

# Macrobenthic functional trait diversity at multiple scales along a subtropical estuarine gradient

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**ABSTRACT:** The capacity of an infaunal organism to exploit resources in a given sedimentary habitat is reflected by its bioturbation-related traits. Functional traits such as body size, mobility, and type of burrowing behavior reflect the species' bioturbation potential and, therefore, their role in affecting sediment characteristics. In this study, we applied a trait-based approach to assess the variation in the functional diversity of intertidal macrobenthic assemblages from Guaratuba Bay, a subtropical estuarine system in Southern Brazil. We expected that changes in sediment characteristics across and within estuarine zones would be followed by detectable changes in the trait diversity of infaunal assemblages. The estuarine gradient strongly affected overall functional diversity, especially on the largest spatial scale, which corresponded to 3 distinct salinity sectors. This was also the case for the variables grain size and depth of the redox discontinuity layer. We identified 3 assemblages with distinct functional trait composition among the sectors. Although the number of species remained relatively constant across sectors, the functional diversity increased from the inner, less saline to the outer, more saline sectors. Our findings emphasize that drivers at the largest spatial scales (i.e. gradients in salinity and sediment texture) play a major role in maximizing functional diversity and shifts in trait composition along an estuarine gradient.

**KEY WORDS:** Biological traits · Functional diversity · Tidal flats · Macrobenthic invertebrates · Estuarine gradients

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## 1. INTRODUCTION

The combined effects of natural and anthropogenic disturbances may affect the structure of marine benthic communities and ecosystem functioning (Elliott & Quintino 2007, Mouillot et al. 2013). Structural indicators, such as species richness, diversity, and abundance, are extensively used to monitor functional states in aquatic environments (Elliott & Quintino 2007, Galván et al. 2016). However, the contribution of individual macrobenthic species in altering or maintaining ecosystem functions and services is directly related to their functional traits

(Bremner 2008, Ellis et al. 2017). Functional traits are any biological trait that can affect the individual performance of an organism and that are related to one or more ecosystem properties or processes (Violle et al. 2007, Mlambo 2014). For instance, the different kinds of bioturbation behavior performed by infaunal organisms are considered functional traits because they directly affect sediment biogeochemical properties and process rates (Reise 2002, Bremner 2008, McLenaghan et al. 2011). Thus, the diversity of functional traits, or simply functional diversity, can be seen as a major component of biological diversity.

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There has been growing interest over the last decade in measuring biodiversity to assess environmental changes and detect changes in assemblage structure, and in understanding the role of functional diversity on ecosystem functioning (Bremner 2008, Mouillot et al. 2013, Dolbeth et al. 2015). Many approaches to assess functional diversity are currently available, including functional diversity indices and biological traits analysis (BTA) (Petchey & Gaston 2006, Bremner 2008, Mouillot et al. 2013). Such approaches have provided novel insights on the relationship between the traits of marine species and environmental gradients, and on the resilience of ecological systems to disturbance (Dolbeth et al. 2015, Gusmao et al. 2016, Bierschenk et al. 2017, van der Linden et al. 2017). However, although widely applied in marine environments worldwide, functional diversity approaches in benthic communities are still scarce in tropical and subtropical estuarine habitats (e.g. Gusmao et al. 2016, van der Linden et al. 2017, Wouters et al. 2018).

Estuarine species are adapted to highly variable physical and geochemical drivers that operate at different spatial and temporal scales. Benthic communities may play a relevant role in regulating the ecosystem's properties and processes in estuarine environments. For example, benthic species can modulate sediment stability and biogeochemistry by speeding up the remineralization of organic matter and nutrient exchange through the sediment–water interface (Reise 2002, McLenaghan et al. 2011). Assessing the functional role of the macrobenthos is especially interesting for management purposes (Dolbeth et al. 2015, Bierschenk et al. 2017) because environmental changes may favor the establishment of different species in the modified environment and cause a shift in the structure of assemblage functional traits (Mouillot et al. 2013). The capacity of macrobenthic species to disturb sediments depends on different bioturbation-related traits, such as feeding behavior, body size, and burrowing capacity (Kristensen et al. 2012). Thus, assessing the diversity and incidence of such traits in macrobenthic assemblages represents an indirect way to address their function along sedimentary ecosystems (Bolam et al. 2016, Gusmao et al. 2016, Douglas et al. 2017).

Due to the high variability in the measurement units characterizing benthic systems and the need to better understand their function (Morrisey et al. 1992, Chapman et al. 2010, Otegui et al. 2016, Wouters et al. 2018), exploratory techniques to identify sources of variability must incorporate appropriate replication at multiple spatiotemporal scales.

Suitable sampling methods are mandatory to understand structural patterns and to obtain true sources of variation, thus avoiding equivocal interpretations of benthic indicators. A hierarchical framework for assessing changes in diversity across multiple spatial and temporal scales has produced promising results on the mechanisms that regulate macrobenthic structures in subtropical estuarine gradients (Gusmao et al. 2016, Morais et al. 2016, Souza et al. 2016).

In this paper, we assessed the spatiotemporal patterns of the functional diversity of intertidal macrobenthic assemblages, expressed by the variation in the number of species, functional trait diversity, and trait composition along a subtropical estuarine system in Southern Brazil. Changes in sediment texture and the quality and quantity of organic deposits along estuarine gradients can affect the recruitment and post-settlement survival rates of infaunal organisms (Reise 2002). The capacity of an infaunal organism to explore resources in a given sedimentary habitat depends on its life history traits. Traits such as body size, mobility, and type of burrowing behavior reflect the species' bioturbation potential and, therefore, their role in affecting sediment characteristics. Thus, it is expected that changes in sediment characteristics across and within estuarine sectors would be followed by detectable changes in the trait diversity of infaunal assemblages. We used a trait-based approach based on bioturbation-related traits to test 4 different hypotheses: (1) Since the infaunal structure is highly driven by sediment characteristics, we expect that the spatial trends in infaunal functional diversity will mostly reflect the gradients in sediment texture and biogeochemistry along the estuarine gradient. (2) As major changes in environmental variables are observed across estuarine sectors (i.e. inner, intermediate, and outer estuary), we expect that most of the variation in functional diversity will be correspondingly observed at the largest spatial scale. Besides, (3) since the infauna can affect and be affected by sediment aeration in sediments (Rosenberg 2001), we expect that variation trends in trait diversity will reflect gradients in sediment redox conditions. Finally, (4) if changes in sediment characteristics can affect the infaunal species differently depending on their traits, we expect that estuarine sectors with increased organic matter and small grain size will be dominated by deposit feeders with increased bioturbating capacity. Distribution patterns of macrobenthic assemblages can be temporally inconsistent (Morrisey et al. 1992, Chapman et al. 2010, Souza et al. 2016). To test the relative importance of short-term events to overall variability, we

also addressed how macrobenthic functional diversity at varying spatial scales respond to changes from fortnights to seasons.

## 2. MATERIALS AND METHODS

### 2.1. Study site

The study was carried out in the Guaratuba Bay (25° 52' S, 48° 38' W), a 15 km long subtropical estuarine system (Bigarella 2001) located in Paraná State, Southern Brazil (Fig. 1). Paraná's coastal zone is characterized by a seasonal humid subtropical climate (Cfa in Köppen's classification) with an annual rainfall of ca. 2200–2500 mm (Alvares et al. 2013). A typical rainy season initiates in late austral spring and lasts through most of the summer (November–March), while the dry season lasts from late autumn to late winter (May–August).

The estuary is shallow at the inner areas, with an average depth of 3 m, and deep at the mouth, with the greatest depths reaching 27 m (Marone et al. 2006, Angulo et al. 2016). The main rivers located in the inner region have a combined annual average discharge of about 80 m<sup>3</sup> s<sup>-1</sup> (Marone et al. 2006). Tides are semi-diurnal with diurnal inequalities and may reach up to 2 m at the mouth of the bay during spring tides (Marone et al. 2006,

Angulo et al. 2016). The estimated tidal current velocity is up to 2.3 m s<sup>-1</sup>, and water residence time is approximately 9.3 d (Marone et al. 2006). The estuary shows high salinity fluctuations with strong horizontal and vertical stratification, especially during the rainy season (Marone et al. 2006, Brandini 2008). Three sectors can be differentiated according to the salinity gradient: an upstream (inner) sector dominated by fluvial processes, with salinity ranging from 0 to 10; a central (intermediate) sector characterized by a mixing zone, with salinity varying between 10 and 27; and a downstream (outer) sector dominated by marine processes, with salinity ranging from 23 to 34 (Brandini 2008, Mizerkowski et al. 2012).

The estuarine system consists of a mosaic of ecosystems such as tidal flats, mangroves, marshes, and seagrass beds. The tidal flats represent 24% of the surface area and are composed of poorly selected sediments characterized by fine sand and silt (Marone et al. 2006, Noernberg et al. 2008, Cotovicz et al. 2014). The Guaratuba Bay is an environmental protection area where the main economic activities are traditional fishing and oyster farming. However, the estuary is affected by anthropic activities with the release of different kinds of contaminants (e.g. sewage and pesticides) in the bay, which, therefore, threatens its environmental quality (Cotovicz et al. 2014, Dauner et al. 2016).

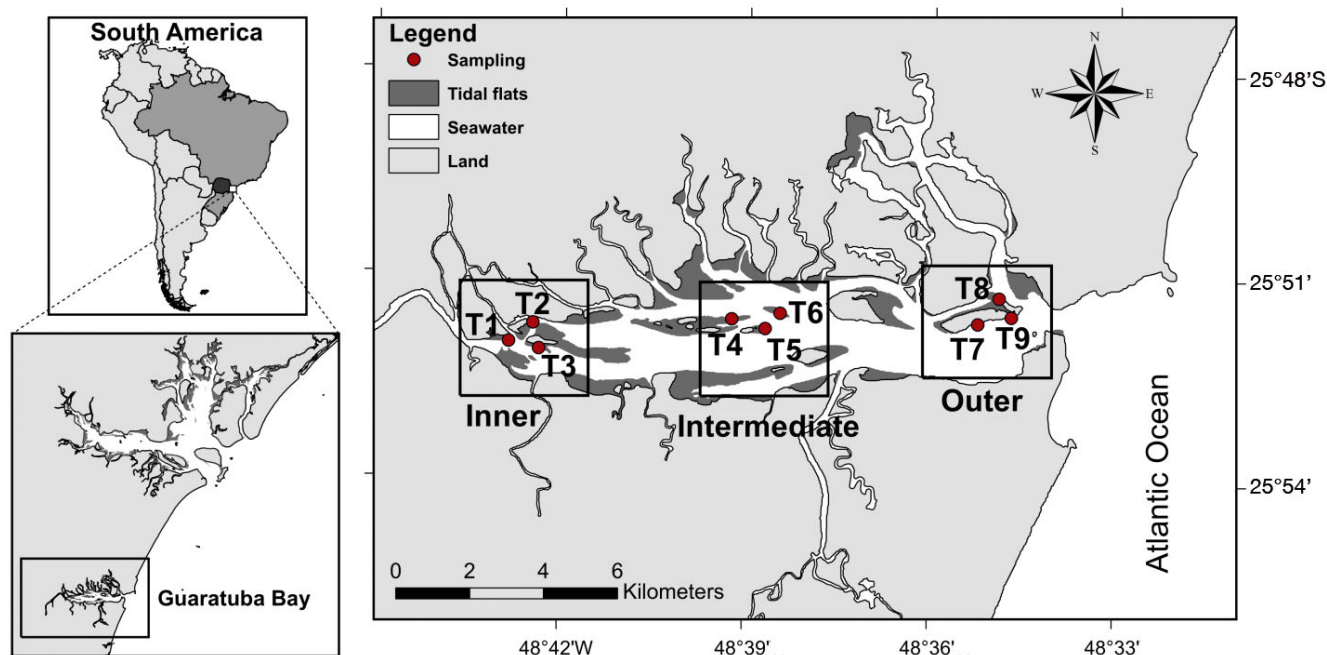


Fig. 1. Study area in Guaratuba Bay, Paraná, Brazil. Sampling surveys undertaken in 3 tidal flats (T1 to T9) within sectors (inner, intermediate, and outer) along the estuarine gradient

## 2.2. Sampling and laboratory procedures

The sampling design was hierarchized and incorporated multiple spatial and temporal scales (Fig. 1; Fig. S1 in the Supplement at [www.int-res.com/articles/suppl/m624p023\\_supp.pdf](http://www.int-res.com/articles/suppl/m624p023_supp.pdf)). Field samplings were conducted in 2012, at the end of the rainy (March–April) and dry (October–November) seasons. The temporally hierarchized sampling design was conducted on 3 or 4 consecutive days within 3 subsequent fortnights in each seasonal period. The spatial design incorporated 3 sampling sectors on the scale of kilometers ( $10^3$  m) following the salinity gradient. The sectors were denominated as inner (oligohaline), intermediate (mesohaline to polyhaline), and outer (polyhaline to euhaline). In each sector, 3 tidal flats, separated by several hundred meters ( $10^2$  m), were selected. The tidal flats were carefully selected across the estuarine gradient to avoid the putative effects of freshwater discharge from rivers and tidal creeks. In each selected tidal flat, 3 sites (at least 10 m apart) were randomly chosen, placed parallel to the water line, at similar tidal levels. These sites measured 4 m<sup>2</sup>, in which 3 sampling plots of 0.25 m<sup>2</sup> were randomly distributed. One benthic sample was taken from each sampling plot using plastic core tubes 15 cm in diameter and 10 cm in height.

The sediment temperature was measured with a precision digital thermometer inserted 10 cm deep in each sampling site. The depth redox discontinuity layer (RDL) of the sediment was measured in triplicate using a ruler. The RDL was determined visually by considering the maximum depth where the sediment color changed from a lighter colored layer (oxygen-rich) to a darker layer (oxygen-poor). Salinity and pH were measured in water that percolated in the sediment using a refractometer and a pH meter.

Additional sediment samples were collected at each sampling site with plastic core tubes (5 cm in diameter and 10 cm in depth) to estimate particle size and organic matter contents. A sample of surface sediment (approximately 10 g in 1 cm depth) was collected for nutrient analysis (total organic carbon, phosphorus, and nitrogen). Another superficial sediment sample (approximately 2 g) was taken for the analysis of chlorophyll *a* (chl *a*) and phaeopigment contents. All sediment samples for biogeochemical analyses were taken to the laboratory under refrigerated and dark conditions for further processing, stored at 20°C, and subsequently dried and macerated.

The sediment samples for faunal analysis were fixed in 4% formaldehyde and preserved in 70%

alcohol solution with Rose bengal. Subsampling and flotation assessment using a sugar solution ( $>1.2$  g ml<sup>-1</sup>) were necessary for samples from the inner sector of the estuary due to the high amount of sand and detritus. Animals were identified to species, or morphotype levels, and counted.

Laboratory procedures for the analysis of granulometry, organic matter, nutrient content (organic carbon, nitrogen, and phosphorus), and photosynthetic pigments (chl *a* and phaeopigments) followed the same techniques and routines described in Morais et al. (2016). Sediment samples for granulometric analyses were treated with a solution of hydrochloric acid (HCl) to dissolve carbonates and with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to eliminate the organic matter. Sediment texture was analyzed by a laser diffraction analyzer (Microtrac, Bluewave) and grain size ranges were classified following the Wentworth scale. The concentration of organic matter was estimated by weight loss after oxidation of ~10 g of dry sediment with a solution of 10% H<sub>2</sub>O<sub>2</sub> (Gross 1971). Sediment total organic carbon was estimated following the methods described by Strickland & Parsons (1972) and Baumgarten et al. (1996). To estimate total nitrogen and phosphorus, a sample of 0.1 g of dried and macerated sediment was treated with potassium persulfate (K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>) in an autoclave at 100°C for 30 min. These samples were then subsampled (25 ml for nitrogen and 15 ml for phosphorus) for colorimetric analyses, as described by Grasshoff et al. (1983). We standardized the analysis procedures by assigning 'zero concentration' to values of absorbance below the limit of detection. To estimate photosynthetic pigments (chl *a* and phaeopigments), sediment subsamples of ca. 0.5 g were treated with 10 ml of acetone (100%) and stored in the dark at 4°C for 24 h. Afterward, the samples were centrifuged at 3000 rpm for 20 min and then analyzed in a Shimadzu UV-1601PC spectrophotometer. Chl *a* and phaeopigments were determined by absorbance reads at 665 and 750 nm, before and after acidification of the sample with 1 ml of 1 N HCl. We used an adaptation of the Lorenzen (1967) equation proposed by Sündback (1983) to calculate the pigment concentrations in sediment samples. Molar N:P and C:N ratios were used to assess the nutrient limitation and relative contribution of autochthonous and allochthonous inputs of organic matter along the estuarine gradient. Organic matter quality was determined by the ratio of phaeopigments to the sum of chl *a* and phaeopigments (PAP ratio) and was considered as an indication of freshness or the degradation conditions of material deposited in the sediment (Boon et al. 1998).

### 2.3. Diversity changes

Macrobenthic taxa were classified into biological traits based on information gathered from regional and international literature (i.e. Arruda et al. 2003, Pagliosa et al. 2012, Jumars et al. 2015), online databases such as BIOTIC, the Biological Traits Information Catalogue (MarLIN 2006) and WoRMS, the World Register of Marine Species (WoRMS Editorial Board 2015), and information from the nearest phylogenetic neighbor or ad hoc opinion of specialists. The classification was carried out considering different functional traits and associated subcategories, which reflected taxa bioturbation capacity. These traits also incorporate aspects of the species' morphology, life history, behavior, and ecological adaptations (Table 1). Rare taxa with <10 individuals in total abundance were not included in the functional diversity analysis. Abundant taxa were scored according to their degree of association with each trait category using fuzzy coding (Chevene et al. 1994). Fuzzy coding uses scores to describe the affinity of taxa to different categories of a given biological trait category based on a fixed score range from 0 to 3. Score values of 0 represent no affinity to a given trait category, while 3 represents total affinity. Intermediate scores between 0 and 3 were used when the same taxon presented multiple biological trait categories (Table S1 in the Supplement). Thus, the sum of all categories of a trait was always 3. This standardization of the scores aimed to avoid giving too much importance to generalist species that present multiple categories of a certain trait.

Functional diversity was computed with Rao's quadratic entropy according to Botta-Dukát (2005). Rao's index for functional diversity incorporates both relative abundances of species and pairwise functional differences between species expressed as species dissimilarities in a multivariate trait space. The multivariate trait space reflect the similarities of the species within an assemblage according to their trait values. It was constructed from a 'trait by species' matrix (Table S1) by estimating the pairwise differences in species traits using Euclidean distances. Thus, it produces a species-by-species distance matrix. Higher values of Rao's index are expected when species are widely overspread in the multivariate trait space (high multivariate dispersion) and when abundances are not concentrated in a group of taxa with similar functional traits. This index was calculated for each sample using the function dbFD of the analytical package 'FD' (Laliberté et al. 2014) that implements the formula proposed by Rao (1982) as follows:

Table 1. Biological traits and categories used to describe the functional diversity of macrobenthic assemblages in the subtropical estuarine system

Biological trait	Categories
Adult body size (according to collected empirical data)	≤5 mm 5 to 10 mm 10 to 20 mm 20 to 40 mm 40 to 80 mm 80 to 160 mm >160 mm
Bioturbation type (processes related to activities of organisms in sediments)	None Biodiffuser Gallery diffuser Upward conveyor Downward conveyor Bio-irrigator
Body design (body shapes)	Soft Soft protected (tube/gallery/tunic cover) Hard exoskeleton Hardshell
Depth in sediment (organisms living in different layers of the sediment)	Surface 0 to 3 cm 3 to 8 cm 8 to 15 cm 15 to 25 cm >25 cm
Feeding type (exhibited modes of feeding)	Deposit feeder Suspension Opportunist/scavenger Grazer Carnivore/predator
Movement type (processes of locomotion)	Sessile/sedentary Swim Crawl Burrow/tube

$$FD_Q = \sum_{ij}^S d_{ij} p_i p_j \quad (1)$$

where  $S$  is a species community characterized by the relative abundance vector  $p = (p_1, p_2, \dots, p_s)$ ,  $d_{ij}$  is the difference (i.e. Euclidean distance) between the  $i^{\text{th}}$  and  $j^{\text{th}}$  species ( $d_{ij} = d_{ji}$  and  $d_{ii} = 0$ ), and  $FD_Q$  expresses the average difference between 2 randomly selected individuals with replacements (Botta-Dukát 2005).

### 2.4. Data analysis

Spatial and temporal variations in the number of species, functional diversity, and environmental variables were evaluated using permutational multi-



variate analysis of variance (PERMANOVA) based on the Euclidean distance because a measure of association makes the univariate test similar to traditional ANOVA (Anderson et al. 2008). The mixed linear model for number of species and functional diversity combines nested and orthogonal design (Fig. S1): Season (fixed; 2 levels; rainy and dry), Fortnight (random; 3 levels; nested in Seasons), Sector (fixed; 3 levels; inner, intermediate, and outer), Tidal flats (random; 3 levels; nested in Sectors), and Site (random; 3 levels; nested in Tidal flats with 3 replicates). A simplified mixed linear model was applied to assess the differences for each environmental variable separately, since samples were not replicated at each site. Our model combines nested and orthogonal design with Season (fixed; 2 levels; rainy and dry), Fortnight (random; 3 levels; nested in Seasons), Sector (fixed; 3 levels; inner, intermediate, and outer), and Tidal flats (random; nested in Sectors with 3 replicates). We used the permutations method to construct a linear combination of mean squares, which avoids problems with terms generated by the linear model without an appropriate denominator mean square for the calculation of *F*-ratios (Season, Sector, Tidal flat within Sector, and interactions: Season  $\times$  Sector, Season  $\times$  Tidal flat). The procedure was described by Satterthwaite (1946), detailed by Blackwell et al. (1991), and thus implemented in the PERMANOVA+ add-on for PRIMER (Clarke & Gorley 2006, Anderson et al. 2008). The test was performed under 9999 permutations and a level of significance at  $p < 0.05$  obtained by the Monte Carlo *p*-value. The PERMANOVA test was followed by a *posteriori* pairwise comparisons on terms, in the model that was found to be significant at  $p < 0.05$ . Moreover, the variance components (VC%) were calculated to estimate the amount of variation for each source of spatial and temporal variability or interactions between them. The significance of a factor (presented in the form of *p*-values) describes how likely the patterns explained by the factor are simply due to random chance, and thus, serves no functional importance to the researcher (Graham & Edwards 2001). Additionally, *p*-values are inherently dependent on the amount of sample, while the determination of fit (measured as VC%) is an estimate of the variance in a response variable that can be explained by the factor. Therefore, the approach adopted here considers the proportion of total variance as the best estimate of the contribution of a given factor to variability in a response variable, as suggested by Graham & Edwards (2001). In the case of negative variance components, its value was set to zero since the term in question had a large *p*-value.

We used RLQ analysis (Dolédéc et al. 1996) to assess the relationship between macrobenthic traits and gradients in environmental variables. This analysis has been applied to different biological models, including marine worms (Wouters et al. 2018). RLQ analysis produces a constrained ordination that incorporates information on the environmental variables at each sampled site (matrix R), species abundance (matrix L), and the trait information of each species (matrix Q). This analysis is designed to maximize the covariance between the traits and the environmental variables mediated by the species abundances (Dray & Dufour 2007). First, a correspondence analysis (CA) is performed on the L-abundance matrix. The scores of the CA are then used as row weights in a principal components analysis (PCA) performed on the R-environmental matrix. Then, a multiple correspondence analysis (MCA) is performed using the Q-trait matrix and the CA site scores as column weights. The resulting constrained ordination shows how environmental gradients are related to different functional traits. This analysis was performed for each season (rainy and dry) separately. Since samples to quantify environmental variables were not replicated at each site (81 samples), the samples of macrofaunal abundances at each site were averaged to perform the RLQ analyses. Highly colinear environmental variables (Pearson correlation coefficient  $> 0.8$ ) were removed from the analyses. The physicochemical variables included temperature, RDL, salinity, pH, chl *a*, phaeopigments, nitrogen, phosphorus, organic matter, mean grain size, molar N:P ratio, molar C:N ratio, and PAP ratio.

Except for PERMANOVA, all statistical analyses and graphs were carried out in the R environment for statistical computing (RStudio Team 2016) using the following packages: 'ade4' (Dray & Dufour 2007), 'FD' (Laliberté et al. 2014), 'ggplot2' (Wickham 2009), and 'vegan' (Oksanen et al. 2013).

### 3. RESULTS

#### 3.1. Environmental variables

The environmental variables displayed distinct variation patterns over space and time in the PERMANOVA analyses (Table 2; Table S2 in the Supplement). Season scale accounted for most of the total variation (43.7%), although temperature did differ among fortnights interacting with tidal flats and sectors (Table 2). Temperature ranged from 24.7 to 31.5°C and from 21.1 to 27.3°C in the rainy and dry

Table 2. PERMANOVA for each measured environmental variable in the subtropical estuarine system. We considered the spatial factors (Sector [S] and Tidal flats [T] nested in Sector) orthogonal to temporal factors (Season [Se] and Fortnights [F] nested in Season) (n = 3). VC%: variance component as percentage of the total; PAP ratio: ratio of phaeopigments to the sum of chl *a* and phaeopigments; RDL: redox discontinuity layer. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001

Source of variation	df	MS	Pseudo- <i>F</i>	VC%	MS	Pseudo- <i>F</i>	VC%	MS	Pseudo- <i>F</i>	VC%
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Temperature					Salinity			pH		
Se	1	343.220	4.84	43.72	919.730	6.16	5.47	0.160	0.88	0
F(Se)	4	70.820	88.33***	33.65	142.050	14.94***	2.79	0.071	1.74	1.11
S	2	30.637	2.87	4.92	8324.600	123.09***	87.08	2.728	12.13***	46.61
T(S)	6	1.790	2.23	0.71	45.920	4.83**	1.15	0.132	3.23*	5.02
Se × S	2	10.074	1.16	0.70	24.636	1.12	0.08	0.085	0.49	0
F(Se) × S	8	9.181	11.45***	12.08	21.790	2.29	0.78	0.096	2.35	6.08
Se × T(S)	6	0.235	0.29	0	8.722	0.92	0	0.158	3.87**	12.91
F(Se) × T(S)	24	0.802	9.23***	3.09	9.506	4.22***	1.38	0.041	1.83*	6.11
Residuals	108	0.087		1.13	2.253		1.28	0.022		22.16
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Chl <i>a</i>					Phaeopigments			PAP ratio		
Se	1	1363.700	2.13	6.06	1019.700	13.89**	17.63	0.048	0.33	0
F(Se)	4	655.210	8.15***	13.64	38.548	3.51*	1.53	0.140	9.47***	10.29
S	2	734.050	1.13	1.14	520.250	3.66	10.68	0.296	1.93	6.12
T(S)	6	412.380	5.13**	11.82	104.880	9.56***	7.79	0.020	1.34	0.63
Se × S	2	1304.400	4.21*	25.05	630.270	8.46**	31.27	0.316	1.72	11.39
F(Se) × S	8	305.860	3.80**	16.05	40.142	3.66**	4.84	0.141	9.54***	31.14
Se × T(S)	6	23.182	0.29	0	35.637	3.25*	4.09	0.051	3.41*	8.79
F(Se) × T(S)	24	80.386	3.78***	12.62	10.967	0.74	0	0.015	1.06	0.58
Residuals	108	21.277		13.63	14.840		22.16	0.014		31.06
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Nitrogen					Phosphorus			N:P ratio		
Se	1	41.061	3.03	4.77	7.506	0.86	0	39.675	2.28	3.53
F(Se)	4	8.700	2.78	2.70	8.841	10.98***	26.42	15.452	8.34***	6.17
S	2	70.181	4.10*	13.40	6.690	1.24	2.40	78.110	3.56	13.05
T(S)	6	3.200	1.02	0.05	2.076	2.58*	6.27	1.276	0.69	0.00
Se × S	2	96.904	4.86*	38.42	3.506	0.91	0	100.960	4.30*	35.79
F(Se) × S	8	14.688	4.70**	16.77	3.957	4.92**	31.09	21.159	11.42***	26.27
Se × T(S)	6	5.885	1.88	4.00	0.805	1.00	0	2.735	1.48	1.20
F(Se) × T(S)	24	3.126	4.33***	10.46	0.805	4.76***	18.81	1.853	2.35**	4.35
Residuals	108	0.722		9.42	0.169		15.01	0.788		9.65
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C:N ratio					Organic matter			Grain size		
Se	1	17075.0	2.39	2.16	147.770	12.45**	10.06	0.83	0.64	0
F(Se)	4	6602.4	0.76	0	4.351	0.71	0	4714.10	1.11	0.24
S	2	28242.0	3.17	5.47	237.050	11.27**	23.62	229 130.00	16.04***	56.24
T(S)	6	4676.0	0.54	0	13.380	2.18	2.31	10312.00	2.43	4.67
Se × S	2	26231.0	3.14	10.29	151.470	9.72**	30.13	7459.40	1.89	2.83
F(Se) × S	8	6971.5	0.81	0	8.204	1.33	1.31	4239.50	1.00	0
Se × T(S)	6	4146.8	0.48	0	8.015	1.30	1.19	1966.00	0.46	0
F(Se) × T(S)	24	8654.5	1.40	9.55	6.150	1.20	2.00	4250.50	2.40**	11.48
Residuals	108	6204.0		72.53	5.105		29.37	1768.20		24.54
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RDL										
Se	1	15.311	1.88	0.31						
F(Se)	4	7.886	1.35	0.19						
S	2	1663.100	27.52***	75.05						
T(S)	6	55.998	9.56***	7.02						
Se × S	2	42.697	6.04**	3.78						
F(Se) × S	8	4.647	0.79	0						
Se × T(S)	6	3.391	0.58	0						
F(Se) × T(S)	24	5.859	1.13	0.55						
Residuals	108	5.199		13.10						

seasons, respectively (Table S2). Significant interactions between space and time were detected for salinity, pH, RDL, and grain size. However, most of the total variation of these variables were related to the largest spatial factor (i.e. sectors), with 87, 46.6, 75, and 56.2%, respectively (Table 2). Salinity and pH increased from the inner to the outer sectors of the estuary ( $p < 0.01$ ), ranging from 0 to 34 and from 6.23 to 7.8, respectively. Grain size and RDL were higher in the inner sector than in other sectors ( $p < 0.01$ ), in both rainy and dry seasons (Table S2). In addition, significant differences in interaction between space and time at small scales were detected for chl *a*, phaeopigments, PAP ratio, nitrogen, phosphorus, and N:P ratio (Table 2). However, most of the total variation of food-related variables (chl *a*, phaeopigments, nitrogen, and N:P ratio) were related to the interaction between sectors and season (25, 31.2, 38.4, and 35.8%, respectively). Chl *a* and phaeopigments differed among sectors mainly in the rainy season ( $p < 0.01$ ), while nitrogen and N:P ratio differed among sectors mainly in the dry season ( $p < 0.05$ , Table S2). A high contribution of the interaction Fortnight  $\times$  Sector was observed for phosphorus and the PAP ratio, with about 31% of the total variability (Table 2). Phosphorus content was higher in the intermediate sector during the rainy season (Table S2), but patterns were not consistent across fortnights ( $p < 0.05$ ). The average PAP ratios in surface sediment samples indicated that the algal organic matter in the inner and intermediate sectors was more degraded in the rainy season (Table S2), but patterns were not consistent across fortnights ( $p < 0.05$ ). A large amount of variation for the PAP ratio was also concentrated at residuals (31%), indicating a high variability and patchy algal organic matter availability in surface sediment along the estuarine gradient. Variations of organic matter content were significant for the interaction between seasons and sectors (Table 2). This interaction and the corresponding residuals accounted for a high percentage of the total variance, with about 30 and 29%, respectively. Organic matter content was significantly higher in the intermediate sector in the rainy season (Table S2), and no significant differences were found between the inner and outer sec-

tors over time ( $p < 0.001$ ). There were no significant differences in individual factors and interactions between spatial and temporal scales for the C:N ratio ( $p > 0.05$ , Table 2). Residuals accounted for most of the total variability (72.5%), indicating that sources of sedimentary organic matter were patchily distributed.

### 3.2. Diversity changes

The number of species (measured as the number of taxa, including unidentified species) and functional diversity displayed marked variation patterns over space and time (Table 3, Fig. 2). PERMANOVA analyses showed that species richness was only significantly affected by the interaction between fortnights and tidal flats (pseudo- $F = 4.2$ ,  $p < 0.001$ ), indicating that tidal flats differences may vary across fortnights and vice versa. Functional diversity was significantly affected by the spatial factors sector (pseudo- $F = 9.66$ ,  $p < 0.01$ ) and tidal flat (pseudo- $F = 3.57$ ,  $p < 0.01$ ). It also was significantly different for the interactions Fortnight  $\times$  Sector, Season  $\times$  Site, and Fortnight  $\times$  Tidal flat (Table 3). These interactions indicate that changes in functional diversity across sectors may vary across fortnights; changes across sites depend on seasons; and tidal flats differences may vary across fortnights. However, the relative importance (VC%) of individual factors and interactions was highly variable. As for the number of species, a large amount of variation was concentrated at

Table 3. PERMANOVA for the number of species and functional diversity of macrofauna in the subtropical estuarine system. We considered the spatial factors (Sector [S], Tidal flats [T] nested in Sector, and Site [Si] nested in Tidal flats within Sector) orthogonal to temporal factors (Season [Se] and Fortnights [F] nested in Season) ( $n = 3$ ). VC%: variance component as percentage of the total. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

Source of variation	df	—Number of species—			—Functional diversity—		
		MS	Pseudo- $F$	VC%	MS	Pseudo- $F$	VC%
Se	1	196.46	1.90	1.93	862.89	2.06	1.3
F(Se)	4	32.51	0.40	0	315.93	1.40	0.6
S	2	281.40	2.89	5.20	12990	9.66**	41.2
T(S)	6	36.44	0.59	0	906.54	3.57**	7.3
Si(T(S))	18	13.28	0.67	0	44.68	0.72	0
Se $\times$ S	2	167.23	1.23	2.03	46.06	0.40	0
F(Se) $\times$ S	8	89.29	1.09	0.93	462.14	2.04	4.9
Se $\times$ T(S)	6	113.79	1.36	4.64	211.31	0.70	0
Se $\times$ Si(T(S))	18	16.19	0.81	0	165.89	2.68**	6.5
F(Se) $\times$ T(S)	24	82.19	4.12***	24.50	226.46	3.66***	10.3
F(Se) $\times$ Si(T(S))	72	19.94	1.27	4.94	61.90	1.37*	3.2
Residuals	324	15.76		55.83	44.93		24.7



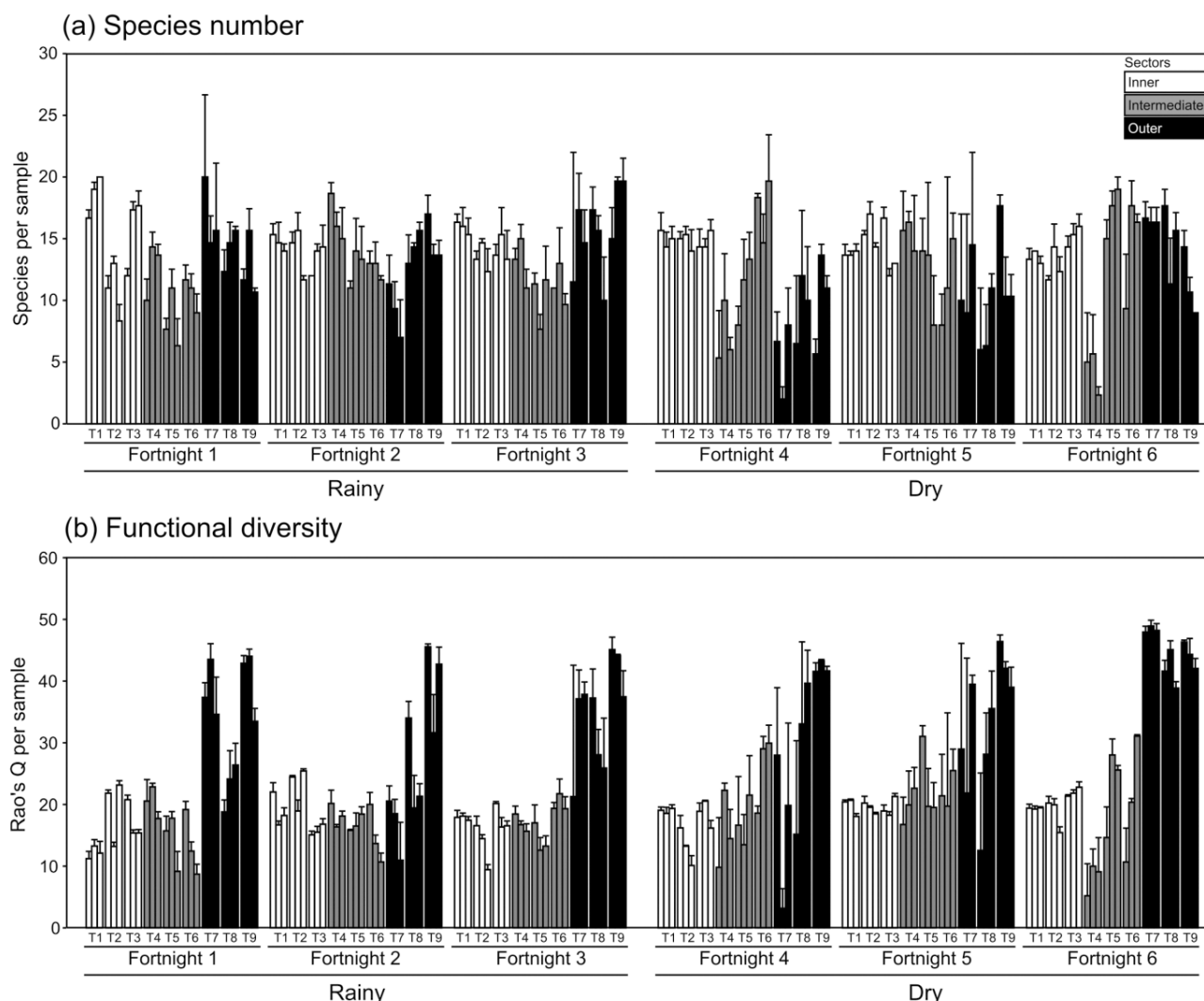
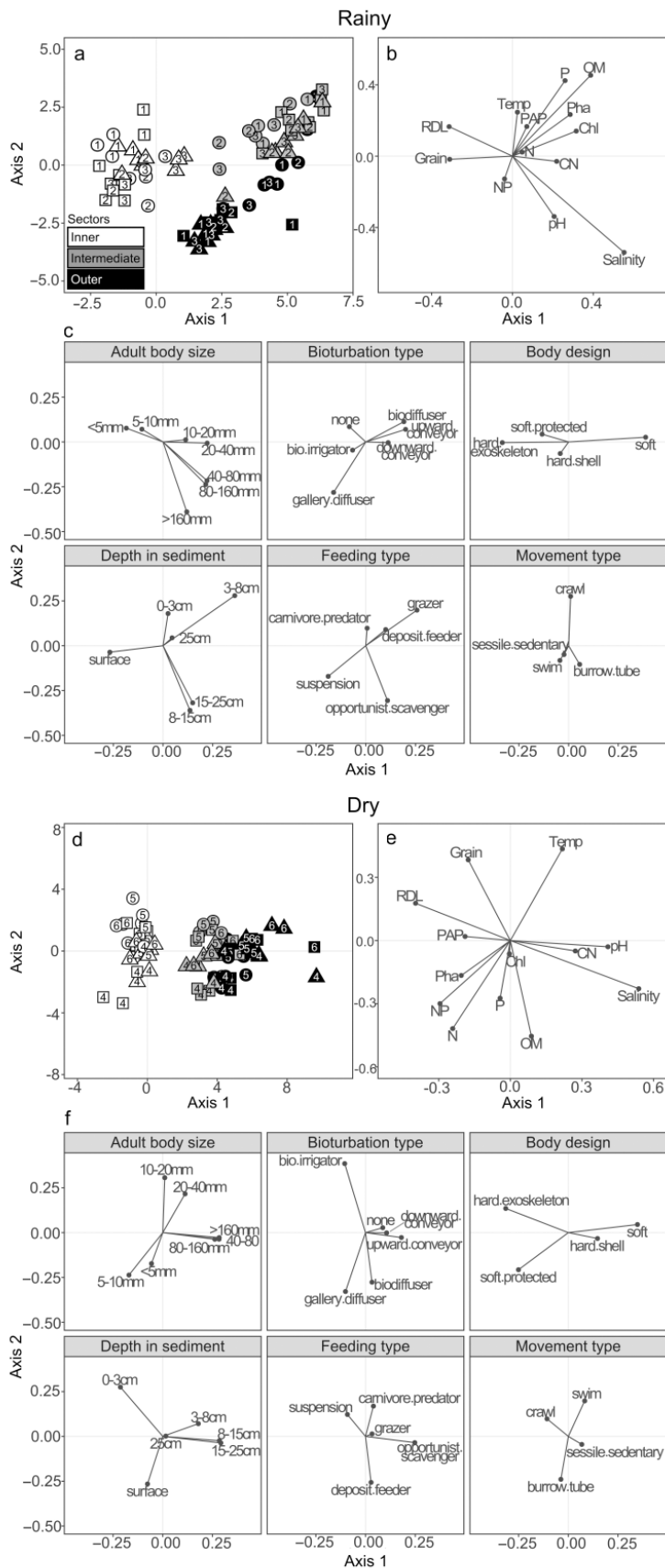


Fig. 2. Mean values ( $\pm$ SE) of (a) number of species and (b) functional diversity of benthic assemblages per sample along the estuarine system for each season (rainy and dry), fortnight (1 to 6), sector (see color key), tidal flat (T1 to T9), and site (bars)

both residuals (55.8%) and the interaction Tidal flats  $\times$  Fortnights (24.5%), whereas for functional diversity, the largest spatial scale (sectors, with 41.2%) and residuals (24.7%) were the most important contributors to the overall variance (Table 3). Functional diversity tended to increase from the inner to the outer sectors of the estuary ( $p < 0.05$ , Fig. 2b), while the number of species showed no evident changes across sectors along the study ( $p > 0.05$ , Fig. 2a).

The results of the RLQ analyses (Fig. 3) performed for rainy and dry seasons showed changes in assemblage structure across sectors separating the inner sector from the intermediate and outer sectors (Fig. 3a,d). For the rainy season, the 1st axis showed high correlation with environmental variables (correlation  $L = 0.74$ ) and explained 89.6% of the total variance (Table S3 in the Supplement). It was mostly ex-

plained by gradients in salinity, organic matter, grain size, and depth of the RDL (Fig. 3b). The 2nd axis explained 8.6% of the total variation (correlation  $L = 0.55$ ) and was mostly explained by gradients in salinity, organic matter, and phosphorus concentrations (Table S3). This axis separated the outer from the intermediate sector (Fig. 3a). For the dry season, the 1st axis explained 88.1% of the total variation (correlation  $L = 0.77$ , Table S4 in the Supplement) and was related to gradients in salinity, pH, and depth of the RDL (Fig. 3e). Changes in assemblage structure across sectors were associated with this axis. The 2nd axis explained 6.8% of the total variation (correlation  $L = 0.33$ , Table S4) and was related to gradients in temperature, organic matter, grain size, and total nitrogen (Fig. 3e). In general, the RLQ ordinations performed for rainy and dry seasons presented simi-



larities in the patterns of trait distribution (Fig. 3c,f). The inner sector was characterized by assemblages with a high incidence of small body size (10 mm or smaller), body with a hard exoskeleton or soft-protected, low capacity to penetrate the sediment (epifaunal or shallow-living infauna), suspension feeding type, and bioirrigator and gallery diffuser bioturbation types. On the other hand, the intermediate and outer sectors were characterized by soft body, large body size (10 mm or larger), active burrowers (biodiffusers and up- and downward conveyors), deep penetration in sediment (3 cm or deeper), and variable feeding types (mostly grazers and opportunistic/scavengers).

#### 4. DISCUSSION

The functional diversity of macrobenthic assemblages changed markedly across the estuarine gradients, and it is clearly related to the largest spatial scales used in this study (sectors). Functional diversity was higher at the outer sector of the estuary and this pattern was temporally consistent across fortnights and seasons. Replicates (i.e. samples, expressed as residuals in Table 3) and sectors accounted for most of the variation in functional diversity. The high amount of variation at the replicate scale just reflects the logistic limitations in sampling macrofaunal patches. Similarly, sediment properties such as grain size and depth of the RDLs also varied significantly across estuarine sectors. These results were in line with the first 3 predictions, indicating

Fig. 3. RLQ ordinations for each season (a–c: rainy; d–f: dry) depicting changes in (a,d) macrofaunal species abundances and (b,e) gradients in environmental variables; and (c,f) functional trait variability. In (a,d): symbol shapes indicate different tidal flats (T1: circle; T2: square; and T3: triangle), and numbers indicate fortnights (1 to 6). In (b,e): Temp: temperature; Grain: grain size sediment; RDL: redox discontinuity layer; OM: organic matter, Chl: chl a; Pha: phaeopigments, PAP: PAP ratio (ratio of phaeopigments to the sum of chl a and phaeopigments); N: nitrogen, P: phosphorus; NP: nitrogen:phosphorus ratio; and CN: carbon:nitrogen ratio

that major changes in functional diversity reflect spatial patterns of sediment characteristics. The largest variations were observed across estuarine sectors (i.e. our largest spatial scale). Although gradients in organic matter content markedly varied across seasons and were mostly related to the variation within sectors (i.e. across tidal flats), grain size and depth of RDL always increased from the outer to the inner sectors of the estuary. The incidence of trait categories related to increased bioturbation capacity (i.e. large size, conveyors, deep borrowing capacity) reflected major changes in salinity and sediment texture. Thus, we did not reject the 4th hypothesis, that increased organic matter and small grain size would be related to traits reflecting increased bioturbating capacity.

The variability in functional trait diversity was congruent with the main estuarine gradient, mainly driven by salinity, pH, grain size, and depth of the RDL. Similar studies also highlighted the salinity gradient as a primary driver of spatial and temporal variations in macrobenthic functional trait diversity along estuarine gradients (van der Linden et al. 2012, 2017, Veríssimo et al. 2012, Darr et al. 2014). However, variations in functional diversity at small scales, expressed by a less consistent spatial pattern mainly in the dry season, indicate that variability within intermediate and outer sectors can also be driven by a distinct set of environmental and biological characteristics. This natural variation at small spatial scales can be confounded with the variation in short-term temporal scales (i.e. fortnights). Thus, any interpretation of the temporal variation within small scales should be made with caution.

Our findings are in contrast with previous studies that found significant spatial changes in species richness along estuarine gradients (van der Linden et al. 2012, 2017, Törnroos et al. 2015, Bierschenk et al. 2017). Many conceptual estuarine models currently available in the literature are based on putative linear variations in diversity, abundance, and distribution along a salinity continuum, from freshwater to marine conditions (Whitfield et al. 2012). In a similar way, predictive statistical models have suggested that the presence or absence of individual macrobenthic species are strongly and directly coupled to physicochemical processes at large scales, which would explain their longitudinal estuarine distribution mostly associated with salinity, depth, current velocity, and sediment texture (Elliott & Quintino 2007, Galván et al. 2016). Caution should be taken in developing such conceptual models and in hoping that they can be generalized. However, a more com-

plex estuarine model emerged from our results regarding the number of species. Although salinity values always varied linearly from the inner to the intermediate and outer sectors, there were non-linear trends in the number of species which was primarily affected by drivers operating at small scales related to the content of photosynthetic pigments, organic matter, and nutrients. The variation patterns in functional diversity did not follow the trends observed for the number of species, which remained relatively constant along the estuarine gradient. This is probably related to the properties of Rao's index, which is an abundance-based index that is relatively independent of species richness and can even decrease as the number of species increases (Botta-Dukát 2005, Laliberté & Legendre 2010, Mouchet et al. 2010).

We identified 3 assemblages with clearly distinct functional trait compositions across estuarine sectors. These differences were detected for all fortnights and seasons, indicating that changes in infaunal trait structure across large spatial scales are temporally consistent. These results are consistent with previously described patterns for taxonomic structure in the same estuarine system (Morais et al. 2016). The segregation of benthic functional groups along environmental gradients is already known in estuaries (van der Linden et al. 2012, 2017, Veríssimo et al. 2012, Darr et al. 2014, Bierschenk et al. 2017). Indeed, environmental factors may impose filters for the establishment of species depending on their biological traits (Mouillot et al. 2013). In our special case, grain size and salinity are the gradients that best explain functional changes in macrofaunal assemblages. Apart from the osmotic constraints that limit the distribution of marine invertebrates upstream, sediment texture directly affects the foraging capacity of infaunal organisms across the entire estuarine gradient (Mermillod-Blondin 2011). Gradients in sediment texture frequently reflect the hydrodynamic regimes within an estuary (Marone et al. 2006). These gradients are highly correlated with other sediment properties, such as the quantity and quality of organic deposits (Mermillod-Blondin 2011). As the mud content increases downstream, organic matter and nutrient supply for microphytobenthic films tend to increase and consequently favor the establishment of a variety of deposit feeders (MacIntyre et al. 1996, Jumars et al. 2015). The food resources available in organic deposits and the physical structure of the sedimentary habitats possibly represent filters for the distribution of infaunal species (Mermillod-Blondin 2011, Ellis et al. 2017, Wouters et al. 2018), which

might explain the observed changes in trait structure along the estuary. The harsh conditions upstream possibly favor a short range of functional traits, which explains why the inner sector is mostly dominated by small-sized filter feeders.

The outer sector presents environmental conditions that favor marine species, especially medium- to large-sized deposit feeders and scavengers. This allows the establishment of functionally contrasting species, resulting in higher functional diversity in the outer sector. The relative importance of the physical structure of the sedimentary habitat and the food quality and quantity in shaping trait structure of infaunal assemblages certainly suggest the need for further studies. Infaunal assemblages with high bioturbation capacity were mostly related to the outer estuarine sector. These assemblages were characterized by a high incidence of traits such as large body size, deep penetration in the sediment, and up- and downward conveyors. This is due to the dominance of polychaetes such as *Aricidea* spp. and *Mediomastus* sp. 1, which are deposit feeders that thrive in sediments with small grain size and high organic matter content (Jumars et al. 2015). On the other hand, the inner sector presented a high incidence of functional traits related to a low bioturbation capacity, such as small body size, shallow penetration in sediment, and epifaunal habit. This was caused by the numerical dominance of crustaceans such as the amphipod *Monocorophium acherusicum*, the tanaids *Monokalliapseudes schubarti* and *Sinelobus stanfordi*, the isopods *Cassidinidea fluminensis* and *Uromunna* cf. *peterseni*, and an unidentified ostracod. The behavioral strategies of such crustaceans play important roles to more effectively compete for food resources in the inner sector. Indeed, these functional characteristics generally reflect an opportunistic life history and have major effects on assemblage secondary production (Dolbeth et al. 2015). The notable differences in trait composition observed between the inner and the outer estuarine sectors is a response to local environmental factors such as low salinity and the prevalence of coarse sand due to the elevated freshwater discharge in the inner sector (Marone et al. 2006, Cotovicz et al. 2014). These environmental characteristics do not provide a suitable habitat for most polychaetes and mollusks in tropical estuaries, explaining why the traits related to increased bioturbation capacity are primarily related to the outer sector (van der Linden et al. 2017).

The correspondence of the variation patterns observed for infaunal functional diversity and the depth of the RDL suggest that the capacity of macrobenthic

assemblages to affect sediment-related processes (i.e. nutrient exchange and oxygen fluxes) via bioturbation varies along the salinity gradient. Specific trait categories are distributed unequally across estuarine sectors, indicating zones of different bioturbation potential across the Guaratuba Bay. Considering that the importance of bioturbation in mediating chemical exchanges across the water–sediment interface is reduced in coarse sediments (Mermillod-Blondin 2011), it is possible that the low incidence of highly bioturbating organisms in the inner sector does not necessarily reflect lower rates of sediment process rates. Thus, as the bioturbation potential decreases towards the inner sectors, the importance of macrobenthic assemblages in modulating sediment-related processes also would decrease. This hypothesis could be tested by experimental studies manipulating the abundance of highly bioturbating macrobenthic species in the 3 estuarine sectors.

Macrobenthic assemblages may vary across different spatial and temporal scales, displaying complex variability (Morrissey et al. 1992, Chapman et al. 2010, Morais et al. 2016, Souza et al. 2016). The resulting patchy distribution of macrobenthos and their variety of biological traits estimated by our hierarchical approach can directly influence functional trait diversity. However, a clear understanding of small-scale variation linking functional traits and environmental drivers along estuarine gradients has not yet emerged in the literature (van der Linden et al. 2012, Veríssimo et al. 2012, Darr et al. 2014, Törnroos et al. 2015). Therefore, it is difficult to carry out comparisons of different estuarine systems to derive predictions or generalizations of the roles of macrobenthic functions in estuaries across space and time.

Linking environmental gradients with species traits represents an efficient way to assess variability in assemblage structure and functions in space and time (Dolbeth et al. 2015, Gusmao et al. 2016, Bierschenk et al. 2017, van der Linden et al. 2017, Wouters et al. 2018). Our trait-based approach does not directly measure how macrobenthic assemblages affect sediment-related ecosystem processes, but the recorded gradient in functional diversity gives insights into the ecological roles played by macrobenthic assemblages across Guaratuba Bay. Although there are differences between species richness and functional diversity, both should be used as complementary tools in estuary management programs. This should be considered by marine managers when assessing potential impacts on macrobenthic assemblages of Guaratuba Bay and other tropical and subtropical estuaries.

## 5. CONCLUSIONS

Functional diversity of macrobenthic assemblages changed along the estuarine gradient and indicated that the highest variation trends along the estuary were related to the largest spatial scales. Such a pattern was not observed for the number of species, suggesting that the functional trait approach is more sensitive in detecting minor or less evident variations in macrobenthic structure. Our results also showed that varying functional trait categories are clearly related to specific estuarine sectors, evidencing the filtering effect of the estuarine gradient on species with specific trait combinations. Although we were unable to assess the direct effects of how individual drivers affect trait distribution, variations in functional diversity and composition were clearly congruent with gradients in salinity, grain size, and RDL. However, significant variations at small spatial and temporal scales highlight the relative importance of changes in other sediment properties, such as the content of photosynthetic pigments, organic matter, and nutrients (carbon, nitrogen, and phosphorus). Our findings provide insights for marine managers on the functional roles of estuarine macrobenthos and the potential consequences of species loss along different estuarine sectors. In terms of practical conservation issues, the loss of vulnerable traits, either from inner or outer sectors, can induce a greater functional alteration in estuarine ecosystem services, including carbon capture and storage related to nutrient cycling, primary production, water quality regulation and bioremediation of waste, and maintenance of biodiversity (Reise 2002, Elliott & Quintino 2007, Bremner 2008, Mermillod-Blondin 2011).

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