

## Original Articles

## Food web attributes to assess spatial–temporal dynamics in estuarine benthic ecosystem

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## ABSTRACT

Threatened benthic ecosystems need urgent tools for effective bioassessment and relevant management. EU Marine Strategy Framework Directive (MSFD) obligates member states to achieve GES (Good Environmental Status) for 11 descriptors of environmental state (MSFD; 2008/56/EC). From all of the descriptors, D4 that focuses on Food Webs is the most functional-oriented indicator, but also the most challenging to implement due to our limited knowledge on benthic interactions. Particularly, it is still unclear how spatially and temporally regulated abiotic variables determine the entire benthic food webs, and which benthic food web attributes best respond to these spatially and temporally derived environmental variations. To fill this gap, we measured the natural isotopic ratios ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) of macrobenthic organisms and their food sources and build twelve food web topologies across three distinct sites (Navigator, Gambia, Tróia) in summer and winter during two consecutive years. To assess these food web topologies, we applied isotopic metrics, further integrated with univariate and multivariate analysis to find food web-based indicators that best respond to these spatial and temporal variability.

We found clear spatial patterns associated to an increase in primary production and quantity and quality of organic matter (OM). Sites with higher organic load and less quality OM (Navigator and Gambia) had simpler food webs, likely associated to high abundance of opportunistic meiobenthic species. Site located inside protected area (Tróia) with high quality OM had the most complex food web characterized by high diversity of specialist consumers that used more efficiently available resources. Similarity metrics were valuable complementary tool that helped to further disentangle the causes of spatial variability, in this case distinguishing between two food webs (Navigator and Gambia) that had similar structures but different resource utilization.

The temporal patterns were not so evident than the spatial patterns, although significant differences were reported between sampling occasions for the same metrics (maximum trophic position and the percentage of carnivores and omnivores,  $p < 0.05$ ). The most complex Tróia's food web demonstrated greater responsiveness in capturing temporal differences in resource use, suggesting that more complex food webs are better equipped to reflect temporal variability. The integration of isotopic metrics complemented with multivariate and univariate analyses proved to be an important tool for the analysis of different aspects of the benthic food web complexity in a spatial–temporal context providing a promising approach to assess the functional integrity of the estuarine ecosystems, especially in the context of the descriptor 4 within MSFD.

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## 1. Introduction

EU Marine Strategy Framework Directive (MSFD) obligates member states to achieve GES (Good Environmental Status) for 11 descriptors of environmental state (MSFD; 2008/56/EC). From all of the descriptors, descriptor 4 (D4) that focuses on Food Webs is the most functional-oriented indicator of the ecosystem status, but also the most challenging to implement due to our limited capacity to quantify functional interactions in marine environment, especially at the base of the food web (Rombouts et al., 2013). Consequently, present indicators under D4 are highly imbalanced, limited to well-studied pelagic habitats or economically important guilds (fish, birds) rarely accounting for the benthic habitats and ecosystem-based processes, hence are not efficient to detect environmental disturbance (Rogers et al., 2010). According to Environmental Report 2020, achievement of GES in marine waters has very little progressed, while 60 % of EU surface waters are still not meeting Water Directive standards, underscoring the urgent need for the development of new tools that would facilitate our understanding of the complex interactions among organisms and their environment to support their wider implementation in the bioassessment strategies.

Estuarine and coastal benthic ecosystems represent one of the major sources of essential services for human well-being (Bonaglia et al., 2014; Schratzberger and Somerfield, 2020). The functional integrity of these ecosystems is maintained by multiple intra and interspecific interactions between organisms that mediate the energy transfer to higher trophic levels (Schratzberger & Somerfield, 2020; Ridall and Ingels, 2021). The proxy to evaluate the efficacy of which this energy is transferred to higher trophic compartments can be assessed by analyzing the food web structure. Food webs reflect structural organization of biota and their interactions, whereas food webs delivered metrics allow to visualize this complex information in a manageable way (Jackson et al., 2012; Gray et al., 2014; Bergamino and Richoux, 2015; Szczepanek et al., 2021). Therefore, they are attractive to managers allowing to account for both direct and indirect effects of environmental disturbance in a single network (Tam et al., 2017).

In estuaries spatially and temporally regulated set of environmental variables determines distribution of biotic communities and their functional traits (Sroczyńska et al., 2021a; Tsikopoulou et al., 2021). However, to date only few studies determined how spatially and temporally regulated abiotic variables determine the entire benthic food webs (Liu et al., 2020; Szczepanek et al., 2021; Ziółkowska and Sokołowski, 2022), and more importantly it is still unknown what are the benthic food web attributes that best respond to these spatially and temporally derived environmental variations. So far, we know that seasonally and spatially regulated inputs of organic matter (OM), primary productivity, as well as physical and environmental variables have a profound impact on the food web structure (Nelson et al., 2015). In most cases, nutrient availability increases primary productivity and the abundance and diversity of primary and secondary consumers, which further reflects in higher complexity of the food web structure (Donazar-Aramendía, 2019; Ziółkowska and Sokołowski, 2022). However, when nutrient input exceeds the oxygen availability, the diversity of primary producers is reduced, promoting a higher abundance of opportunistic species at the lower trophic levels (Guen et al., 2019; Xu et al., 2022). The consequence of this shift is a reduction of the prey diversity, ultimately resulting in the diminishment of trophic connections and shrinking of the trophic niche (Thompson et al., 2012; Burdon, 2021; Xu et al., 2022).

It becomes evident that more hypothesis-driven studies are needed to understand how benthic food webs respond to environmental drivers over spatial and temporal scales, and which food web attributes best reflect these changes. To fill this gap, we measured the natural isotopic ratios ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) of macrobenthic organisms and their food sources and constructed twelve food web topologies across three distinct sites and over 4 different sampling occasions. To assess these food web topologies, we applied isotopic metrics (Cucherousset and Villéger, 2015; Jackson et al., 2012; Layman et al., 2007) that were demonstrated to be

successful in quantifying multiple anthropogenic impacts on food webs (Donazar-Aramendía, 2019; Sroczyńska et al., 2021a) as well as inferring important information on trophic diversity, food web stability or trophic resilience (Layman et al., 2012; Jackson et al., 2012). These metrics were further integrated with univariate and multivariate analysis to find benthic food web-based indicators that best respond to these spatial and temporal variability. Finally, this study aims to contribute to a new paradigm of analyzing ecological data based on empirically derived integrated functional (food web) attributes for better assessment of the benthic ecosystem status (Baiser et al., 2019).

### 1.1. Study conceptualization and hypothesis

The Sado estuary, situated in SW Portugal, offers an ideal study location due to its unique blend of anthropogenically disturbed regions and areas of high ecological significance. Previous studies on this estuary demonstrated strong spatial and temporal differences in the distribution of meio- (Sroczyńska et al., 2021a, Vieira et al., 2023) and macrobenthic (Caeiro et al., 2005; Brito et al., 2023) species composition as well as functional traits (Sroczyńska et al., 2021a). The main variables responsible for the spatial-temporal differences in community distribution patterns were related to sediment grain size and OM inputs, whereas differences in oxygen concentration were demonstrated to be decisive for the functional traits composition patterns. Sites with clay predominance and an increased organic input demonstrated to have less diverse communities with the predominance of small opportunistic taxa.

Spatial and temporal changes in food web structure are usually associated with seasonal variations of primary production and fluvial discharge of terrestrial OM (Szczepanek et al., 2021) that together determine the trophic conditions of the sediments. Therefore, the sampling sites selected for this study (Navigator, Gambia and Tróia) present divergent conditions described by differences in biogeochemical properties of the sediment, mainly related to sediment grain size and quality and quantity of OM. The temporal scale of the study included winter and summer months along two consecutive years.

Given that the structuring of benthic food webs in spatial and temporal contexts depends on organic matter inputs and environmental conditions, we anticipate that communities inhabiting sites with substantial organic matter input (e.g., Gambia and Navigator), but limited oxygen availability, are likely to exhibit a less diverse range of available food sources. This, as observed in our earlier studies, has led to a decrease in benthic community diversity and has promoted a greater abundance of opportunistic species at the lower trophic levels. Drawing from above, our first hypothesis (H1, Table 1) presumes that sites with larger OM input (Gambia and Navigator) will present a narrower trophic niche size and reduced trophic diversity, reflected in lower isotopic and diversity metrics (Table 1). At the same time, we hypothesize that communities at these sites will have a lower species richness, but with more scattered isotopic values, which will be reflected in an increase in the isotopic divergence and uniqueness metrics.

We further hypothesize that we find greater similarity, reflected in the overlap metrics (H2, Table 1), between sites with similar trophic conditions (Gambia and Navigator). Regarding temporal scale, in colder seasons, the reduced primary productivity and the increased terrestrial and freshwater OM inputs produces less diverse benthic communities with few top predators, which, overall, should reduce trophic interactions. Therefore, we hypothesize that winter food webs will present reduced isotopic diversity including maximum trophic position (H3, Table 1) in comparison to summer seasons. Instead, summer seasons that present an increase in primary productivity and peak in species reproduction will have a higher resource diversity and will host a more diverse community of benthic species, which will translate into an increase of maximum trophic position, diversity and redundancy. We also hypothesize (H4, Table 1) that we will observe more similar food webs during the same season (e.g., win19/win20 or sum20/sum21) than when comparing food webs across different seasons.

Table 1

Summary of the selected metrics in relation to the hypothesis tested in this study (H1–H4).

| Scale                                                                                                                                                                                                                                                                                                                                                                                                                                               | Metric | How the metric is measured                                                                                                                                                                                                                                                                                             | Ecological meaning of the metrics                                                                                                                                                             |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>H1:</b> Communities at sites with higher organic matter input (Gambia and Navigator), but little oxygen availability will have less diverse availability of food source what will impact benthic community diversity, promoting higher abundance of opportunistic species at the lower trophic level decreasing trophic diversity metrics (CR, NR, TA and Max TP, Iric, Ieve and % of omnivores and carnivores) but will increase Idiv and IUni. |        |                                                                                                                                                                                                                                                                                                                        |                                                                                                                                                                                               |
| Spatial                                                                                                                                                                                                                                                                                                                                                                                                                                             | CR     | <b>Carbon range-</b> difference between the most <sup>13</sup> C depleted and enriched species ( Jackson et al. 2012)                                                                                                                                                                                                  | Diversity of the basal resources                                                                                                                                                              |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                     | NR     | <b>Nitrogen range</b> – difference between the most <sup>15</sup> N depleted and enriched species ( Jackson et al. 2012)                                                                                                                                                                                               | Extent of vertical food web structure                                                                                                                                                         |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                     | TA     | <b>Total Area</b> encompassed by the consumers food web using mean <sup>13</sup> C and <sup>15</sup> N consumers isotopic values that includes all species in the isotopic space (Layman et al. 2007)                                                                                                                  | Diversity of the resource use by the consumers (trophic diversity)                                                                                                                            |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                     | Iric   | <b>Isotopic richness</b> – corresponds to TA (Volume of the minimum convex hull that includes all species), but considers scaled (δ15N-δ13C) isotopic values ( Cucherousset and Villéger, 2015)                                                                                                                        | Diversity of the resource use by the consumers (trophic diversity)                                                                                                                            |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                     | Max TP | <b>Maximum trophic position</b> of the species at a given site using the site mean δ <sup>15</sup> N of basal resources as a baseline (Winemiller et al. 2007)                                                                                                                                                         | Realized food chain length                                                                                                                                                                    |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                     | IEve   | <b>Isotopic evenness</b> – Regularity in the distribution of taxa in the isotopic scaled space (Cucherousset and Villéger, 2015)                                                                                                                                                                                       | Equitability in the resource use                                                                                                                                                              |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                     | IUni   | <b>Isotopic uniqueness</b> – Average closeness of organisms in the isotopic scaled space ( Cucherousset and Villéger, 2015)                                                                                                                                                                                            | inverse of the trophic redundancy, proxy for the ecosystem resilience                                                                                                                         |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                     | Idiv   | <b>Isotopic divergence</b> – accounts for the distribution of consumers, within the convex hull area, Idiv is close to 1 when most of the organisms have extreme isotopic values and is close to 0 when most of the organisms are close to the centre of gravity of the convex hull (Cucherousset and Villéger, 2015). | Balance in the distribution between different trophic groups in the community (primary producers and top predators), i.e., high Idiv can be sign of the presence of large invasive predators. |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                     | IDis   | <b>Isotopic dispersion</b> – weighted-deviation to the average position of points in the stable                                                                                                                                                                                                                        | Is a scaled multidimensional variance accounting for both the convex hull area                                                                                                                |

Table 1 (continued)

| Scale                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | Metric                     | How the metric is measured                                                                                                                                                                                                                    | Ecological meaning of the metrics                                                 |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | omniv_car                  | isotope space divided by the maximal distance to the centre of gravity ( <a href="#">Cucherousset and Villéger, 2015</a> ).<br><b>Omnivore and Carnivore percentage</b> – % of omnivores and carnivores relative to all of the trophic guilds | and the isotopic divergence.<br><br>Proxy for ecosystem stability                 |
| <b>H2:</b> Greater overlap reflected in Ines and Isim will be found between similar sites (Gambia and Navigator).                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                            |                                                                                                                                                                                                                                               |                                                                                   |
| Spatial (isotopic overlap)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | Isotopic nestedness (Ines) | Ratio between shared area of two food webs (communities) and the smallest convex hull area ( <a href="#">Cucherousset and Villéger, 2015</a> )                                                                                                | Complementarity or redundancy in resource use by two communities                  |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | Isotopic similarity (Isim) | Ratio between the volume shared and the volume of the union between two convex hull areas between two communities ( <a href="#">Cucherousset and Villéger, 2015</a> )                                                                         | Similarity in filling the isotopic space by the consumers between two communities |
| <b>H3:</b> Colder seasons (winter) have a reduced primary productivity, but increased terrestrial and freshwater OM input which will produce less diverse benthic communities, being responsible for reduced isotopic diversity metrics especially reflected in Max TP. Summer seasons with an increase in primary productivity and peak in species reproduction will have a higher resource diversity and will host more benthic species, which will translate into an increase of Max TP and higher % of carnivores and omnivores as well as their trophic diversity metrics (CR, NR, TA, Iric, IEve) and reduced IUni. |                            |                                                                                                                                                                                                                                               |                                                                                   |
| Temporal                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | Metrics as above           |                                                                                                                                                                                                                                               |                                                                                   |
| <b>H4:</b> Greater similarity in food webs during the same season (e.g., win19/win20 or sum20/sum21) than when comparing food webs across different seasons.                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                            |                                                                                                                                                                                                                                               |                                                                                   |
| Temporal (isotopic overlap)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | Metrics as above           |                                                                                                                                                                                                                                               |                                                                                   |

Along with each metric name and hypothesis, the spatial and temporal scale, how the metric is measured, and the ecological meaning of the metrics are presented.

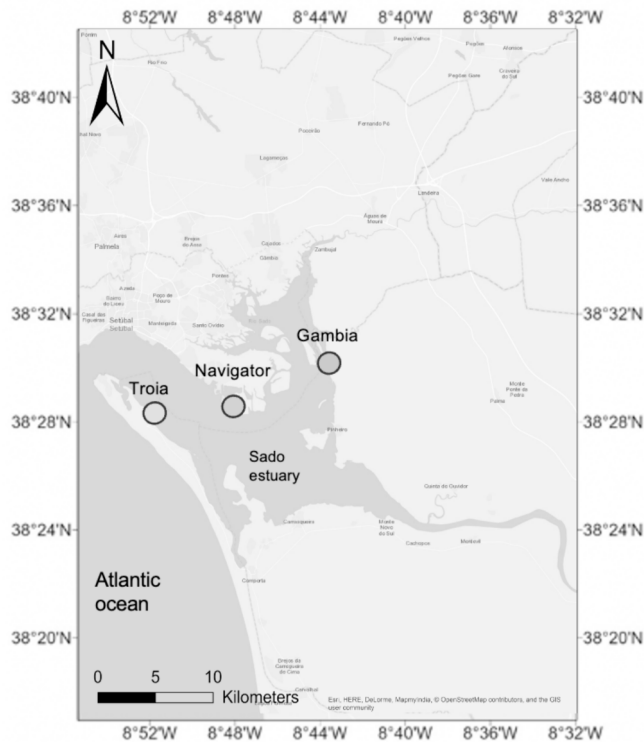
2. Material and methods

2.1. Study area

Sado estuary is the second largest estuarine system in Portugal, with an area of approximately 240 km<sup>2</sup>, and is one of the most important wetlands in Europe (Bettencourt et al., 2004) (Fig. 1). The intertidal areas comprise approximately 78 km<sup>2</sup>, of which 30 % are salt marshes and intertidal flats (Caeiro et al., 2005), with semi-diurnal mesotidal regime and tidal amplitude varying between 0.6 m and 1.6 m during spring and neap tides, respectively. Salinity is influenced by the Sado river flow (annual mean of 40 m<sup>3</sup>s<sup>-1</sup>) changing with seasonal and inter-annual conditions and temperature can range from 10 to 26 °C (Bettencourt et al., 2004). The Sado Estuary (SW coast, Portugal) comprises large adjacent urban and heavy industrial areas with many polluting activities, although parts of the Estuary are environmentally protected, reflecting the importance of ecological conservation and biomonitoring (Vieira et al., 2022).

2.2. Sampling design

The sampling sites were selected based on the expected differences in biogeochemical and trophic conditions of the sediments according to water hydrodynamics within an estuary (high/low water residence time), salinity gradient and the type of neighboring anthropogenic activities (Caeiro et al., 2005; Sroczynska et al., 2021a). Based on these



**Fig.1.** Sado estuary located at southwest of Portugal (38° 31' 14" N, 8° 53' 32" W). The selected sampling sites: Navigator (38.486502, -8.795191), highly industrialized area; Gambia (38.537263, -8.742584) with high organic inputs from aquacultures; Tróia (38.461421, -8.857838) located at mouth of estuary.

criteria three sampling sites were proposed: (1) Navigator is located in the proximity of an industrial area, dominated by fine sand, clay and high organic contents (Vieira et al., 2023); (2) Gambia is located within the borders of the Sado Nature Reserve, affected by the surrounding aquaculture activities with the predominance of clay-fine sediments (Brito et al., 2023); (3) Tróia is located close to the estuary mouth, directly exposed to hydrodynamic forces, that bring well oxygenated water, and with predominance of sandy sediments (Sroczynska et al., 2021aa). All three sites were sampled during four campaigns: winter 2019 (win19), summer 2020 (sum20), winter 2020 (win20) and summer 2021 (sum21).

### 2.3. Sediment biogeochemistry and environmental parameters

Sediment samples were collected by core (141 cm<sup>2</sup>) to the depth of 10 cm and were stored at -20 °C until further analysis. Total organic matter (OM<sub>per</sub>) (%), grain size (%), elemental Carbon (C<sub>total\_per</sub>) and elemental Nitrogen (N<sub>total\_per</sub>) was determined as described in Vieira et al., (2023) according to Costa et al., (2011) and Teixeira et al., (2020). Chlorophyll *a* (Chla<sub>mg\_g</sub>) and phaeopigments (Phaeo<sub>mg\_g</sub>) were determined as described by Lorenzen (1967). Approximately 0.5 g of sediment samples were extracted with 3 mL of ice-cold spectrophotometric grade of 90 % (v/v) acetone. Samples for extraction were placed in an ultrasound bath for 24 h at -20 °C in the dark. After that period, samples were centrifuged for 15 min, at 4.000 rpm and at 4 °C, and the supernatant was used for the analysis. Concentration values for Phaeo<sub>mg\_g</sub> was obtained after acidification of the supernatant with 0.5 M Hydrochloric acid (HCl).

The concentration of 14 elements (Li, Sr, Mn, Ni, Cr, Be, U, Ba, Co, Cu, Zn, As, Pb and Hg) were quantified from the sediment samples collected at each site (mg/kg). Mercury was quantified by thermal

pyrolysis atomic absorption analysis (LECO 254 Advanced Mercury Analyser, AMA), described by Costley et al. (2000). Whereas the elements Li, Sr, Mn, Ni, Cr, Be, U, Ba, Co, Cu, Zn, Pb and metalloid As were analyzed applying the procedure described in Catry et al., (2021). The metals were quantified by Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) (Perkin Elmer NEXION 2000C) after the total acid decomposition of organic debris, carried out in a closed Teflon vessel microwave assisted system (CEM MARS 5). The quality control of this procedure was assured using procedural blanks, duplicate samples (coefficient of variation < 10 %), and the analysis of the MESS-4 CRM, which were prepared using the same analytical procedure and reagents.

At each sampling site, sediment interstitial water parameters were measured such as salinity (Sal), Oxygen (O<sub>2</sub>) (mg/L), pH and Temperature (T) (°C) using a VWR pHenomenal® MU 600H.

### 2.4. Sample collection for stable isotope analysis

At each sampling site, basal food sources and macrobenthic organisms were sampled during a period of low tide. The sampling was performed in win19 (November), sum20 (June); win20 (December) and sum21 (May).

Basal food sources such as macroalgae, seagrass and marine plants were randomly hand-picked including entire sampling area. Samples for microphytobenthos (MPB) were collected at three randomly chosen locations (within each site). It was collected approximately 2000 mL of sediment from the first sediment layer (~1–2 cm), to ensure enough concentration of microphytobenthic cells for isotopic analysis. Samples for Particulate Organic Matter (POM) were collected from small pools, when the pools were not available the water was sampled directly from the adjacent channel.

Macrofauna was collected: 1) quantitatively with a core (141 cm<sup>2</sup>) to 30 cm depth and sieved over a 1 mm mesh at three random locations within each site, and 2) qualitatively by hand picking the organisms from the sampling area. For quantitative sampling, to collect representative number of individuals, two replicates were used at each location, with a total of 6 cores per site. Macrobenthic taxa that were hand-picked were chosen to account for a wide spectrum of functional groups and trophic positions. Both types of samples (quantitative and qualitative) were further transported to the lab in the cold containers for further analysis.

### 2.5. Sample processing in the lab

Samples of hand-picked fresh macroalgae, seagrass and plants were gently washed, separated, identified and dried in the oven for 48 h at 60 °C. The epipellic fraction of MPB was collected via migration through the lens tissue method (Eaton & Moss, 1966). For the POM analysis, 1.5 L of seawater was filtered over pre-combusted Whatman GF/F filters and oven dried for 48 h at 60 °C.

The sediment samples, collected for the quantitative approach, were washed with sea water and sieved. Each organism that was picked, rinsed with sea water and identified to the lowest possible taxonomic level according to specialized monographs and literature (e.g., Fauvel, 1927; Hayward & Ryland, 2017). The online database World Register of Marine Species (<http://www.marine-species.org>) was used to further check the validity of species names. After identification, all macrofauna organisms were incubated for 4–5 h in filtered habitat water to allow gut clearance, further were frozen in liquid nitrogen and placed in the oven to dry for 48 h at 60 °C. For larger individuals, the muscle tissue was used for isotopic analysis, while for the smaller individuals, the entire body was used. Before weighing samples into pre-combusted tin cups (8 x 5 mm. Elemental Micro-analysis Ltd.), all samples (food sources, MPB and macrobenthos) were grounded to a homogeneous powder, while the filters containing POM were gently scraped directly into the cups.



## 2.6. Stable isotope analysis

Around 1–2 mg of the pre-weighed samples were combusted into CO<sub>2</sub> and N<sub>2</sub> in an elemental analyzer (EA, Flash 2000HT, Thermo Fisher Scientific), which provided carbon and nitrogen contents (%C and %N). Isotopic ratios of carbon and nitrogen ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) were obtained on an isotope ratio mass spectrometer (IRMS, Delta V Advantage, Thermo Fisher Scientific) coupled to the EA via ConFlo IV interface. The raw data was normalized by three-point calibrations for C isotopes, using international reference materials IAEA-CH6 (sucrose,  $\delta^{13}\text{C}$ : −10.45 ‰), IAEA-600 (caffeine,  $\delta^{13}\text{C}$ : −27.77 ‰), as well as EEZ-20 ( $\delta^{13}\text{C}$ : −12.3 ‰), and a three-point calibration for N isotopes using IAEA-N-1 (ammonium sulfate,  $\delta^{15}\text{N}$ : +0.43 ‰), IAEA-600 (caffeine,  $\delta^{15}\text{N}$ : +1‰), and IAEA-N-2 (ammonium sulfate,  $\delta^{15}\text{N}$ : +20.3 ‰) composition. Calibrated in-house standards L-alanine ( $\delta^{13}\text{C}$ : −18.39 ± 0.16 ‰;  $\delta^{15}\text{N}$ : +0.91 ± 0.18 ‰) was used as check standard. Two-point calibration was used for C isotopes, involving either Rice flour IRF01 ( $\delta^{13}\text{C}$ : −27.44 ‰) or casein ( $\delta^{13}\text{C}$ : −20.81 ‰) with glucose ( $\delta^{13}\text{C}$ : −10.96 ‰), and Rice flour IRF01 ( $\delta^{15}\text{N}$ : 4.32 ‰) or casein ( $\delta^{15}\text{N}$ : 5.6 ‰) with IAEA-600 (caffeine,  $\delta^{15}\text{N}$ : +1‰). The standard uncertainty calculator provided in Szpak et al., (2017) was used to calculate precision ( $u(\text{Rw}) = 0.24\%$  [AAFM2] for  $\delta^{13}\text{C}$  and 0.27 ‰ for  $\delta^{15}\text{N}$ ) and accuracy ( $u(\text{bias}) = 0.22\%$  for  $\delta^{13}\text{C}$  and 0.33 ‰ for  $\delta^{15}\text{N}$ ) combining all data. Total analytical uncertainty ( $u_c$ ) was estimated to be 0.32 ‰ for  $\delta^{13}\text{C}$  and 0.43 ‰ for  $\delta^{15}\text{N}$  (see Table A.1. C and N isotopic signatures of consumers).

## 2.7. Data analysis

### 2.7.1. Isotopic metrics

All isotopic metrics are presented in Table 1. The community-wide metrics:  $\delta^{15}\text{N}$  range (NR),  $\delta^{13}\text{C}$  range (CR) were calculated as the difference between the maximum and minimum value of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  respectively. Total area encompassed by the consumers (TA) was calculated for each site using mean consumer species  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values. *Max.TP* for an individual from each site was calculated according to Winemiller et al., (2007) where:

$$TP_{SI} = \lambda + (\delta^{15}\text{N}_{sc} - \delta^{15}\text{N}_{baseline}) / \Delta n$$

gamma represents the trophic level of the baseline (1 for basal resources),  $\delta^{15}\text{N}_{sc}$  is the nitrogen isotope signature of the consumer being evaluated and  $\delta^{15}\text{N}_{baseline}$  is the mean nitrogen isotope signature of basal resources (POM, MPB, algae and aquatic plants). For  $\Delta n$ , which is the trophic level enrichment of  $\delta^{15}\text{N}$  value, the value 2.3 ‰ was used (Zanden and Rasmussen, 2001). The isotopic metrics (*Iric*, *Ieve*, *IUni*, *Idiv*) and overlap metrics (*Ines* and *Isim*) were calculated according to Cucherousset and Villéger (2015). Prior to metric calculations, mean raw isotopic values for each  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  were corrected by a community centroid approach to have the same range (0–1) in order to correct for the natural variability in the isotopic values among sites with different isotopic baselines (Villéger et al., 2008). This was achieved by estimating the spatial values for isotope data by taking the mean distance of macrobenthic taxa from the community mean at each site, following Schmidt et al., (2011). Isotopic richness (*Iric*), isotopic divergence (*Idiv*), isotopic evenness (*Ieve*), isotopic dispersion (*Idis*) and isotopic uniqueness (*IUni*) were measured as described in Table 1. For further description of these metrics, see Cucherousset and Villéger (2015). Overlap metrics (*Isim* and *Ines*) were derived from functional ecology that are based on the volume of the intersection between two convex hulls (Villéger et al., 2011, 2013). Isotopic similarity (*Isim*) is the ratio between the volume of the intersection and the volume of the union of the two groups of organisms in the stable isotope space (Villéger et al., 2011). *Ines* is the complementary metric to *Isim*, which is the ratio between the volume of the intersection and the minimal volume filled by a group. The index of *omniv\_car* was calculated for each site separately as the % of all of the consumers classified as carnivores and

omnivores (according to Fauvel, (1927); Hayward & Ryland, (2017)) relative to all of the trophic groups present.

### 2.7.2. Multivariate and univariate analysis

Principal component analysis (PCA) was applied to a dataset with all variables from Table A.2, appendix A and isotopic diversity metrics (*CR*, *NR*, *TA*, *Iric*, *Max.TP*, *Idiv*, *Idis*, *IUni*, *Ieve*, *car\_perc* and *omniv\_car\_perc*) (Table 2). Some environmental and biogeochemical variables (except for pH) were log10 transformed (all metals, *Chla\_mg\_g*, *Phaeo\_mg\_g*, *Chla\_Phaeo*, *Temp*, *Sal*, *O<sub>2</sub>*); variables that were expressed as % (% of clay, % of sand, % of gravel, % of OM, % of total carbon, % of total nitrogen, % of CaCO<sub>3</sub>), were transformed using arcsine square root transformation. PCA was first done including all the transformed variables; afterwards, the variables that had the lowest contribution to the PCA axes, as well as those that had high (>0.9) correlation with other variables were removed. The variables removed were: (*N\_total\_per*, *Phaeo\_mg\_g*, *Chla\_phaeo\_ratio*, *Li*, *Sr*, *Mn*, *Ni*, *U*, *Zn*, *As*, *Co*, *Cu*, *T*, *Sal*, *O<sub>2</sub>* and *pH*). To check for the significance of the PCA, a PCA test (R package “PCAtest”, Camargo, (2022)) was applied with the following parameters: number of random permutations: 1000; number of bootstrap replicates to build 95 %-confidence intervals of the observed statistics: 1000; alpha level for statistical tests: 0.5. PCA plot was done using the function “fviz\_pca\_biplot” using R package “FactoMineR” (Lê et al., 2008). After applying a PCAtest, only variables that were correlated more than 0.5 to PCs were considered. The variables considered were: *CR*, *NR*, *TA*, *Max.TP*, *Iric*, *omniv\_car*. PCAtest was applied in the same way and with the same input parameters as done for the biogeochemical and environmental variables.

All the isotopic and trophic diversity metrics were tested between sites and sampling occasions using two-way analysis of variance (ANOVA, fixed factors were “site” with three levels and “sampling occasion” with four levels). Prior to analyses all the metrics were normalized and checked for the normality and homogeneity of the variance using Shapiro-Wilk normality test and Levene’s Test for Homogeneity of Variance (center = median,  $p < 0.05$ ). The Tukey’s test was performed to the metrics that obtained significant differences, considering 5 % significance. Analysis was performed in R studio using the “Vegan” package, function “aov” (Chambers et al., 1992).

## 3. Results

### 3.1. Biogeochemical and environmental characteristics of the study sites

The sediment biogeochemistry and the environmental variables measured *in situ* are summarized in Table A.2. The sediment biogeochemical analysis revealed differences between all three sites. Sediment at Navigator was predominantly muddy, characterized by the highest mean values of *Clay\_per* ( $20.9 \pm 1$  to  $35.2 \pm 6.6\%$ ), enriched in *OM\_per* ( $2.1 \pm 0.1$  to  $3.9 \pm 0.6\%$ ) and *C\_total\_per* ( $0.9 \pm 0.03$  to  $1.1 \pm 0.1\%$ ). Navigator had also the highest content of metals. Sediment in Gambia was mostly characterized by the highest proportion of *Gravel\_per* ( $14.9 \pm 5.6$  to  $16.9 \pm 2.1\%$ ) and intermediate values of *OM\_per* ( $0.6 \pm 0.04$  to  $1.7 \pm 0.3$ ). In contrast, predominance of *Sand\_per* and *CaCO<sub>3</sub>\_per* ( $91.6 \pm 2.8$  to  $97.3 \pm 0.1$  and  $3.5 \pm 0.3$  to  $4.6 \pm 0.9\%$ , respectively) as well as reduced contents of *Clay\_per* and *OM\_per* ( $1.5 \pm 0.5$  to  $2.9 \pm 0.4$  and  $0.4 \pm 0.02$  to  $1.9 \pm 0.3\%$ , respectively) were found in Tróia sediments. Navigator and Gambia had high content of *Chla*, whereas Tróia sediments had the highest values of *Chla\_phaeo* representing the proxy for the freshness and quality of the phytodetrital organic matter. Oxygen (*O<sub>2</sub>*) that was measured at each site showed the lowest values in Navigator and Gambia varying between  $5.3 \pm 0.6$  to  $9.6 \pm 0.4$  and  $8.8 \pm 0.4$  to  $17.9 \pm 0.4$  mg/L, respectively, while Tróia registered the highest values varying between  $8.6 \pm 0.2$  and  $13.9 \pm 0.3$  mg/L.

PCA analysis clearly differentiated all three sites demonstrating clear differences in biogeochemical sediment condition between sites. Both axes were significant according to PCAtest (1000 bootstrap replicates,

**Table 2**

Food web isotopic and diversity metrics calculated for each community sampled in three sampling sites (Navigator, Gambia and Tróia) of Sado Estuary across 4 sampling occasions (win19, sum20, win20 and sum21).

| Sampling occasion | Site      | Isotopic and diversity metrics |      |       |        |      |      |      |      |      |          |           |
|-------------------|-----------|--------------------------------|------|-------|--------|------|------|------|------|------|----------|-----------|
|                   |           | CR                             | NR   | TA    | Max TP | Iric | Idiv | IDis | IEve | IUni | car_perc | omniv_car |
| win19             | Navigator | 7.10                           | 4.00 | 17.84 | 1.08   | 0.29 | 0.73 | 0.57 | 0.63 | 0.24 | 0        | 65.00     |
|                   | Gambia    | 5.90                           | 2.50 | 10.68 | 1.98   | 0.17 | 0.80 | 0.47 | 0.69 | 0.32 | 0        | 75.00     |
|                   | Tróia     | 6.90                           | 6.80 | 20.45 | 2.82   | 0.33 | 0.69 | 0.42 | 0.71 | 0.28 | 33.00    | 50.00     |
| sum20             | Navigator | 7.10                           | 4.80 | 18.90 | 3.13   | 0.22 | 0.73 | 0.52 | 0.61 | 0.24 | 18.00    | 71.00     |
|                   | Gambia    | 7.40                           | 5.50 | 31.38 | 2.99   | 0.37 | 0.73 | 0.58 | 0.75 | 0.21 | 24.00    | 67.00     |
|                   | Tróia     | 10.30                          | 5.70 | 35.94 | 3.17   | 0.42 | 0.71 | 0.46 | 0.68 | 0.18 | 9.00     | 39.00     |
| win20             | Navigator | 6.80                           | 3.00 | 14.15 | 2.30   | 0.10 | 0.80 | 0.47 | 0.69 | 0.29 | 19.00    | 38.00     |
|                   | Gambia    | 5.50                           | 4.20 | 36.46 | 2.56   | 0.26 | 0.74 | 0.63 | 0.63 | 0.30 | 18.00    | 32.00     |
|                   | Tróia     | 14.20                          | 6.30 | 66.21 | 3.39   | 0.47 | 0.68 | 0.43 | 0.62 | 0.23 | 9.00     | 27.00     |
| sum21             | Navigator | 11.50                          | 5.50 | 35.15 | 3.30   | 0.30 | 0.71 | 0.62 | 0.75 | 0.62 | 38.00    | 57.00     |
|                   | Gambia    | 8.80                           | 4.60 | 28.78 | 3.04   | 0.24 | 0.81 | 0.63 | 0.73 | 0.45 | 33.3     | 47.60     |
|                   | Tróia     | 9.60                           | 3.50 | 24.80 | 4.08   | 0.21 | 0.76 | 0.49 | 0.65 | 0.24 | 8.30     | 33.30     |

Isotopic and diversity metrics: Carbon Range (CR); Nitrogen Range (NR); Total Area (TA); Maximum Trophic Position (*Max TP*); Isotopic Richness (*Iric*); Isotopic divergence (*Idiv*), Isotopic dispersion (*IDis*), Isotopic evenness (*IEve*), Isotopic uniqueness (*IUni*), Carnivorous percentage (*car\_perc*) and omnivores and carnivorous ratio (*omniv\_car*).

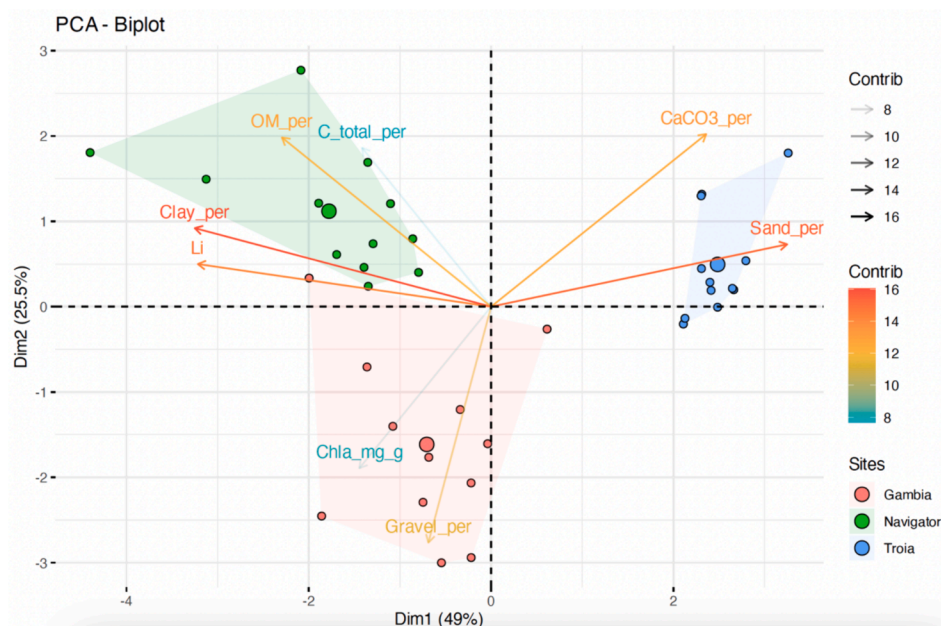
1000 random permutations). The first PC accounted for 49.0 % (95 %-CI:43.9–55.7) of the total variation. The second PC axis accounted for 25.5 % (95 %-CI:20.7–32.8) of the total variation and all together both PC axes explained 74.5 % of the variation observed (Fig. 2). All of the 8 variables had significant loadings on either first (Clay\_per, Sand\_per, OM\_per, CaCO<sub>3</sub>\_per, Li, Chla\_mg\_g) or second (Gravel\_per, C\_total\_per) PC axes. The contribution of these variables for each site was: Clay\_per (−0.94 with PC1) and OM\_per (−0.66 with PC1) in Navigator, Gravel\_per (−0.80 with PC2) and Chla\_mg\_g (−0.54 with PC2) in Gambia and Sand\_per (0.94 with PC1) and CaCO<sub>3</sub> (0.68 with PC1) in Tróia (numbers in parentheses represent correlations of empirical PCs with variables).

### 3.2. Spatial variation of the benthic food web structure

The convex hull biplots clearly reveal distinct isotopic niches between Navigator, Gambia, and Tróia. Navigator and Gambia presented smaller trophic niches and chain lengths compared to Tróia (Fig. 3 and Fig. A.1, appendix A). This pattern was supported by the metrics CR, NR,

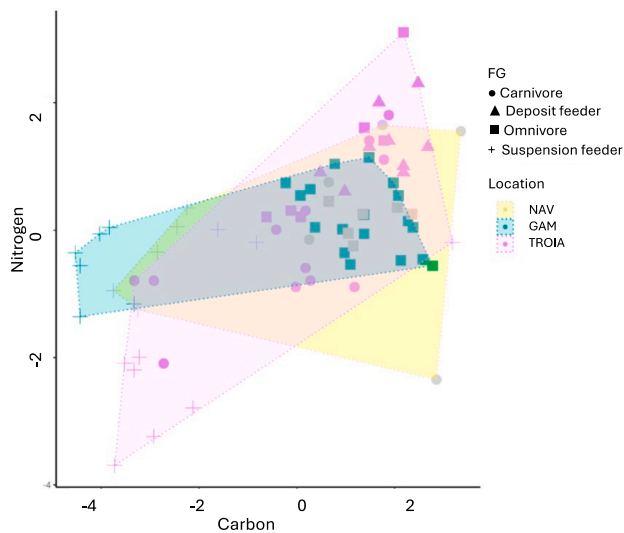
TA, *Max TP* and *Iric* that reached the highest values for Tróia in three sampling occasions (win19, win 20 and sum 20) (Table 2). Besides Navigator and Gambia had fewer consumers (mostly suspension feeders and omnivores) (Fig. 3, winter 19 and summer 20), they occupied distinct edges of the isotopic space, which was reflected in an increased isotopic divergence (*Idiv*) and isotopic dispersion (*IDis*). High proportion of omnivores also contributed to high observed *omniv\_car* ratio (Table 2).

In contrast, Tróia had a larger number of consumers occupying a broader isotopic space, mainly deposit feeders and omnivores (Fig. 3 and Fig. A.1, appendix A), that contributed to higher *Max TP* and *Iric* values (except at sum21) (Table 2). Large number of consumers occupying similar trophic levels in Tróia increased redundancy in Tróia's food web structure (decreased values of *IUni*, Table 2). Additionally, biplots highlighted that Tróia food web was characterized by the presence of secondary consumers and the high number of top predators (Fig. 3, Fig. A.1, appendix A), represented by the highest values of *Max TP* in comparison to remaining sites (Table 2). Nevertheless, the only significant differences in *Max TP* were detected between Navigator and Tróia

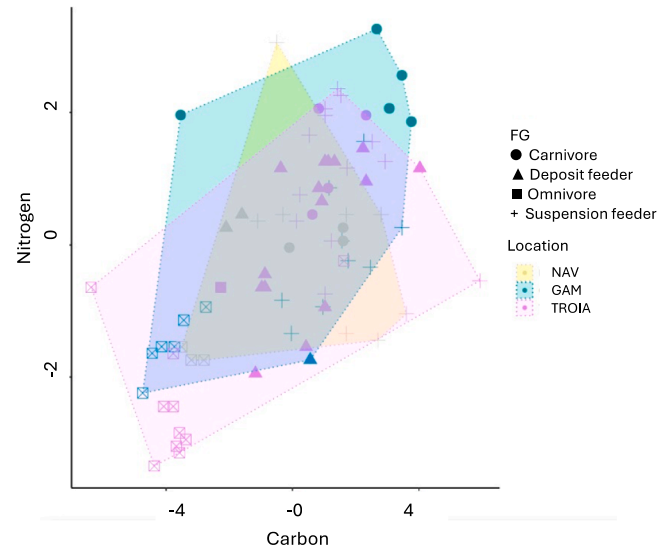


**Fig. 2.** Principal component analysis (PCA) biplot based on scaled environmental and biogeochemical variables measured at three study sites in Sado estuary, coloured by estuary “confidence” convex type. Variable’s vectors are presented based on their contributions to the principal components (gradient colours and transparency of vectors) with red representing high contributions, yellow intermediate and blue representing very low contributions. The dots represent the cluster centroids for group variables. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

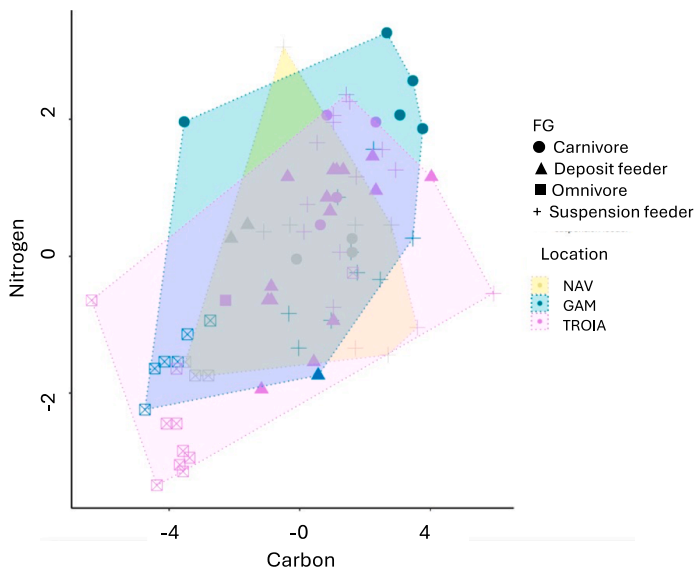
Winter 19



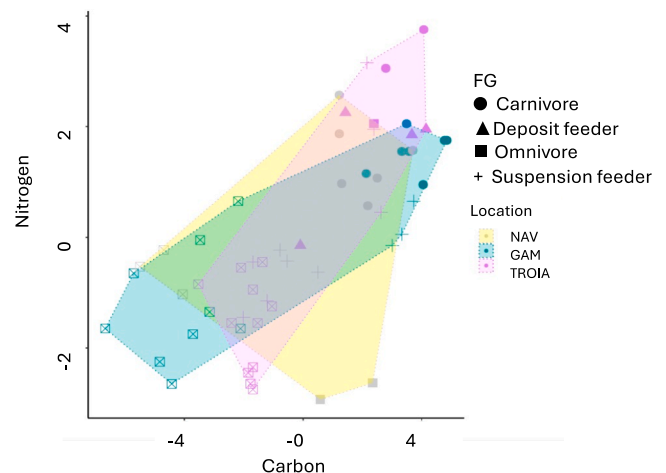
Summer 20



Winter 20



Summer 21



**Fig. 3.** Plots with all sites pooled per sampling occasions (winter 2019 and 2020 and summer 2020 and 2021). In the x-axis and y-axis are the isotopic signatures of carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ), respectively. The convex hull volume represented by the yellow, green, and purple areas, correspond to Navigator, Gambia and Tróia, respectively. The Feeding guilds (FG) are represented by geometric shapes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

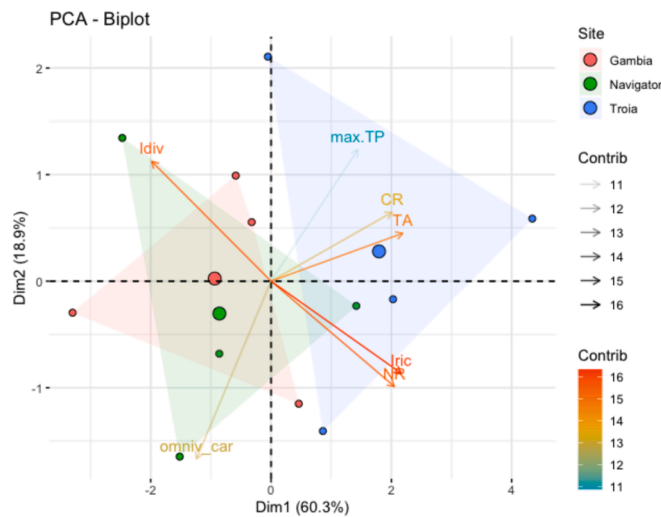
( $p = 0.037$ ), but not Tróia and Gambia as shown in [appendix A, Table A.3](#). The abundance of top predators in Tróia exceeded that observed in Navigator and Gambia in win19, revealing high complexity of Tróia's food web structures as indicated by high observed isotopic evenness and isotopic richness ([Table 2](#)).

PCA based on food web metrics demonstrated a clear separation, along the first PC axis, of Tróia food web from those of Gambia and Navigator ([Fig. 4](#)). According to PCAtest only the first PC axis was significant accounting for 60.3 % (95 %-CI:40.9–79.6) of the total variation. Besides the second PC axis did not appear to be significant it accounted for another 18.9 % of the variation. The main variables that significantly contributed to this separation were *CR*, *NR*, *TA*, *Iric* and *Idiv* that had significant loadings on the first PC axis. Tróia's food web was distinct from the remaining two sites by having higher correlation values for *NR* (0.83), *Iric* (0.88), *TA* (0.89), *CR* (0.82) and *Max.TP* (0.59).

Whereas *Idiv* (−0.81) and *omniv\_car* (−0.51) were associated to Navigator and Gambiás food webs as indicated by the respective correlations with the first PC axes (in brackets). It is worth to note that only *NR*, *TA* and *Iric* had significant correlations with the first PC axis, according to PCAtest.

Regarding isotopic overlap, Navigator exhibited the most distinct food web structure, compared to Gambia and Tróia, registering the lowest similarity (*Isim*) values (varying between 0.007 and 0.329) when compared with other two sites ([Fig. 5](#)). However, Gambia and Tróia presented the highest *Isim* (varying between 0.18 and 0.551), between each other, consistently for all of the sampling occasions ([Fig. 5](#)). The highest similarity between Gambia and Tróia was observed in win20, as reflected in both *Ines* and *Isim* metrics indicating that a large part of the Gambiás food web overlaps with that of Tróia ([Fig. 5 iii](#)).





**Fig. 4.** Principal component analysis (PCA) biplot based on scaled metrics (*CR*, *NR*, *TA*, *Iric*, *Idiv* and *Omniv\_car*) used to characterize the food web structure analyzed at three study sites (Navigator, Gambia and Tróia in Sado estuary, coloured by estuary “confidence” convex type. Variable’s vectors are presented based on their contributions to the principal components (gradient colors and transparency of vectors) with gray representing high contributions, yellow intermediate and blue representing very low contributions. The dots represent the cluster centroids for group variables. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.3. Temporal variation of the benthic food web structure

Throughout the seasons, the convex hulls of Tróia and Gambia were consistently more similar than that of Navigator (Fig. 6). Navigator vs Gambia presented the smallest overlap between each other in all of the sampling occasions with the lowest observed values of *Isim* and *Ines*. These divergences become even more evident in win19 and sum21, suggesting a distinct resource use composition in each of these food webs (Fig. 6). Tróia’s trophic niche was the least variable along the seasons, consistently registering the highest values of *Max.TP*, isotopic redundancy and trophic diversity.

In winter of 2019, Gambia and Navigator food webs exhibited similar trophic guild compositions, predominantly consisting of omnivorous and suspension feeders. In subsequent seasons, its diversity increased with the presence of more predators and other type of consumers, such as deposit feeders, herbivores and carnivores (Fig. 3). Despite these variations, the presence of omnivores and suspension feeders in the food web was consistently maintained throughout the seasons. The flattening structure of the food web is a result of high isotopic dispersion combined with low values of *Max.TP*, *Iric* and high values of *Idiv* throughout the seasons.

Concerning the convex hulls overlap, Navigator and Gambiás food webs revealed a similar pattern by presenting the highest similarity between win19 vs win20 and win20 vs sum20, while the lowest similarity was registered in win19 vs sum21 (Fig. 6a and Fig. 6b). Along the analyzed sampling occasions, an increase in the isotopic space and complexity at both sites was observed, with the appearance of other consumers belonging to distinct trophic groups (e.g., herbivores and deposit feeders). This increase was also reflected in the increase in Omnivore and Carnivore ratio (*omniv\_car*)  $p = 0.00492$ , whose Tukey’s test reported significant differences ( $p < 0.05$ ) between the win19 vs win20 and win20 vs sum20 levels (Table A.3).

Tróia’s food webs throughout the sampling occasions were the least variable, registering consistently the highest values of *Max.TP*, high isotopic redundancy and trophic diversity. This was also evident in very high nestedness values for Tróia food webs between sampling occasions

(89.5–96.3 %, Fig. 6c). Nevertheless, a seasonal pattern was observed in Tróia food web, indicating differences in food web structure between summer and winter seasons (Fig. 6c).

PCA based on isotopic and diversity metrics demonstrated a partial seasonal separation of win19 and sum20 in the first axis (Fig. 7). Metrics that contributed to this separation were *NR* and *Iric*, associated with sum20, and *omniv\_car* that was positively associated with win19. There was a clear annual pattern along the second axes that separated win19 and sum20 from win20 and sum21. The metrics responsible for this differentiation were *TA*, *Max.TP*, *CR* and *Idiv* that were associated with win20 and sum21, while *NR*, *Iric* and *omniv\_car* were associated with win19 and sum20. This separation could be associated with the general increase of the isotopic niches and isotopic diversity metrics in Navigator and Gambia (by hosting more predatory consumers) in win20 and sum21. Nevertheless, according to PCAtest, the second PC axis was not significant indicating that temporal effect, especially the one associated to interannual variation is less strong than spatial pattern that was observed between Tróia and remaining two sites.

## 4. Discussion

### 4.1. Spatial variation of the benthic food web structure

Isotopic and diversity metrics, coupled with multivariate analysis, were useful in discriminating spatial patterns in benthic food webs. In accordance with our first hypothesis (Table 1), we found smaller trophic niches and chain lengths at Navigator and Gambia, as indicated by the respective metrics, compared to Tróia. Navigator and Gambia are both located in the inner estuary, where limited oxygen exchange and site-specific anthropogenic activities (e.g., aquaculture activities, paper factory respectively) are directly responsible for the observed organic enrichment. The simpler food webs in Navigator and Gambia are corroborated by the very low diversity of predatory and omnivory macrofauna consumers found at these two sites compared to Tróia. As demonstrated elsewhere (Dorgham, 2014; Hale et al., 2016) organic enrichment affects food web structure directly through siltation (increase in turbidity), habitat modification and oxygen reduction, leading to the disappearance of sensitive species. Similarly, a direct consequence of the organic load in Navigator and Gambia might have negatively affected the communities of more sensitive taxonomic groups, such as echinoderms, sponges and other vulnerable species. This has resulted in lower taxonomic and trophic diversity found at these two sites compared to the remarkably high benthic diversity in Tróia.

Indirectly an excessive organic enrichment may promote hypoxic conditions, triggering shifts in primary production and benefitting opportunistic species at the base of the trophic level (Zheng et al., 2020). For example, lower quality OM (estimated by *Chla:pheo ratio* values) at Navigator and Gambia was previously demonstrated to be highly influential for meiofauna distributional patterns in Sado estuary (Vieira et al., 2023). This contributed to a high biomass of small opportunistic species and fewer predators at sites with high OM loads (Sroczyńska et al., 2021b). Since many macrofauna species directly feeds on meiofauna, a less diverse meiofauna community previously found at Navigator and Gambia can be directly responsible for the lower observed diversity of intermediate consumers, such as deposit feeding polychaetes (Vafeiadou et al., 2013). This resulted in less benthic omnivores and predators in Navigator and Gambia compared to Tróia. Similar findings were reported for the Baltic Sea, where the authors demonstrated that the available biomass of primary consumers (meiofauna and small macrobenthic consumers) determines the abundance of large omnivores and carnivores (Szczepanek et al., 2021).

In contrast Tróia benefits from the tidal dynamics and higher oxygen exchange between the inner estuary and the marine environment, characterized by predominantly sandy sediments with low organic matter, reflecting an environment with high-quality food sources, as evidenced by *Chla:pheo ratio* values. Tróia food web reported high



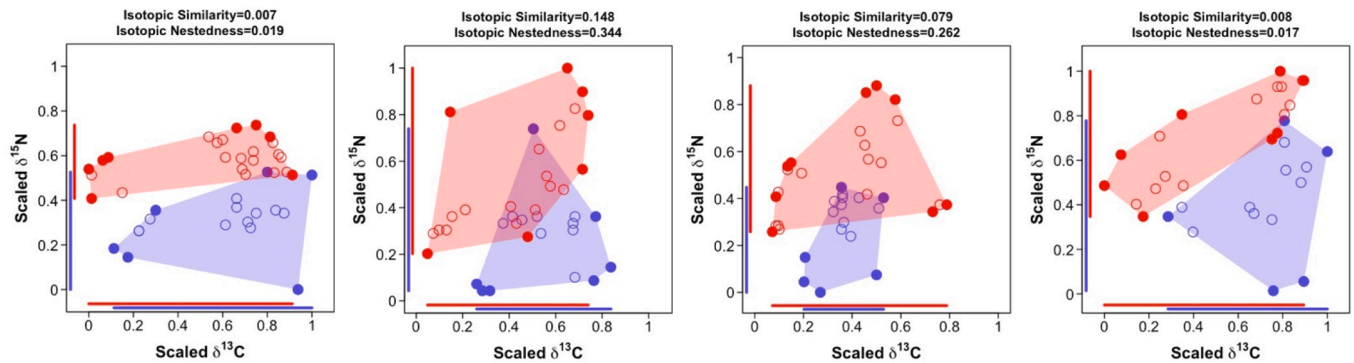
Winter 19

Summer20

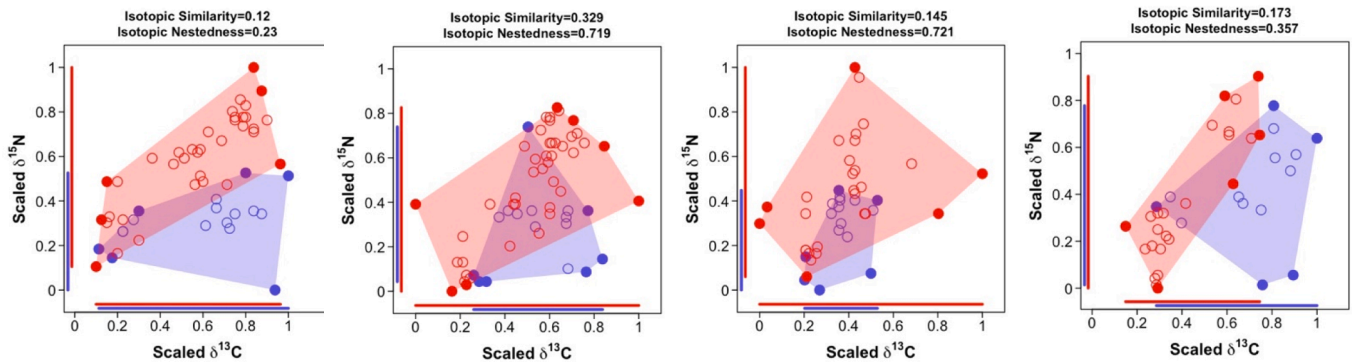
Winter 20

Summer21

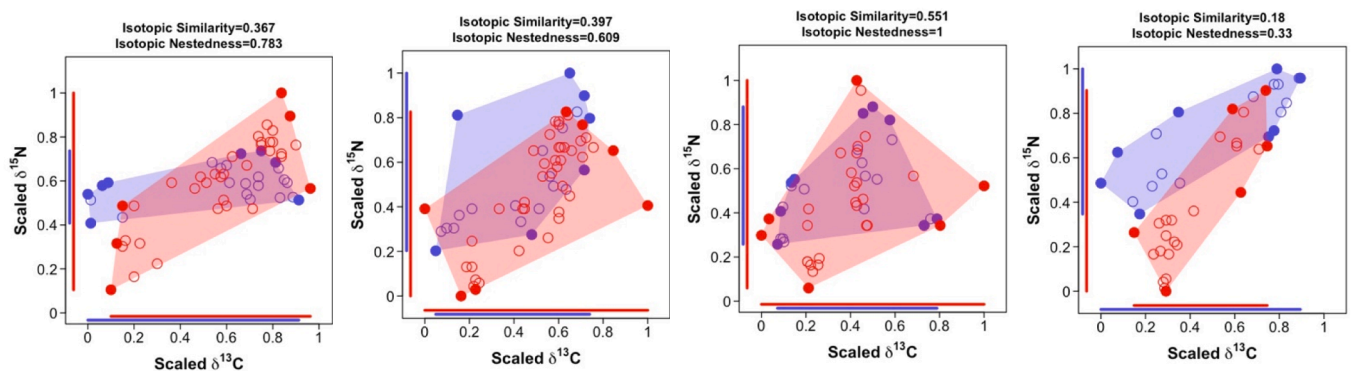
## i) Navigator (blue) vs Gambia (red)



## ii) Navigator (blue) vs Tróia (red)



## iii) Gambia (blue) vs Tróia (red)

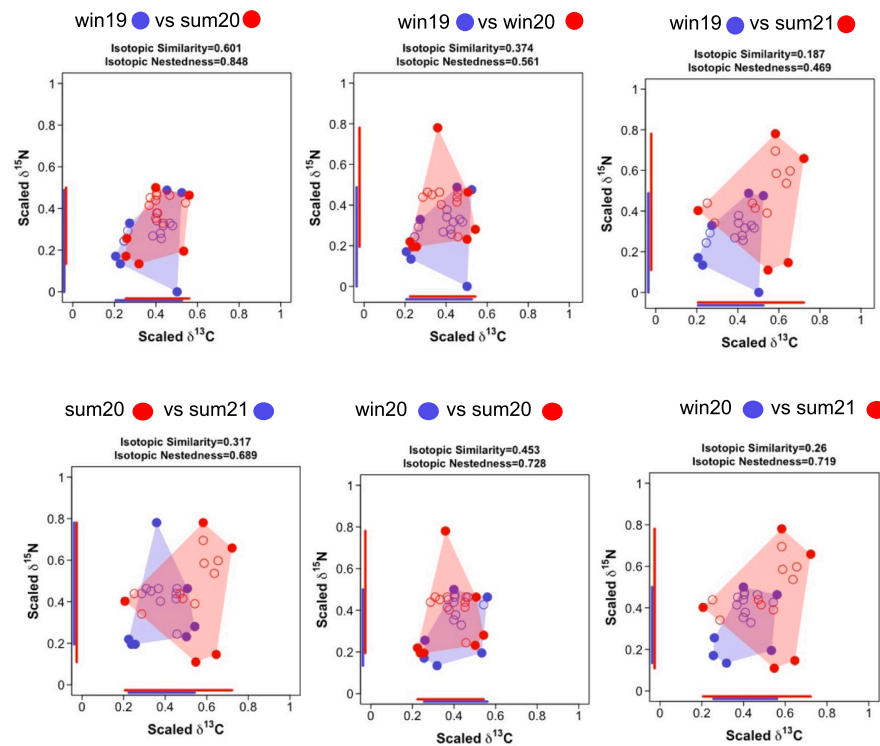


**Fig.5.** Isotopic overlap metrics in a two-dimensional isotopic space ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ), between two sites across the 4 sampling occasions (win19, sum20, win20 and sum21). *i)* Navigator vs Gambia (blue and red, respectively); *ii)* Navigator vs Tróia (blue and red, respectively); *iii)* Gambia vs Tróia (blue and red, respectively). Isotopic overlap metrics were measured using the isotopic richness of the two sites (i.e., convex hull volume represented by the red and blue areas, respectively) and the volume of isotopic space they shared. Isotopic similarity is the ratio between the volume shared (purple area). Isotopic nestedness is the ratio between the volume shared and the volume of the smallest convex hull (in blue). Isotopic overlap on each stable isotope axis is showed by the overlap of the colored segments representing range of scaled values for each site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

number of pathways and interactions, composed of secondary consumers, and a high number of top predators. This higher taxonomic and trophic diversity of benthic consumers in Tróia has led to a broader use of resources by primary consumers, as evidenced by a wider species distribution along the carbon axis.

Despite Navigator and Gambia sharing more similar sediment biogeochemical conditions, we did not observe a higher overlap between them, contrary to our second hypothesis (H2, Fig. 1). In fact, Navigator and Gambia exhibited very low isotopic niche overlap, with the lowest similarity values (*Isim* and *Ines*). Instead, Gambia food web

## a) Navigator



**Fig. 6.** Isotopic overlap metrics in a two-dimensional isotopic space ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ), between seasons: win19 vs sum20 / win20/ sum21; win20 vs sum20/ sum21 and sum20 vs sum21 in each site. **a)** Navigator; **b)** Gambia and **c)** Tróia. Isotopic overlap metrics were measured using the isotopic richness of the two sites (i.e., convex hull volume represented by the red and blue areas, respectively) and the volume of isotopic space they shared (i.e., volume of their intersection, delimited by the purple line). Isotopic similarity is the ratio between the volume shared (purple area) and the volume of the union of the two convex hulls. Isotopic nestedness is the ratio between the volume shared and the volume of the smallest convex hull (in blue). Isotopic overlap on each stable isotope axis is shown by the overlap of the colored segments representing a range of values for each site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

showed greater similarity to Tróia food web. The low similarity and nestedness between Gambia and Navigator were attributed to a better efficiency in the use of resources by the consumers in Gambia (estimated by the high values of chlorophyll *a*, pheopigments and ratio of both, Table S.1; and the high values of *Idisp* and *Iric*, Table 2). This can be attributed to the emergence of new macrobenthic consumers in Gambia, particularly more carnivores and deposit-feeders (since summer 2020), which have used more efficiently the available food resources and, thereby, increased the isotopic space occupied by the consumers.

However, we observed that consumers within the same trophic guilds in Gambia occupied higher positions on the trophic level compared to the same consumer species in Navigator (Fig. 3 and Fig. A.1). This could be explained by the abrupt increase in Chla (5 orders of magnitude), phaeopigments (10 orders of magnitude) and carbon range values from summer 2020 in Gambia (Table S.1), which stimulated primary production and likely triggered shifts in resources, leading to increased energy transfer to higher trophic levels (Zheng et al., 2020). Similar findings were reported for the Polish coastal area, where the authors found that in sites with increased organic input from riverine discharge, the omnivores occupied higher trophic levels, likely due to an increased availability of meiobenthic prey (Szczepek et al., 2021).

In summary, the quantity and quality of OM proved to be an important variable in shaping spatial patterns in benthic food web structure. Moreover, similarity metrics were a valuable complementary tool that helped to further disentangle the causes of spatial variability, in this case distinguishing between two food webs (Navigator and Gambia) that had similar structures but different resource utilization.

#### 4.2. Temporal variation of the benthic food web structure

Summer increase in temperature and photoperiod stimulates primary productivity, increasing secondary and tertiary production, increasing the complexity of the food webs (Humphries et al., 2017). Contrary to our hypothesis (H3, Table 1), no seasonal pattern was observed in food webs concerning primary productivity. The highest Chla and phaeopigments were detected in win20 and sum20, indicating that primary productivity is not directly influenced by the seasonal effects. Instead, various factors potentially contribute to the abundance and diversity of food sources in Sado estuary. As previously demonstrated, increases in organic inputs to the system are not always congruent with the seasonal variations, they can also occur on a microscale (Moens & Beninger, 2018; Young et al., 2021), or may be linked to the spatially heterogeneous nature of the estuary itself (Elliot & Quintino, 2007).

Previously studies demonstrated that spatial differences in sediment conditions, often associated with anthropogenically mediated variations in local OM exert primary control on community distribution in this estuary (Vieira et al., 2023). These spatial differences are more influential for community structure than temporally derived variations in basal resources. Similarly, any seasonal patterns in food web structure are likely masked by more pronounced spatial differences, which are characteristic of each site within the estuary.

We also hypothesized (H4, Table 1) to find more similar food webs at the same season (win19/win20 vs sum20/sum21) than among different seasons. Contrary to our hypothesis, we observed a gradual increase in trophic diversity, particularly with the emergence of herbivores and

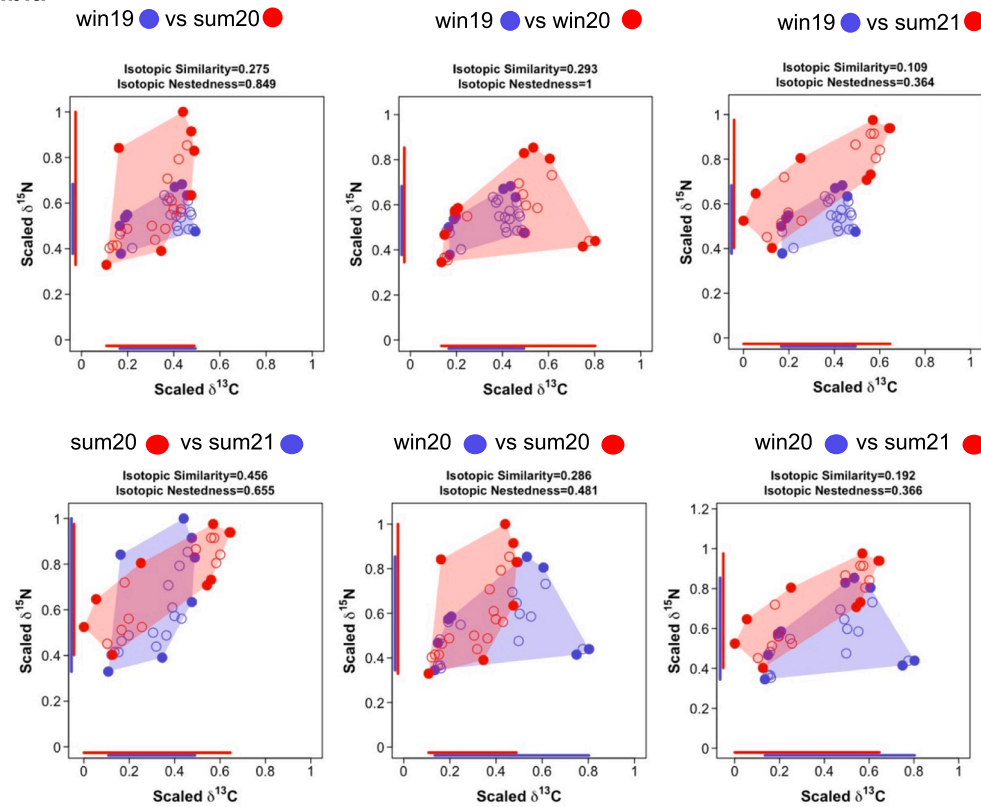
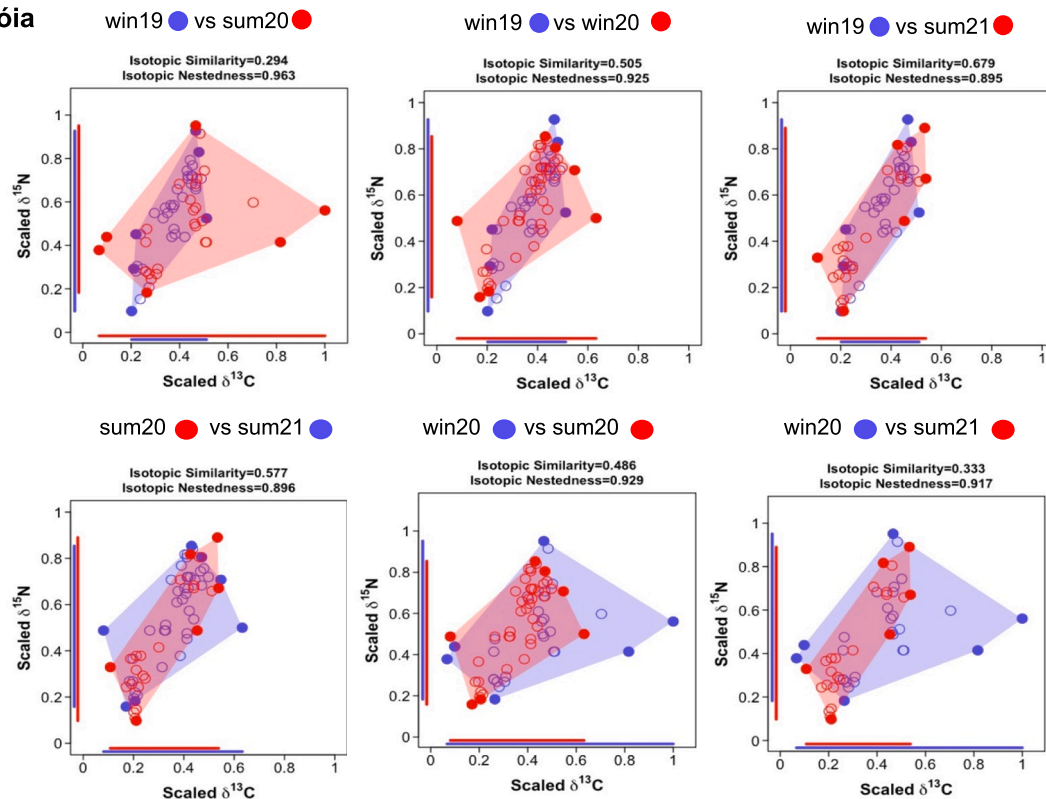
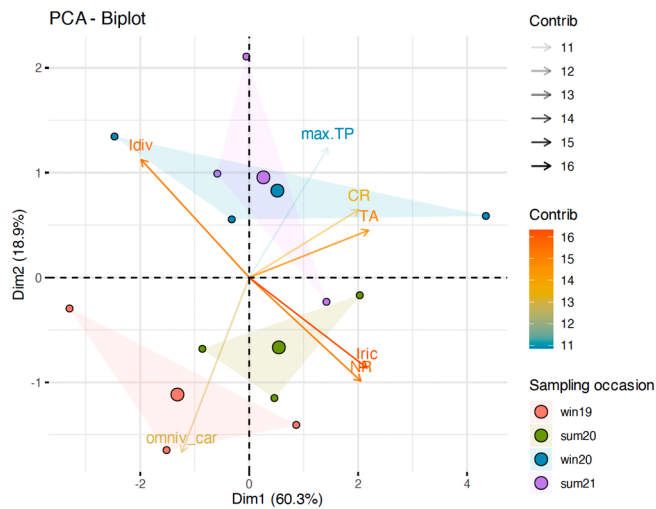
**b) Gambia****c) Tróia**

Fig.6. (continued).

other carnivores, along with a concomitant increase in food web complexity (as indicated by isotopic metrics) in Navigator and Gambia throughout the seasons. Significant differences were found for

maximum trophic position and the percentage of carnivores and omnivores between winter 19 and other seasons, regardless of the site. Seasonal variations were more evident in Tróia's food web, as evidenced by



**Fig. 7.** Principal component analysis (PCA) biplot based on scaled metrics (*CR*, *NR*, *TA*, *Iric*, *Idiv*, *Max.TP* and *Omniv.car*) used to characterize the food web structures analyzed across 4 seasons (win19, sum20, win20 and sum21) in Sado estuary, coloured by estuary “confidence” convex type. Variable’s vectors are presented based on their contributions to the principal components (gradient colours and transparency of vectors) with grey representing high contributions, yellow intermediate and blue representing very low contributions. The dots represent the cluster centroids for group variables. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the lowest observed similarities between seasons (win19 vs sum 20 and win20 vs sum21), reflecting temporal differences in the availability and diversity of food sources. We propose that the more complex food webs in Tróia exhibit better responses to seasonal shifts. These food webs are composed of more specialist consumers, that more efficiently use the available resources, hence, any temporally derived variability in food sources leads to a corresponding shift in isotopic position of these consumers (Ziółkowska & Sokolowski, 2022). In contrast, the food webs in Navigator and Gambia, are composed of consumers with more generalistic behavior and high plasticity, enabling them to quickly adapt to local variations in OM availability (Szczepeanek et al., 2021), which is reflected in more flexible positioning of these consumers in the isotopic space. As a result, temporal shifts associated with resource utilization are more difficult to capture in such webs.

## 5. Concluding remarks

The integration of isotopic metrics complemented with multivariate and univariate analyses proved to be an important tool for the analysis of different aspects of the benthic food web complexity in a spatial-temporal context. Isotopic diversity metrics were useful to examine the structure of the food web, while similarity metrics provided insight into the differences in resource utilization. Tróia food web demonstrated greater responsiveness in capturing temporal differences in resource use, suggesting that more complex food webs are better equipped to reflect temporal variability.

Multivariate analyses were useful in identifying spatial and temporal patterns demonstrating the strong influence of OM on spatial benthic food web discrimination. Meanwhile, univariate analyses revealed significant differences between maximum trophic position and the percentage of carnivores and omnivores between sites, indicating the potential for these metrics to serve as indicators of the ecosystem change in the future.

In summary, we concluded that the combination of different isotopic and diversity metrics coupled with univariate and multivariate analyses is a very promising approach to assess the functional integrity of the

estuarine ecosystems, especially in the context of the descriptor 4 within MSFD. The suggested analysis of benthic food web attributes can be easily applied to any ecosystem or particular type of disturbance, potentially improving the accuracy of assessing GES under D4. Knowing that current indicators under D4 of MSFD are mostly focused on economically important guilds (fish, birds), often overlooking benthic habitats and ecosystem-based processes, this study offers valuable insights for developing new strategies to assess benthic ecosystems.

## Authors’ contributions

The first draft of the manuscript was written by Soraia Vieira, and all authors commented on previous versions of the manuscript and approved the final manuscript. She also performed data analysis. Kasia Sroczyńska contributed to the study conception and design, sample collection, data analysis and the writing of the manuscript. Helena Adão contributed with the scientific idea and the conception design, she participated in field and lab work and in the writing of the manuscript, she got the financial support. Anne-France Maurer and Cristina Barrocas Dias participated in the lab work, analyzing stable isotope data and in the writing of the manuscript. Jorge Lobo-Arteaga and Joana Neves contributed to taxonomic identification of macrofauna. All authors participated in the sampling collections. Sediment analyses were performed by Joana Neves and Marta Martins.

## Ethics in publishing

Not applicable.

## Declaration of generative AI in scientific writing

Not applicable

## CRedit authorship contribution statement

**Soraia Vieira:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Anne-France Maurer:** Writing – review & editing, Data curation. **Cristina Barrocas Dias:** Writing – review & editing, Data curation. **Joana Neves:** Writing – review & editing, Data curation. **Marta Martins:** Writing – review & editing. **Jorge Lobo-Arteaga:** Writing – review & editing, Data curation. **Helena Adão:** Writing – review & editing, Validation, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. **Kasia Sroczyńska:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecolind.2024.112243>.

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