinguish the juveniles from sub-adults (Fig. 5a).

$$Wg = \beta_0 x (Lt)^{\beta 1} \tag{6}$$

Where, *Wg* and L_t is the fish weight and total length respectively. β_0 is the intercept and β_1 is the fish growth coefficient [17]. The second criterion was length at first maturation (L_{50}) calculated [17] and used to distinguish sub-adults from adults (Fig. 5b).

To distinguish sub-adults from adults, it was used the average size at first maturation (L_{50}) [5,6,17]. The equation (Eq. (7)) model to estimate size at first maturity (L_{50}) is:

$$F = -1x \left[1 + e^{(\beta 0 + \beta 1 xL)} \right]^{-1} \tag{7}$$

The *F* represents the frequency of maturated individuals of each size class interval, *L* the pivotal point of each size class interval and β_0 and β_1 are the parameters estimated by the least squares of the linearized form the Eq. (8):

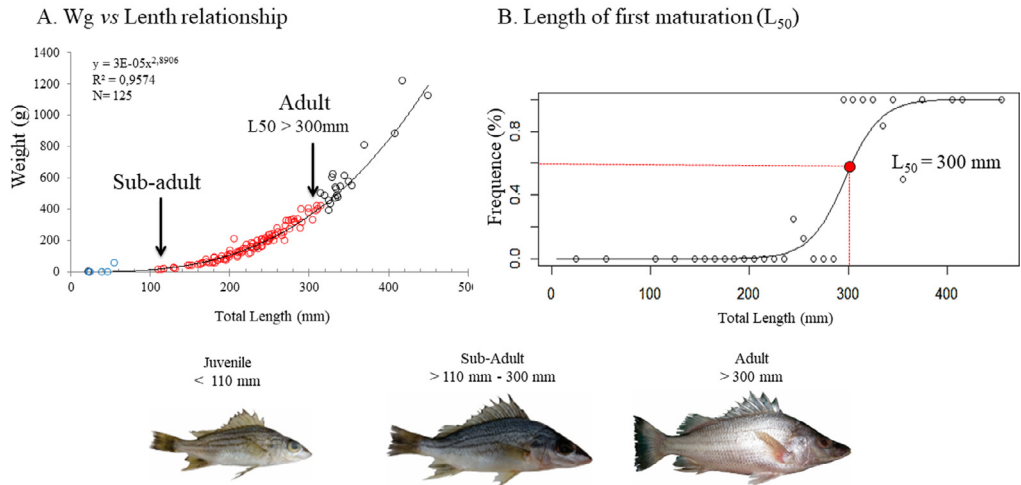$$-\ln \left[\left(\frac{1}{F} \right) - 1 \right] = \beta_0 + (\beta_1 x L_T) \tag{8}$$

Therefore, the size at first maturation (L_{50}) is estimated by Eq. (9):

$$L_{50} = -\beta_0 / \beta_1 \tag{9}$$

where, F is the proarea or ratio of reproductive female for each size class; L_T is the total length (cm) and L_{50} is the size of first maturity (cm).

Ontogenetic Phase Determination

Pomadasys ramosus

A. Wg *vs* Lenth relationship

$y = 3E-05x^{2.8906}$
$R^2 = 0.9574$
$N = 125$

B. Length of first maturation (L_{50})

Fig. 5. Length−weight relationship (arrows indicate size at inflection point) for *Pomadasys ramosus* (a), and (b) relative frequency of adults (lines and arrow in maturation curve indicate length at first maturity [L_{50}]).

Feeding ecology and MPs contamination

The stomach contents of each size class for each area (upper, middle and lower estuary) and season (early and late dry seasons, late and rainy seasons) were examined using a stereomicroscope (Zeiss x 50) (Fig. 2c). All dietary and non-dietary items (*e.g.*, microplastics) were separated, identified and counted.

In the laboratory, precautionary measures are recommended to avoid airborne and inter-sampling contamination. Blanks and open Petri dishes should be used to evaluate contamination. This is recommended at all times. To avoid airborne and inter-sampling contamination the working station and all equipment used in the evisceration must be cleaned with distilled water and absolute ethanol, prior to the procedures for the identification of digestive tracts contents [2,3]. Then, tweezers, scissors, scalpels and Petri dishes were also oven dried and double checked for contamination under stereomicroscope before the next use.

Laboratory procedures

After sampling, fishes were stored on ice and subsequently frozen. In the laboratory, each fish was identified, weighed, and measured (standard length) and the stomachs were removed and frozen at −24 °C. Individuals of each of the fish food web target species within different families (Ariidae, Gerreidae, Sciaenidae, Haemulidae, Lutjanidae, Centropomidae, Clupeidae and Engraulidae) was grouped into three size classes corresponding to different ontogenetic stages. The stomach contents of each size class for each area and season was examined and analysed using a stereomicroscope (Zeiss × 50) and microscope (Zeiss 15 × 40). All dietary and non-dietary items (*e.g.*, plastics, nylon) were separated. Prey were sorted and identified to the lowest possible taxonomic level. The items found in the stomachs were washed with distilled water, dried with tissue paper, and weighed with a precision scale (0.001 g). To assert the presence of plastics in the gut contents, supposed non-natural particles were oven dried at 70 °C for 48 h. Withered fragments were considered non-synthetic organic matter, while those fragments without significant changes in their physical characteristics, not easily cut or broken were classified as plastics [2]. Plastics were photographed and measured with the aid of the Remote Capture DC and Axiovision-LE 4.7.2 software respectively. Fragments <5 mm were

classified as MPs, and characteristics such as color and type (hard, soft or filament) were noted. The quantification of food items ingested followed three criteria [20,21]:

The percentage frequency of occurrence (%F) of each prey according to the Eq. (10):

$$\%Fi = \left(\frac{Fi}{Ft}\right) x100 \qquad (10)$$

Where, Fi is the number of stomachs containing the food item i and Ft is the total number of stomachs examined. The advantages of the frequency of occurrence method are that provided food items are readily identifiable and it is quick and requires minimum apparatus [22,23].

The percentage of prey abundance in number (%N) according to the Eq. (11):

$$\%Ni = \left(\frac{Ni}{Nt}\right) x100 \qquad (11)$$

Where, Ni is the numbers of food item i and Nt is the total number of food item in the stomachs examined. The numerical method is relatively fast and simple to operate, provided that identification of prey items is feasible [22].

The percentage of prey abundance in weight (%W) according to the Eq. (12):

$$\%Wi = \left(\frac{Wi}{Wt}\right) x100 \qquad (12)$$

Where, W_i is the weight of food item i and W_t is the total weight of food item in the stomachs examined. The volumetric technique (weight) probably gives the most representative measure of bulk and may be applied to all food items [20].

The Index of Relative Importance (IRI) [17,21], which incorporates frequency of occurrence (%F_i), percentage by number (%N_i), and percentage by weight (%W_i), was used according to the Eq. (13):

$$IRI_i = \%F_i x(\%N_i + \%W_i) \qquad (13)$$

This index was expressed in percentage of each prey according to the following Eq. (14) proposed by [17,21]:

$$\%IRI = 100 x \left(\frac{IRI}{\sum_{i=1}^{n} IRI}\right) \qquad (14)$$

Where, n is the total number of categories of food items.

Schoener's index (C) was used to evaluate food niche overlap between the different size classes of both species [24]. The index was calculated following the Eq. (15):

$$C = 1 - 0.5\left(\sum |Wxi - -Wyi|\right) \qquad (15)$$

Where, W_{xi} is the mean proarea of the biomass of food item i used by the specific size classes of fish sp_x and W_{yi} is the mean proarea of biomass of food item i used by the specific size classes of fish sp_y. Zero indicates no overlap, while 1 indicates complete overlap. The index value is generally considered biologically significant when >0.6 (Table 2) [22].

Statistical analysis

Two-way ANOVA was used to determine whether significant differences in fish density, biomass and number of species of fish occurred in space and time. ANOVA was used to test differences in fish community variables among areas (upper, middle and lower estuary) and seasons (early-dry, late-dry, early-rainy and late-rainy). All data were log10(x + 1) transformed to increase the normality of distribution. The Levene's test was used to check the homogeneity of the variances. Whenever significant differences were detected the Bonferroni Test was used *a posteriori* [25].

Canonical correspondence analysis (CCA) was used to measure ecological interactions among environmental data and both the color of microplastics and food groups ingested by fish predator [dependent variables as values of I_{RI} (Index of relative importance: Eq. (12)) or numbers of the

Table 2

Cathorops spp. feeding habitat overlaping for the prey Ostracoda and Caladoid Copepode. Cathorops spixii (Cs) and C. agassisii (Ca). Juvenile (1), sub-adult (2) and adult (3).

Season	Area	Spp(Phase) x	Spp(Phase) i	Schoener's Index (C)
Early Dry	Upper	$Cs(2)$	$Cs(3)$	0.63
	Middle	$Cs(2)$	$Cs(3)$	0.69
Late Dry	Upper	$Cs(3)$	$Ca(1)$	0.60
		$Cs(3)$	$Ca(3)$	0.62
Early Rainy	Upper	$Cs(1)$	$Ca(1)$	0.60
		$Cs(2)$	$Ca(2)$	0.62
		$Ca(1)$	$Ca(2)$	0.72
Late Rainy	Middle	$Cs(1)$	$Cs(2)$	0.62
		$Cs(1)$	$Ca(2)$	0.67
		$Cs(2)$	$Ca(2)$	0.79
	Lower	$Cs(1)$	$Ca(1)$	0.64
		$Cs(2)$	$Ca(2)$	0.64

Ostracoda

Calanoid-Copepode

individuals, ingested prey and MPs. Significant differences were accepted when α <0.05. CCA is a direct gradient analysis where the species (juvenile, subadult and adult) composition, or distribution, their prey and microplastics, is directly related to environmental variables [26]. To perform CCA, a multiple least-squares regression was computed with the site scores (derived from weighted averages of species' ontogenetic phases) as the dependent variables and the environmental variables as the independent variables [26]. The density data of some target fish species (*e.g.* Ariidae [5,12], Gerreidae [13], Haemulidae [16], Sciaenidae [6,10] and Centropomidae [2,3]) were analysed to extract patterns of variation in relation to the environmental data (direct gradient analysis). Species with <1% of the total density and biomass were excluded from the analyses. These analyses focused on symmetric and biplot scaling, and the environmental and biological data were log10(x + 1) transformed. A Monte-Carlo permutation test was used to determine which environmental variables were significant to the variability of the dependent variable. An ordination diagram was computed (triplot: with environmental variables), where the density of species (predator and prey) and MPs (or another contaminant) were represented by triangles, sites during each season by circles and environmental variables (salinity, water temperature, dissolved oxygen and Secchi depth) by arrows (Fig. 6) [26]. The diagram shows how the main pattern of variation in community composition can be influenced by environmental variables, and the distributions of the species (predator and prey) and MPs contamination, along each environmental variable [26].

Conclusions

Standard sampling protocols to obtain information about estuarine ecology and food web contamination (MPs, metals and others pollutants) are important when realizing comparisons between and within estuarine habitats to detect changes. Although several studies have focused on the contamination of fishes by plastic debris, few attempts have been made to understand spatio-temporal patterns of availability and ingestion of MPs. Both the distribution patterns of fishes and

Hamulidae Species Spatial and Temporal Distribution and MPs Contamination
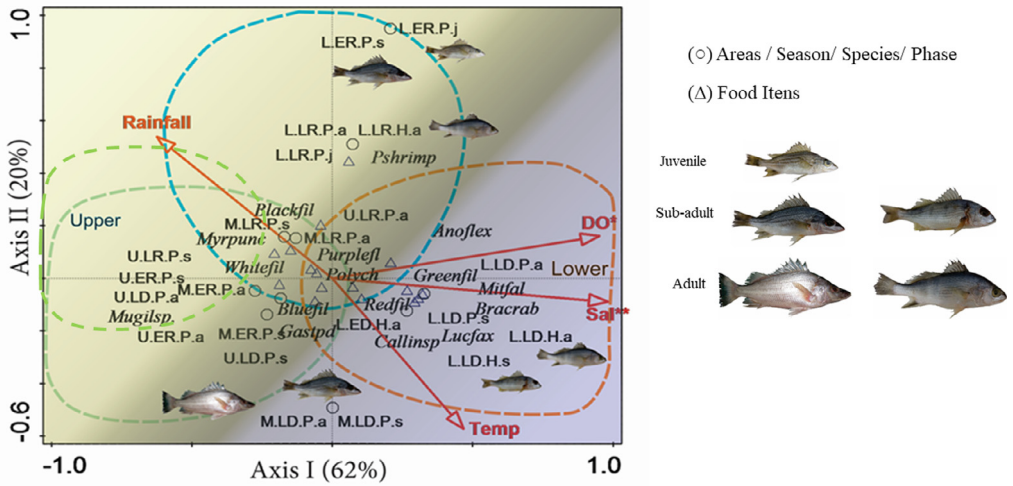
Fig. 6. Canonical correspondence analysis (CCA) triplot for the ecological correlations between the index of relative importance (%IRI) of ingested items and the environmental variables. Circles (B) represent areas (U: upper, M: middle, L: lower), seasons (ER: early rainy, LR: late rainy, ED: early dry, LD: late dry), species (P: *P. ramosus*, H: *H. corvinaeformis*) and ontogenetic phases (j: juvenile, s: sub-adult, a: adult). Triangles (D) represent items (Bluefil: blue microfilaments; Redfil: red microfilaments; Geenfil: green microfilaments; Blackfil: black microfilaments; Purplefil: purple microfilaments; Whitefil: white microfilaments; Myrpunc: *Myrophis punctatus*; Mugilsp.: *Mugil* sp.: Polych: Polychaeta; Pshrimp: Panaeidae shrimp: Lucfax: *Lucifer faxoni*; Gastpd: Gastropoda; Callinssp.: *Callinectes* sp.; Bracrab: Brachyuran crab Mitfal: Mitella falcata; Anoflex: *Anomalocardia flexuosa*).
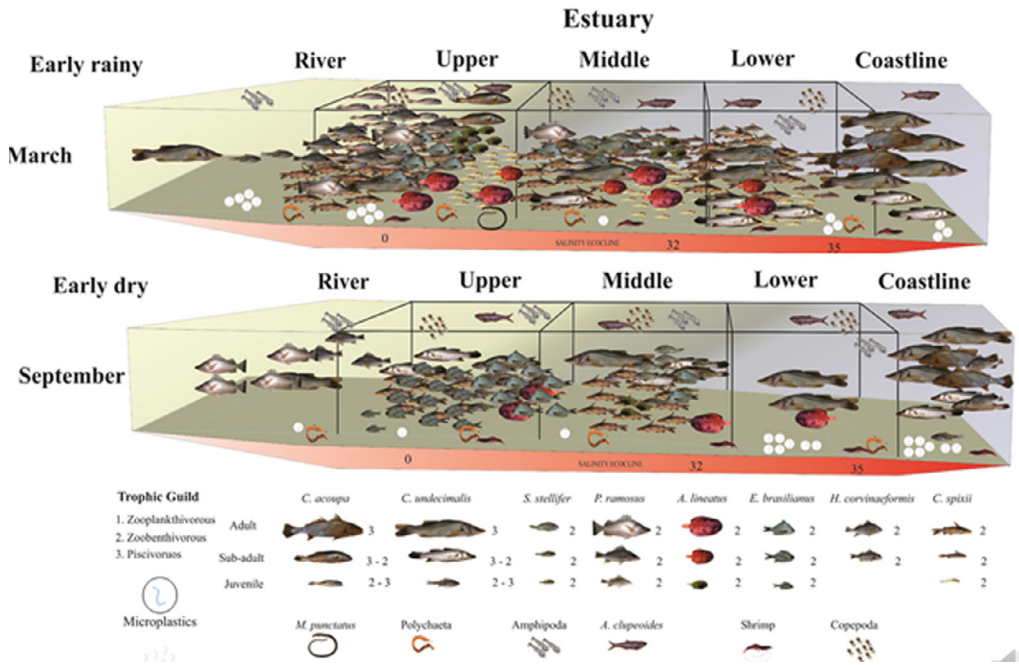
Fig. 7. Estuarine fish species conceptual model representing predator and prey densities and contamination by MPs movement in different area of estuary at rainy and dry seasons.

MPs availability vary with the spatial and seasonal changes of environmental factors typical of tropical and sub-tropical estuaries (Fig. 7). Any investigation aiming at determining MPs (and/or other pollutants) impacts on estuarine environments must include the role of the estuarine ecocline on fish ecological behavior and chance of interactions with MPs. This approach is important to detect which environmental variables are associated with patterns of MPs exposure and ingestion through the life cycle of fish species. In addition, changes in fish and other fauna patterns of use within the estuary will be more precisely detected.

Acknowledgements

Declaration of Competing Interest

The authors declare no conflict of interests.

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