DEPARTAMENTO DE CIÊNCIAS DA VIDA

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The functional composition of the benthic invertebrate community of the Mondego estuary: insights within the functioning of this ecosystem.

Pieter van der Linden

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Acknowledgements

First of all, I would like to say that I immensely enjoyed my stay here in the beautiful country of Portugal, were the sun always shines and the waves are good. During the two years in Portugal, I made many friends and all of you contributed in some way or another to the realization of this thesis, many thanks to you all. Furthermore, I would like to thank some people in particular. My supervisor, João Carlos Marques, who gave me the opportunity to do my thesis' research at IMAR - Institute of Marine Research, accepting me as his student. Gabi, for all your administrate help. My lovely co-supervisor, Joana Patrício who helped me a great deal. Agnese Marchini (University of Pavia, Italy), without you I could not have done it. You taught me about biological traits analysis and gave me the perspective I needed. You put in so much (positive) energy in helping me. Núria Cid, you gave me the inspiration and you helped me a great deal with the analysis. Luísa Marques and Heliana Teixeira, for your help with the species identification. João Franco, Pedro Vinagre and Nuno Leite, for your help during the field sampling. Pedro Vinagre and Tânia Fernandes for your help with the sorting of the samples. And last but not least, I want to thank my family; Wil, Miek, Michel, Mats & Naud, I feel so lucky with you around me! Many thanks. You are all greatly appreciated.

Abstract

The main aim of this study was to investigate the functional composition of the subtidal benthic invertebrate communities of the Mondego estuary and thus contributing to a better understanding of the response of these communities to the pressures within this type of ecosystem.

We characterized the environmental conditions and investigated the spatial and seasonal distribution of the communities by means of traditional taxonomic analysis and BTA. Moreover, we investigated the relation between taxonomic and functional composition by comparing indices of taxonomic and functional diversity.

Traditional taxonomic analysis highlighted spatial and seasonal variations of community composition (*i.e.*, a general reduction of species richness, Shannon-Wiener diversity, and an increase of species density towards the upper parts of the estuary). BTA analysis highlighted the functional characteristics of the benthic community within the estuary and common features across species that have appeared or disappeared among seasons. Furthermore, BTA allowed to investigate interactions among traits (*i.e.*, the strong influence of the trait 'salinity preference') and was used to calculate functional diversity (FD). Indices of taxonomic diversity (Shannon-Wiener index) and FD (RQE index) allowed to explore the relation between taxonomic and functional composition. This relation might potentially have indicated that the community at the upper most reaches of the estuary exhibits lower functional redundancy than the downstream assemblages. This study represents one of the first attempts to investigate the functional composition of the benthic invertebrate community in an estuarine environment by means of BTA.

BTA in addition to traditional taxonomic analysis provided a more comprehensive understanding of the functioning of this ecosystem. Therefore, the inclusion of BTA is highly recommendable for estuarine ecological studies, but more research is needed to improve its effectiveness regarding the following issues: (i) the set of selected biological traits should be optimized towards the inclusion of more relevant ecological information. This objective can be achieved by improving the knowledge on single species biological traits, which is at present still fragmentary; (ii) the biological traits of species should be linked with the services these environments provide. This trait-service association will be a crucial step in ecosystem service monitoring and management.

The present study is submitted to the 'Ecological Indicators' journal.

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Contents

1. Introduction

Human impacts have pushed estuarine ecosystems far from their historical baseline of rich, diverse, and productive ecosystems (Lotze *et al.*, 2006). Centuries of overexploitation, habitat destruction and pollution have increased the rates of species invasions and species extinctions (Loreau *et al.*, 2001; Hooper *et al.*, 2005; Lotze *et al.*, 2006; Worm *et al.*, 2006). These changes in biodiversity have a strong potential to alter the functioning of these ecosystems (Hooper *et al.*, 2005; Lotze *et al.*, 2006; Worm *et al.*, 2006). Ecosystem functioning is a broad term, which includes the processes (*e.g.* nutrient cycling), the services that these processes provide to humanity (*e.g.* fisheries, nursery habitat and filtering capacity), as well as the resilience and resistance of these factors over time or in response to disturbance (Díaz & Cabido, 2001; Bremner 2008).

Ecological experiments, observations and theoretical developments show that ecosystem functioning depend greatly on biodiversity in terms of the "functional characteristics" of organisms present in the ecosystem and on their distribution and abundance over space and time (Diaz & Cabido 2001; Loreau *et al.*, 2001; Hooper *et al.*, 2005; Elliott & Quintino 2007). However, in transitional/estuarine environments, ecosystem functioning has been mostly investigated with "traditional" analysis based on the taxonomic composition of the communities (Mouillot *et al.*, 2006; Elliott & Quintino, 2007).

Functional analysis of benthic invertebrates, in these environments, has been mostly limited to the feeding habits (*e.g.* Fano *et al.*, 2003; Dolbeth *et al.*, 2003; Cardoso *et al.*, 2004; Grilo *et al.*, 2011) and size-structure (*e.g.* Warwick 1984; Basset *et al.*, 2004; Mouillot *et al.*, 2006; Reizopoulou *et al.*, 2007). However, these traits are just

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two biological traits that can indicate some aspects of functioning; other traits, which (also) refer to the life strategy- and behavioral characteristics, have received far less attention, despite addressing other important aspects of functioning.

Biological traits analysis (BTA), which was largely developed in terrestrial and freshwater ecology, is a useful analytical approach to describe different aspects of functioning based on 'multiple' biological traits of aquatic invertebrates (*e.g.* mobility, feeding type, size, life span, and reproductive technique) (Bremner *et al.*, 2003). Similar to traditional taxonomic analysis, which includes taxonomic diversity indices and multivariate methods based on the taxonomic composition, BTA includes functional diversity (FD) indices and multivariate methods based on the functional composition. FD was originally defined by terrestrial ecologists as the value and range of biological traits of the organisms present in a given ecosystem (Díaz & Cabido, 2001). However, FD has been measured in different ways capturing different components of this diversity (Petchey & Gaston, 2002), thus a more general definition of functional diversity was needed embracing these different components (Mouillot *et al.*, 2006). Mason *et al*. (2005) redefined FD as a measure (or group of measures) of the distribution of the species and abundance of a community in functional attribute space that represents: (a) the amount of functional attribute space filled by species in the community (functional richness) (b) the evenness of abundance distribution in filled niche space (functional evenness) and (c) the degree to which abundance distribution in niche space maximizes divergence in functional attributes within the community (functional divergence).

BTA has discriminated the effects of disturbance on biological traits of invertebrates in freshwater ecosystems (*e.g.* Dolédec *et al.*, 1999; Statzner & Bêche, 2010). In

marine environments, BTA has been successfully applied to assess fishing effects on benthic fauna (*e.g.* Bremner *et al*., 2003; Tillin *et al*., 2006) and to investigate the effects of climate change (Neumann & Kröncke, 2010). Furthermore, this approach has been used in Mediterranean lagoons in order to identify the dominant traits in different transitional environments (Marchini *et al*., 2008). BTA was used to assess functional diversity in different species assemblages (*e.g.* Bremner *et al*., 2003; Bady *et al*., 2005; Mermillod-Blondin *et al*., 2005; Schratzberger *et al*., 2007; Mouillot *et al*., 2007; Hewitt *et al*., 2008), as well as for management and conservation purposes (Bremner, 2008; Frid *et al*., 2008). The wide range of traits used by BTA, the strong link between them and ecosystem processes (Díaz & Cabido, 2001), as well as the sound theoretical framework (see Bremner *et al*., 2003 and Statzner & Bêche, 2010) are a considerable advance over traditional methods dealing with ecosystem functioning (Neumann & Kröncke, 2010).

The main aim of this study was to investigate the functional composition of the subtidal benthic invertebrate communities of the Mondego estuary and thus contributing to a better understanding of the response of these communities to the pressures within this type of ecosystem.

We characterized the environmental conditions and investigated the spatial and seasonal distribution of the communities by means of traditional taxonomic analysis and BTA. Moreover, we investigated the relation between taxonomic and functional composition by comparing indices of taxonomic and functional diversity.

2. Material & Methods

2.1 Study area

The Mondego estuary (Fig. 1) is a relatively small warm-temperate polyhaline intertidal system (21 km long and 860 ha surface area) located on the NW coast of Portugal. The last 7 km, near the mouth, consist of two arms separated by Murraceira island.
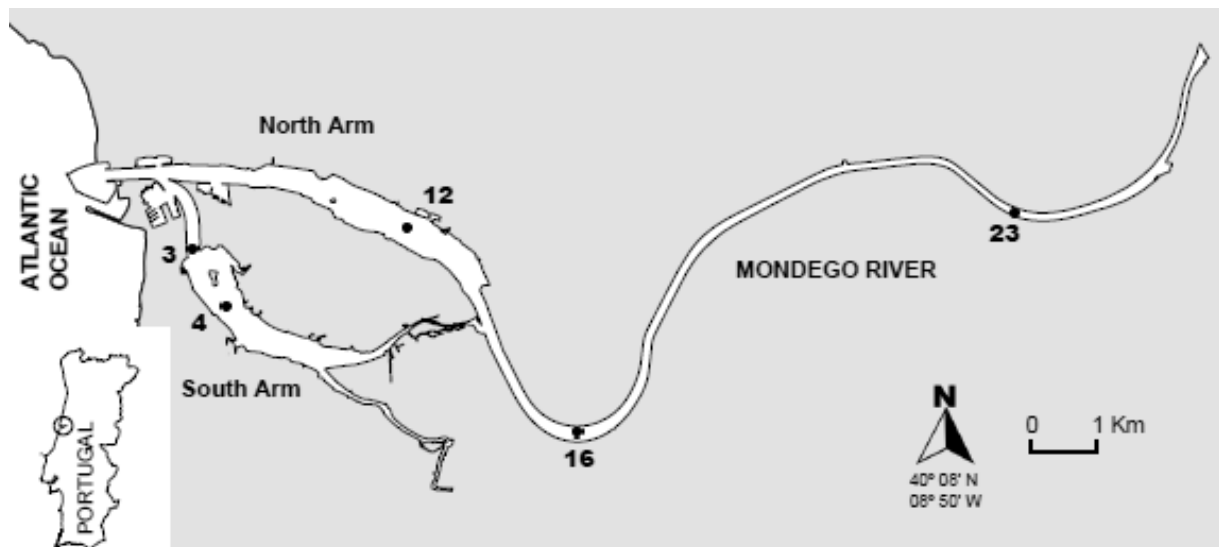
Figure 1: The Mondego estuary (Portugal), with the sampling locations: stations 3, 4, 12, 16 and 23.

The northern arm of the estuary is deeper (4-10 m during high tide) and is the most hydrologically altered; it constitutes the main navigation channel and is the location for the Figueira da Foz harbor. The southern arm is shallower (2-4 m during high tide) and lies within a more 'natural environment'; 75 % of its area are intertidal flats, where in some locations seagrass (*Zostera noltii*) meadows are present. The hydraulic circulation in the South arm depends mostly on tides and on the connection with the North arm. The main human induced pressures in the Mondego estuary are

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the nutrient-loadings coming from agriculture (mainly corn and rice fields), harbor activities, fish farms located on Murraceira island and wastewater coming from Figueira da Foz and other upstream locations (Teixeira *et al*., 2009; Veríssimo *et al*., 2011).

2.2 Data collection

2.2.1 Biological data

Benthic samples were collected at five subtidal stations located in the estuary (Fig. 1), in two different seasons: summer 2009 (September) and winter 2010 (March). The location of stations tried to encompass the variety of benthic communities that inhabit the Mondego estuarine gradient, from the most brackish reaches to marine-like conditions. St 3 and st 4 are located in the South arm of the estuary, st 12 in the North arm and st 16 and st 23 are located further upstream. In each station, three sediment samples (replicates) were taken with a van Veen grab (0.1 m^2). Samples were stored in 4% buffered formalin solution and washed in the laboratory through 0.5, 1 and 2 mm mesh sieves. Afterwards, animals were sorted and preserved in 70% ethanol and, subsequently, identified and counted to the species level when possible, or to the lowest taxonomic level. For each taxon, the number of individuals found in all three samples (replicates) per station was summed and divided by 3, which gave the average amount of individuals per 0.1 m^2 (density) per station. The taxa density in each station was displayed in a numerical matrix (matrix 'taxa by stations'). This was done for both seasons.

2.2.2 Environmental data

During the collection of the sediment samples, environmental variables were measured at the bottom and surface of the water column. Since benthic invertebrates are facing mostly the environmental conditions at the bottom, only the environmental variables at the bottom were considered in this study; depth (m), transparency (m), salinity, temperature (°C), pH, dissolved oxygen (%), chlorophyll *a* (mg m^{-3}), N-NH$_4$ (mg L^{-1}), N-NO$_3$ (mg L^{-1}), N-NO$_2$ (mg L^{-1}), P-PO$_4$ (mg L^{-1}), SiO (mg L^{-1}), TSS (total suspended solids, g L^{-1}), and granulometry data (%) for clay (<0.038 mm), silt (0.038-0.063 mm), fine sand (0.063-0.25 mm), medium sand (0.25-0.5 mm), coarse sand (0.5-2 mm) and gravel (>2 mm).

2.3 Compilation of data matrices

Biological traits analysis (BTA) requires three different numerical matrices: (1) taxa density in each station (matrix 'taxa by stations'); (2) biological traits of the taxa (matrix 'taxa by traits'); and (3) a combination of the previous two, biological traits in each station (matrix 'traits by stations'). In order to see the seasonal effects, these three matrices were built with summer and winter taxa. Data of taxa density in the first matrix was transformed by log (1+x) in order to reduce the influence of dominant taxa on the samples without losing density effects. After choosing the biological traits and the categories to be considered in the analysis, the 'taxa by traits' matrix was compiled by gathering traits data from a variety of published sources; species identification guides (*e.g.* Barnes, 1994), journal papers and online databases (*e.g.* MarLIN BIOTIC, 2011). Each trait was subdivided in different 'categories' that display the organisms' behavior/strategy into more detail (*e.g.* the four considered categories

of the trait 'feeding' for benthic invertebrates were: predator, herbivorous, detritus feeder and filter feeder). These 'taxa by traits' matrices were fulfilled with the affinity of each taxon (*i.e.* species or genus) to the trait categories by using a 'fuzzy coding' approach (Chevenet *et al.*, 1994). An affinity score of '0' indicates no affinity of a taxon to a trait category, whereas a score of '3' indicates a high affinity to the trait category (note that traits score is '0' for all categories if information is not currently available). For example, the polychaete, *Nephtys cirrosa* is solely a 'predator'; therefore, the affinity for this category will be '3'. Another taxon, for example, the crustacean *Carcinus maenas,* could be mostly predating and occasionally feeding on plants, then the affinity scores would be '2' for 'predator' and '1' for 'herbivorous-feeder'. Thus, fuzzy coding procedure allows capturing variation in the affinity of a given taxon to the categories of a given trait, thereby addressing spatial or temporal differences in the traits of a given taxon (Statzner & Bêche, 2010). To give the same weight to each taxon and each biological trait in further analysis, affinity scores are standardized so that their sum for a given taxon and a given trait equals 1 (or 100%). If information on a given trait is currently not available for a taxon, it takes the mean trait profile of all other taxa in subsequent trait analyses (*i.e.* such a taxon does not contribute to potential patterns of that given trait) (Statzner & Bêche, 2010). The number of traits selected for BTA is related to the ability of the analysis to study the functioning of species assemblages (Petchey & Gaston, 2006). Studies that include many traits can provide an informative picture of ecological functioning, whereas those including a few traits may produce a misleading view of species assemblage functioning (Bremner *et al.*, 2006). However, parsing literature information regarding several traits is difficult and time consuming, and gaps in species knowledge make it

impossible to completely describe traits for all taxa, particularly in the case of large taxonomic lists (Marchini *et al*., 2008). Moreover, the relationship between biological traits and ecological functioning is not always clear; thus, not all traits are as easy to interpret in the context of BTA (Petchey & Gaston, 2006; Marchini *et al*., 2008). Choosing traits to use in a BTA is a compromise between these aspects. In our study, we considered only traits that could be easily coded, without having to use '0' for all categories. Eight biological traits were chosen related to aspects of life history, behavior and feeding habits: 'feeding', 'mobility', 'habitat', 'body size', 'life span', 'reproductive technique', 'reproductive frequency' and 'salinity preference'. The eight traits were subdivided into twenty-eight categories (see Table 1). The matrix 'traits by stations' was used to compute the proportion of individuals of a given category that appeared in a given site: the standardized affinity scores for each taxon (matrix 'taxa by traits') were multiplied by its density at each station (matrix 'taxa by stations') and the standardized categories were summed over all taxa; this has been repeated for each of the five stations and for summer and winter. For this computation, R-2.12.2 (R Development Core Team, 2011) and ade4 library (Chessel *et al*., 2004) were used.

Table 1: Biological traits and their categories.

Biological traits	Traits categories
Feeding	Predator Herbivore Deposit-feeder Filter-feeder
Mobility	Sessile Swim Burrow Crawl Walk
Habitat	Infaunal Epifaunal
Body size	Very-small Small Medium Large
Life span	Short Medium Long
Reproductive technique	Gonochoristic Hermaphrodite
Reproductive frequency	Twice every year One's per year (extended period) One's per year (distinctive period) One's per 2 years (extended period) Semelparous
Salinity preference	< 5 5 - 20 > 20

2.4 Measures of taxonomic and functional diversity

The peculiar environmental conditions of estuaries, characterized by strong gradients
and high variability of environmental factors, are known to produce effects on
community structure, namely, they select communities with low diversity and high
dominance of the few species that are adapted to such critical environments (Elliott &
Quintino, 2007). Therefore, this peculiar taxonomic composition therefore is expected

to affect the functional composition too (Botta-Dukát, 2005; Petchey & Gaston, 2002). With this in mind, we decided to investigate the relation between taxonomic and functional composition, by comparing indices of taxonomic and functional diversity.

2.4.1 Taxonomic diversity

We selected three measures based on taxonomic community composition, namely richness (number of species), evenness (uniformity in the distribution of individuals among species) and density (total number of individuals in a given area) (Hill, 1973). One of the most frequently used diversity indices, the Shannon-Wiener Index (Shannon and Weaver, 1963) was calculated. This index accounts for density and evenness of species (see Marques *et al.*, 2009 for details). Shannon-Wiener was computed with log base 2.

2.4.2. Functional diversity

The functional composition was evaluated using functional diversity (FD) as a diversity measure. To compute FD we used the Rao's Quadratic Entropy (RQE) index as in Champely & Chessel, (2002):

$$\text{RQE}_k = \sum_{i,j=1}^{S_k} p_{ik} \times p_{jk} \left[d_{ij} \right]^2$$

with S_k being the taxonomic richness in station k, and p_{ik} and p_{jk} being the relative abundances of the ith and jth taxa in station k, respectively. $[d_{ij}]$ is the traits dissimilarity between taxa i and j (*i.e.*, measured by Euclidean distance). The biological distance matrix $[d_{ij}]$ was computed separately for each trait. Since there are eight traits, this was done eight times. The 'taxa by stations' matrix was combined

with the 'taxa by traits' matrix in the RQE calculation. The average of those eight distance matrices served to compute the overall RQE.

For the computation of Shannon-Wiener diversity and FD, for all stations and for both summer and winter samples, R-2.12.2 (R Development Core Team, 2011), 'ade4' library (Chessel *et al*., 2004) and 'vegan' library (Oksanen *et al*., 2011) were used.

2.5 Multivariate data analysis

2.5.1. Environmental data analysis

The environmental data was analyzed by means of a Principal Correspondence Analysis (PCA). PCA's for summer and winter data were made separately, in order to explore seasonal and spatial patterns. The environmental variables were transformed by log (1+x) and by square root, whenever data was moderately skewed in distribution. The redundant variables were removed from the analysis so that the first two axes account for the maximum variability in the data set. The variables that were retained in the model were acting as proxy for the ones that were eliminated. The remaining variables were then normalized and subjected to PCA for ordination. A lower triangular Euclidean distance matrix relating to the ordination was constructed (Clarke & Green, 1988). Granulometry data for st 16, in winter, was lacking and therefore excluded from the analysis. For this analysis, R-2.12.2 (R Development Core Team, 2011) and ade4 library (Chessel et al., 2004) were used.

The following environmental variables were plotted: salinity, dissolved oxygen (O_2), pH, TSS, $N-NH_4$, $N-NO_3$, $N-NO_2$, $P-PO_4$ and granulometry data for clay, fine sand, medium sand, coarse sand and gravel. Depth and transparency were not plotted because they were of no interest for this study and some variables were not plotted

because of their high correlation with other variables: temperature (correlated with salinity), SiO (correlated with salinity and the nutrients), silt, in summer, (correlated with clay), medium sand, in summer, (correlated with coarse sand) and coarse sand, in winter, (correlated with gravel). All the nutrients were highly correlated to each other, however, we decided to plot all of them.

2.5.2. Biological traits analysis: co-inertia and fuzzy correspondence analysis

To explore taxa density patterns and the traits displayed by those taxa, we conducted a co-inertia analysis (Dolédec & Chessel, 1994) on the two sets of matrices; 'taxa by station' matrix and the 'taxa by traits' matrix both for summer and winter data. This analysis, simultaneously, ordinates the two matrices maximizing both the variance from the individual matrices and the correlation between them (Dolédec & Chessel, 1994; Dray *et al*., 2003). High co-inertia values indicate that the data structures are correlated, whereas low co-inertia means that they vary independently or do not vary (Dray *et al*., 2003). The statistical significance of the co-structure was examined by means of a Monte-Carlo random permutation test (999 permutations) (Dolédec & Chessel, 1994). The observed co-inertia value was then compared with the 999 permutated pairs of the two matrices to assess significance of the outcome value. For this analysis, R-2.12.2 (R Development Core Team, 2011) and ade4 library (Chessel *et al*., 2004) were used.

Fuzzy Correspondence Analysis (FCA) allowed us to identify which traits were relevant in distributing the species within the estuary and between seasons. FCA is a correspondence analysis method appropriate for fuzzy coded data (Chevenet *et al*., 1994). FCA was performed on the 'traits by stations' matrix; Euclidean distances

between samples, calculated from the relative frequencies of abundance-weighted biological traits in each station, were used to ordinate the stations on a multidimensional space. FCA provided the variability contained in every axis and the correlation ratios of every biological trait along the principal axes. It also allowed us to plot the scores of each station and each trait category on two-dimensional factor maps. In the resulting plots, stations were located at the weighted average of the trait categories presented in those stations, and *vice versa*. Therefore, stations close to one another in the context of the plot coordinates have similar patterns of density across trait categories. In order to identify the traits most responsible for the variation along the principal factorial axes for all stations, and between seasons, FCA was repeated for two sets of 'traits by stations' matrix, one for the summer and one for the winter. For this analysis, R-2.12.2 (R Development Core Team, 2011) and ade4 library (Chessel *et al*., 2004) were used.

3. Results

3.1 Environmental conditions

Fig. 2A and B shows the PCA plots of the Mondego estuary environmental data for summer and winter, respectively.

In summer, the first two axes of the PCA accounted for 84 % of the total variability; 67 % was explained by axis 1 and 17 % by axis 2 (Fig. 2A). St 3, st 4 and st 12 were separated from the other ones mainly by: higher salinity, dissolved oxygen concentrations, and fine sand sediments. St 16, was mainly separated by higher pH, TSS and clay sediment, and st 23 by higher nutrients concentrations, chlorophyll *a*, and course sand to gravel sediments.
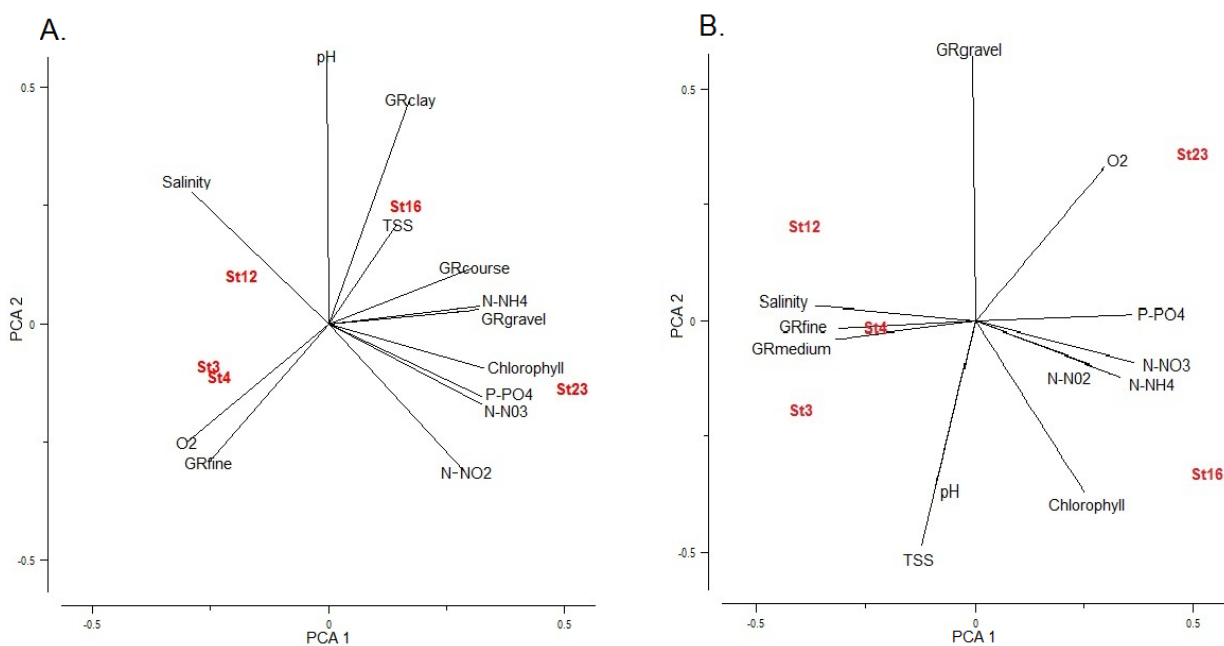

Figure 2: Principal Component Analysis of the relevant environmental variables in the Mondego estuary, in A. summer and B. winter.

In winter, the first two axes of the PCA accounted for 80 % of the total variability. 59 % was explained by axis 1 and 21 % by axis 2 (Fig. 2B). St 3, st 4 and st 12 were separated from the other ones by; higher salinity, and fine to medium sand sediments. St 16 and st 23 were mainly separated by higher nutrient, oxygen and chlorophyll *a* concentrations.

The most evident seasonal variations were caused by: salinity, oxygen, chlorophyll and nutrient concentrations. In summer, salinity and dissolved oxygen concentrations were higher at the South arm stations of the estuary (st 3 and st 4) whereas, chlorophyll *a* concentrations were higher (in comparison to winter), within the whole estuary, especially at st 23 (eight times the concentration of the lower estuary). In winter, salinity was much lower (in comparison to summer), at st 3, st, 4, st, 12 and st 16, whereas dissolved oxygen concentrations were higher in the upper estuary. Nutrient concentrations were especially high at st 16 during this season.

3.2 Taxonomic composition of the benthic community

3.2.1 Spatial and seasonal distribution of taxa

Table 2: Taxa collected in the Mondego estuary, in summer and winter.

#	Species (Author)	#	Species (Author)
1	*Abra* sp. (Leach in Lamarck, 1818)	23	*Mediomastus* sp. (Hartmann, 1944)
2	*Abra tenuis* (Montagu, 1803)	24	*Melita palmata* (Montagu, 1804)
3	*Bathyporeia pilosa* (Lindström, 1855)	25	*Microphthalmus* sp. (Mecznikov, 1865)
4	*Bathyporeia sarsi* (Watkin, 1938)	26	*Modiolus modiolus* (Linnaeus, 1758)
5	*Carcinus maenas* (Linnaeus, 1758)	27	*Mysta picta* (Quatrefagues, 1865)
6	*Cerastoderma edule* (Linnaeus, 1758)	28	*Nephtys cirrosa* (Ehlers, 1868)
7	*Corbicula fluminea* (Müller, 1774)	29	*Nephtys hombergii* (Savigny in Lamarck, 1818)
8	*Corophium multisetosum* (Stock, 1952)	30	*Paragnathia formica* (Hesse, 1864)
9	*Crangon crangon* (Linnaeus, 1758)	31	*Pisione remota* (Southern, 1914)
10	*Cyathura carinata* (Krøyer, 1847)	32	*Pomatoceros lamarcki* (Quatrefages, 1866)
11	*Eurydice* sp. (Leach, 1815)	33	*Pygospio elegans* (Claparède, 1863)
12	*Gammarus chevreuxi* (Sexton, 1913)	34	*Scoloplos armiger* (Müller, 1776)
13	*Gastrosaccus spinifer* (Goës, 1864)	35	*Scrobicularia plana* (da Costa, 1778)
14	*Glycera* sp. (Savigny, 1818)	36	*Sphaeroma serratum* (Fabricius, 1787)
15	*Goniada* sp. (Audouin & Milne-Edwards, 1833)	37	*Spio filicornis* (Müller, 1776)
16	*Halicyclops* sp. (Norman, 1903)	38	*Spisula elliptica* (Brown, 1827)
17	*Harpinia* sp. (Boeck, 1876)	39	*Streblospio shrubsolii* (Buchanan, 1890)
18	*Hediste diversicolor* (Müller, 1776)	40	*Tapes pullastra* (Montagu, 1803)
19	*Heteromastus filiformis* (Claparède, 1864)	41	*Tellina tenuis* (da Costa, 1778)
20	*Hydrobia ulvae* (Pennant, 1777)	42	*Tharyx* sp. (Webster & Benedict, 1887)
21	*Idotea pelagica* (Leach, 1815)	43	*Venerupis decussata* (Linnaeus, 1758)
22	*Lekanesphaera levii* (Argano & Ponticelli, 1981)		

A total of 43 different taxa were identified for both seasons (see Table 2). In summer, a total of 36 different benthic invertebrate taxa were identified, and in winter, a total of 23 different taxa were recorded. The distribution of taxa changed between stations and seasons (see Fig. 3).

The upper estuary (st 16 and st 23) and the lower estuary (st 3, st 4 and st 12) were characterized by different taxa assemblages. The non-native bivalve *Corbicula. fluminea*, the amphipod *Corophium multisetosum*, the isopod *Cyathura carinata* and the polychaete *Hediste diversicolor* characterized the upper estuary, whereas the gastropod *Hydrobia ulvae*, the polychaetes *Nephtys cirrosa* and *Microphthalmus* sp.,

the amphipod *Bathyporeia sarsi* and the bivalves *Tellina tenuis* and *Cerastoderma edule* characterized the lower estuary.
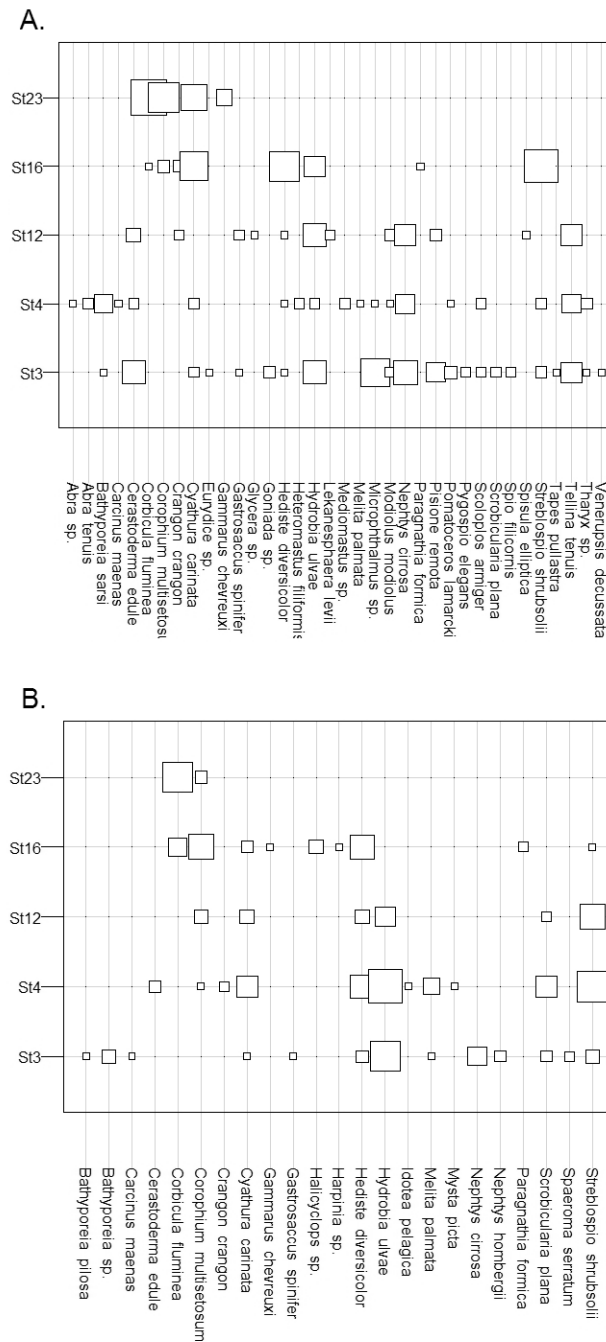

Figure 3: Taxa distribution within the Mondego estuary, in A. summer and B. winter. The size of the squares is proportional to the density (bigger squares, higher density).

The taxa that showed clear seasonal changes were *C. multisetosum, Streblospio shrubsolii, H. diversicolor, C. carinata* and *H. ulvae*. Apart from *H. ulvae*, these taxa were only found in the upper estuary in summer, while in winter, they were also found at the lower estuary. For example, in winter, the species *S. shrubsolii* was dominant at st 12, while in summer it was absent from the same station. Another example is the gastropod *H. ulvae*, which was dominant at the South arm (st 3 and st 4) in winter, while being much less abundant in summer.

A total of 20 different taxa were not found in winter, amongst which: *Microphthalmus* sp*., T. tenuis* and *Pisione remota*. However, in winter, also 5 other taxa, that were not present in summer, were collected: the amphipod *Harpinia* sp., the copepod *Halicyclops* sp., the polychates *Mysta picta* and *Nephtys hombergii*, and the isopod *Sphaeroma serratum.*

3.2.2 Taxonomic diversity

In general, species richness, species density and Shannon-Wiener diversity was higher in summer (Fig. 4). Considering the spatial distribution, the highest species richness and Shannon diversity was found in the South arm (st 3 and st 4) and decreased towards the upper parts of the estuary (st 12, st 16 and st 23), the only exception was st 16, displaying higher species richness and Shannon-Wiener diversity in winter than in summer. In general, species density increased towards the upper parts of the estuary, the only exception was st 4, in winter, displaying the highest density within the estuary. This increase in density was mainly caused by the density increase of *H. ulvae* in winter (see fig 3B).
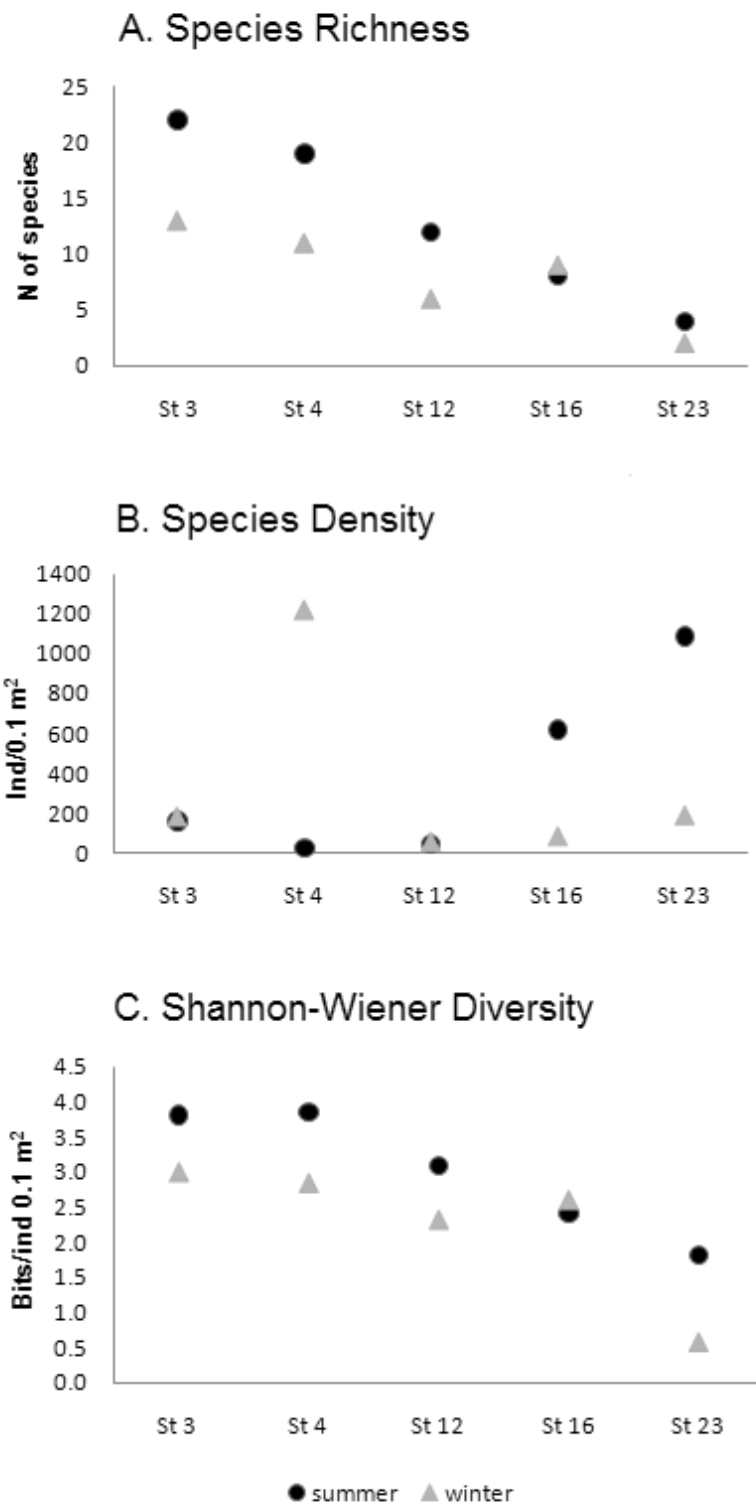
Figure 4: Spatial (st 3, 4, 12, 16 and 23) and seasonal (summer and winter) distribution of A. Species Richness, B. Species Density and C. Shannon-Wiener Diversity.

3.3 Functional characteristics of the benthic community

3.3.1 Spatial and seasonal distribution of traits (categories)

In summer, a total of 36 different taxa displayed 28 traits categories and the 23 different taxa in winter displayed 26 traits categories (mobility 'sessile' and body-size 'large' were absent) (Fig. 5).

Salinity preference (Fig. 5A, B):

Species that prefer low salinities (<5) were found in the upper parts of the estuary and species that prefer the higher salinities were found in the lower parts of the estuary. However, seasonal differences were observed: in summer (Fig. 5A), the species *C. multisetosum, S. shrubsolii, H. diversicolor* and *C. carinata* were mainly found at the upper estuary, but they also appeared at the lower estuary in winter (Fig. 5B).
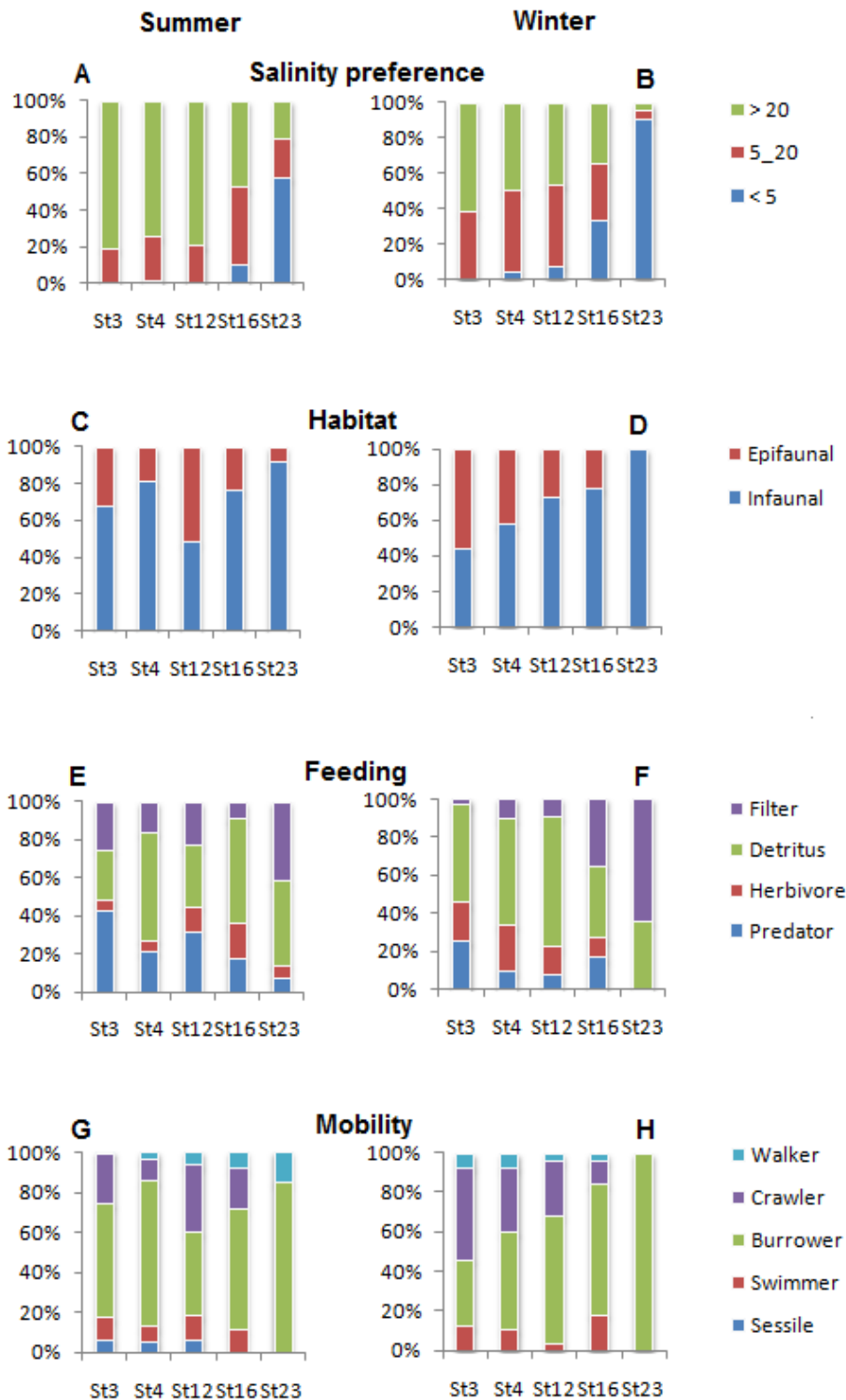
Figure 5: Percentage of a given trait category (A and B – salinity preference; C and D – habitat; E and F – feeding; G and H – mobility; I and J – body size; K and L – life span; M and N – reproductive technique; O and P – reproductive frequency) at a given station, for summer and winter. Figure to be continued on the next page.
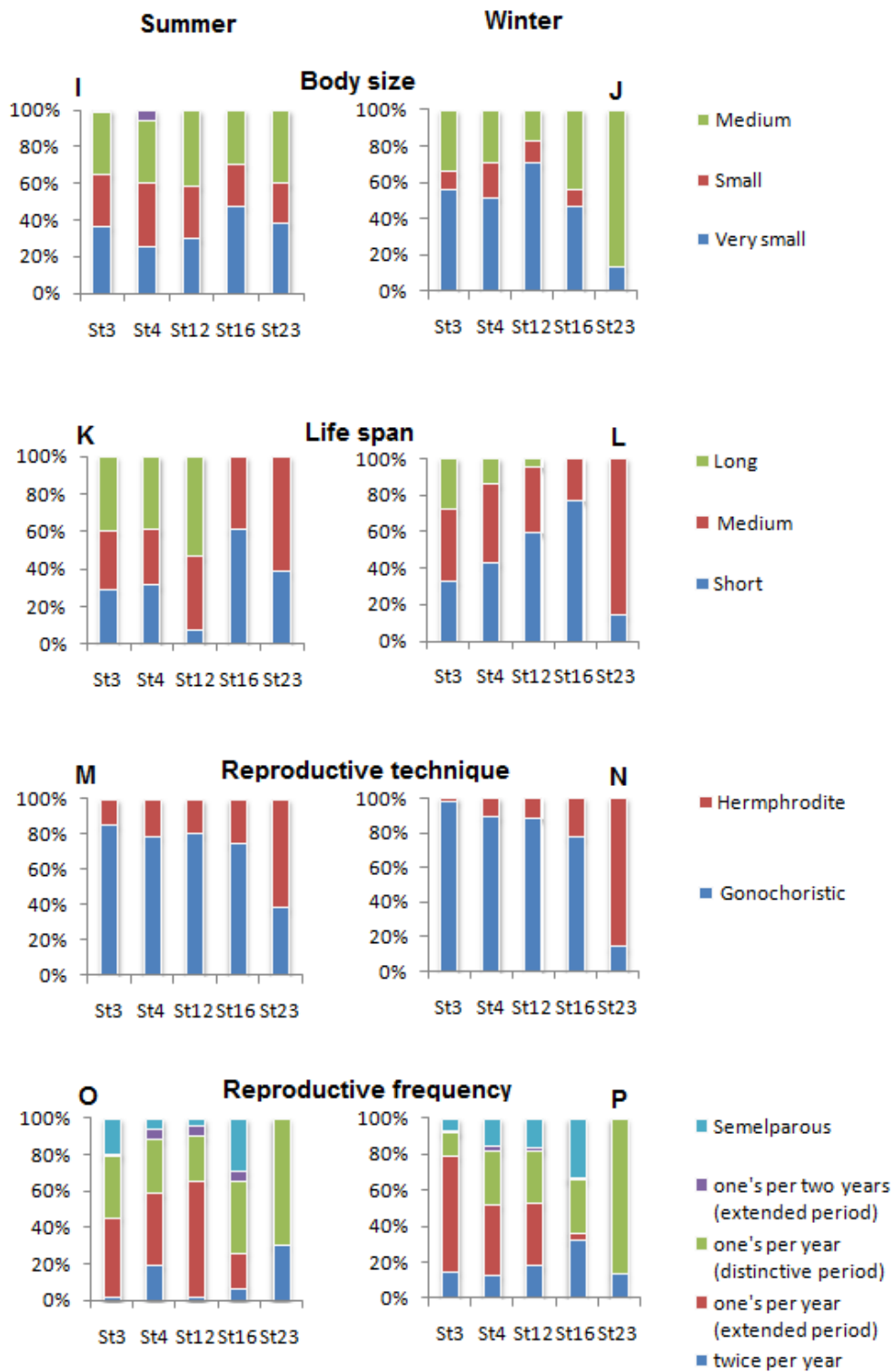
Figure 5 (cont.)

Habitat (Fig. 5C, D):

Almost in all stations and at both seasons, infaunal species were dominant over the epifaunal species. In winter, the dominance of infaunal individuals increases towards the upper parts of the estuary. In summer, the community in the South arm, contained a relatively high percentage of infaunal individuals, whereas in winter epifaunal species were more abundant. This change can be explained by the epifaunal gastropod *H. ulvae*, which showed lower abundances in summer than in winter. In the lower part of the North arm (st 12), epifaunal individuals were more abundant in summer, while in winter, infaunal species were dominant. This seasonal change can be explained by the dominance of the infaunal polychaete *S. shrubsolii,* in winter, while in summer, this species was completely absent from this station.

Feeding (Fig. 5E, F):

In winter, filter feeders became increasingly more important towards the upper parts of the estuary, while deposit-feeders dominated the lower parts of the estuary. In summer, we were not able to see this pattern. This was mainly due to the shift of *S. shrubsolii* from the upper parts of the estuary in summer, towards the lower parts in winter. In winter, the importance of filter feeders at the upstream stations was due to the dominance of *C. fluminea*, which displays this feeding behavior. Furthermore, predatory species were more abundant at the South arm, in summer, while herbivorous species were dominating over the predatory species in winter. This can be explained by the herbivorous and detritus feeder *H. ulvae*, which was more abundant in winter than in summer. In fact, in summer this species was totally absent from st 4, while in winter it was the dominant species. Moreover, the decrease of

predatory species from summer to winter is related to the absence of *Microphthalmus* sp. and *N. cirrosa* during the winter period.

Mobility (Fig. 5G, H):

Although burrowing species were the most abundant, seasonal differences were observed for some parts of the estuary. In summer, sessile species (*e.g.* *Pomatoceros lamarcki* and *Modiolus modiolus*) were only found at the lower estuary, while in winter they were absent. Furthermore, in winter, crawling species increased at the South arm (st 3 and st 4), while the percentage of burrowing species decreased. This increase in crawling species was mainly due to the increase of *H. ulvae* in winter.

Body size (Fig. 5I, J):

The spatial distribution of the different body size categories changed between seasons. In summer, all categories were quite evenly distributed along the estuary; on the other hand, individuals with a very-small body size were more abundant in the lower parts of the estuary during winter. The only large-sized species, *Tharyx* sp., disappeared completely from the South arm stations in winter.

Life span (Fig. 5K, L):

Species with a long life span were only found at the lower parts of the estuary (st 3, st 4 and st 12), while the upper parts showed species with a short to medium life span. Species with a long life span were more abundant in summer. This change could be explained because most bivalve species (*i.e. C. edule, Tapes pullastra. Venerupsis*

decussata, T. tenuis, Spisula. elliptica) and the polychaete *N. cirrosa* have long life spans, however, these species were scarcer or even absent in winter. Furthermore, in winter, species with a short life span showed a relative increase at st 12, because almost all species with a long life span were absent at this station.

Reproductive technique (Fig. 5M, N):

Species with a gonochoristic reproductive-technique were dominant in most of the estuary, except for st 23, where the hermaphrodite *C. fluminea* was dominant. There were no clear differences between the two seasons.

Reproductive frequency (Fig. 5O, P):

In general, most species in the estuary breed once a year. Species that breed over an extended period were found mostly in the lower estuary. No seasonal differences were observed.

3.4 Functional diversity compared to taxonomic diversity

In general, at both seasons (although clearer in summer), the Shannon-Wiener values (H), decreased from downstream towards the upper estuary. The FD showed a similar value between all stations, except at st 23, where it increased in summer and decreased in winter (Fig. 6). The ratio between these two indices (FD / H), showed a similar value at all stations, except at st 23, where it slightly increased.
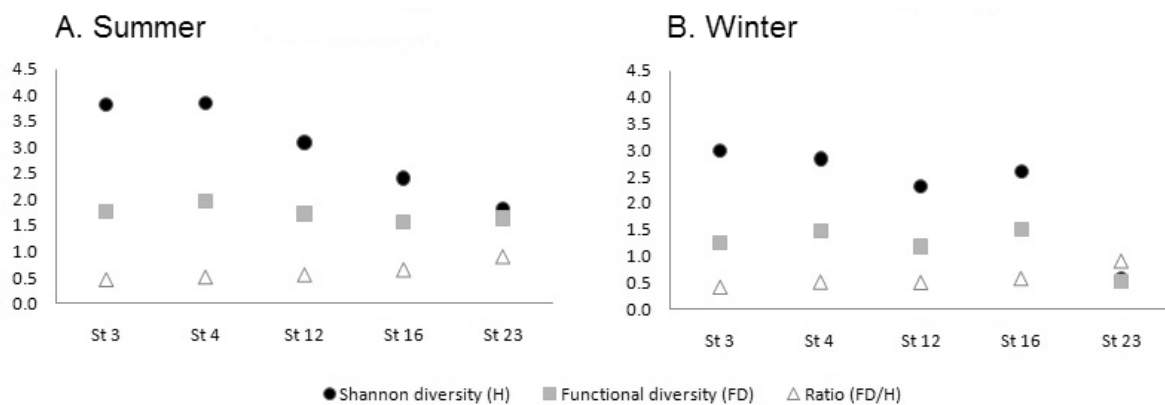

Figure 6: Spatial and seasonal distribution of functional diversity (FD), Shannon diversity (H) and the ratio between the two diversity indices (FD / H) in A. Summer and B. Winter.

3.5 'Multivariate' biological traits analysis

3.5.1 Co-inertia analysis

The co-structure between the taxa table and the traits table was investigated using co-inertia analysis. This analysis tests the hypothesis that the species traits array (*i.e.* the matrix fulfilled with literature data) matches the species distribution array of the Mondego estuary. In other words, it tests whether the two matrices are correlated and display a high co-inertia. The observed co-inertia values were 0.23 for summer and 0.31 for winter. The Monte-Carlo test then compared the observed co-inertia values with 999 random permutations of the two arrays, in order to test significance of correlations. In summer, 69 out of 999 permutations were above the observed co-inertia value (P=0.069). In winter, 57 out of 999 permutations were above the observed value (P=0.057). Both results were just slightly above the 5% significance level, thus did not allow excluding that the traits and species data varied independently, despite existing also signs that the co-variation was not random. Therefore, the hypothesis that species traits are not randomly distributed along the Mondego estuary cannot be accepted, but neither strongly refused.

3.5.2 FCA

FCA on the traits by stations matrix was performed for both seasons separately (Fig. 7). In summer, the first two axes of the FCA accounted for 87% of the total variability. 69% was explained by axis 1 and 18% by axis 2. The contribution of each trait to this variability was reflected in the correlation ratios (Table 3). The categories of the traits 'salinity preference', 'reproductive frequency' and 'life span' separated more on axis 1

whereas those for the traits 'life span and 'reproductive frequency' separated more along axis 2.
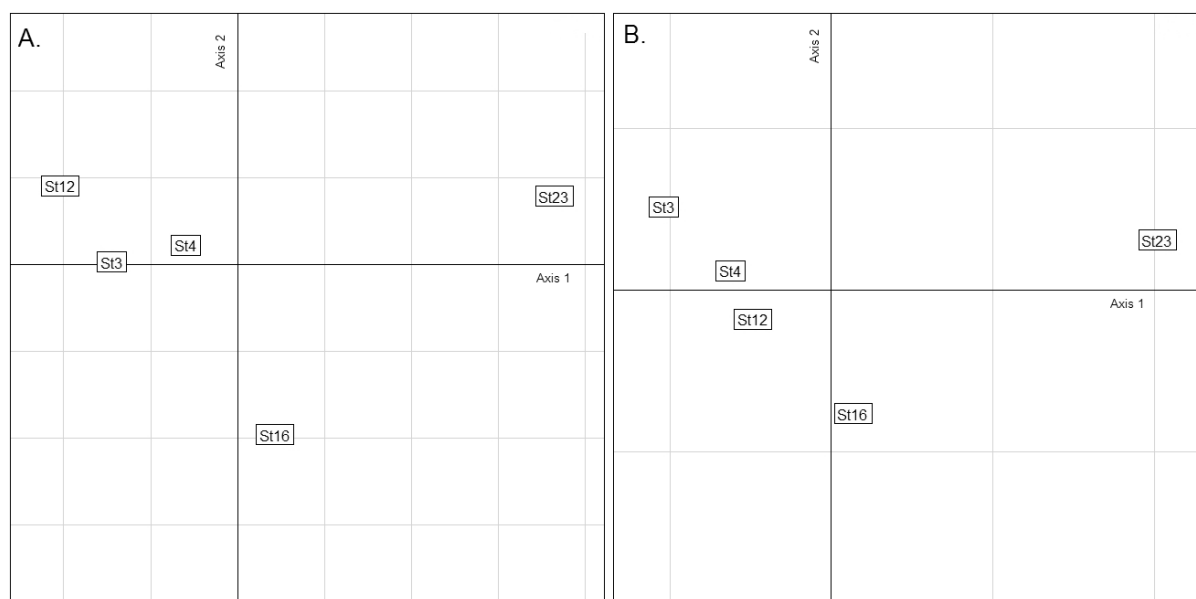

Figure 7: FCA "traits by stations" plot with the position of the stations according to the distribution of the traits categories, for A. summer and B. winter.

Table 3: Percent contribution of each FCA axis, and correlation ratios between each axis and the biological traits for both seasons.

	SUMMER		WINTER	
Traits	Axis 1 (69%)	Axis 2 (18%)	Axis 1 (80%)	Axis 2 (14%)
Salinity preference	0.40	0.04	0.57	0.00
Reproductive frequency	0.28	0.10	0.31	0.16
Reproductive technique	0.13	0.01	0.46	0.01
Life span	0.19	0.11	0.14	0.17
Mobility	0.14	0.01	0.19	0.03
Habitat	0.08	0.00	0.15	0.01
Feeding	0.07	0.05	0.29	0.01
Body size	0.01	0.01	0.20	0.01

In winter, the first two axes of the FCA accounted for 94% of the total variability, with 80% explained by axis 1 and 14% by axis 2 (Table 3). The categories of the traits

'salinity preference', 'reproductive technique' and 'reproductive frequency' separated more on axis 1, whereas those for the traits 'life span' and 'reproductive frequency' separated more along axis 2.

At both seasons, the ordination of the traits by stations clearly separated the lower estuary (st 3, st 4 and st 12) from the upper most reaches of the estuary (st 23) on axis 1, while st 16 was mainly separated by axis 2 (see Fig. 7). 'salinity preference' was the most relevant trait in distributing the stations along axis 1. In addition, the separation of st 16 along axis 2 was mainly explained by the traits 'reproductive frequency' and 'life span', with respectively the trait categories 'semelparous' and 'short life span'.

4. Discussion

The benthic community of the Mondego displays features that are typical of estuarine environments, namely the low diversity and high dominance of the few species that are adapted to these peculiar environments. The strong gradients and the high variability of environmental conditions greatly affected the spatial and seasonal distribution of the benthic community within the Mondego estuary. These findings are congruent with other studies that observed similar patterns of spatial and seasonal distribution of benthic invertebrates in this estuary (*e.g.* Chainho *et al.*, 2006; Teixeira *et al.*, 2009; Veríssimo *et al.*, 2011).

Traits distribution: the key role of 'salinity preference'

The results of the BTA showed quite similar trait distributions within the lower estuary, whereas, the trait distribution in the upper parts differed considerably. Within the estuary, the traits distribution at st 16 changed substantially between seasons. As the FCA pointed out, the most important trait that distributed the benthic community along the estuary was 'salinity preference'. In fact, the distribution of most of the other traits was strongly affected by this trait, which confirms the important environmental filtering of salinity (McLusky, 1971). This became especially clear when we investigated the traits distribution between the two seasons. For example, the shift of species such as *S. shrubsolii* towards the lower estuary in winter, is probably a response to osmotic stress due to higher freshwater inputs from the Mondego river in winter causing a drastic dip in salinity (Chainho *et al.*, 2006). This species is known to tolerate salinities ranging from 5 to more than 20 (MarLIN BIOTIC, 2011), however, the salinity at st 16, in winter, dropped below 5. As a consequence, the shift of this

species caused a reduction in detritus feeders and a subsequent dominance of filter feeders in the upper estuary. This is just one example of a species that was probably affected by osmotic stress and caused a different distribution of traits between seasons. Other examples of species that were probably affected by osmotic stress were; *C. multisetosum, H. diversicolor* and *C. carinata.* All these species tolerate salinities higher than 20 (MarLIN BIOTIC, 2011) and shifted towards the lower estuary in winter. There are also species which have been completely absent or almost disappeared from the estuary in winter; *P. lamarcki, M. modiolus, T. pullastra, V. decussate, T. tenuis* and *Spisula eliptica* completely disappeared and *N. cirrosa* was only found at st 3, while in summer, it also inhabited st 4 and st 12. Except for *S. eliptica,* which tolerates salinities as low as 5, the other species only tolerate salinities higher than 20 (MarLIN BIOTIC, 2011). *S. eliptica* was only present in summer at st 16, and since salinity dropped to almost zero in winter, the disappearance of this species due to osmotic stress seems plausible as well.

All the above-mentioned examples showed that the functional composition of a number of traits depended on the species preference to salinity, suggesting that the trait 'salinity preference' may strongly affect other traits.

The traits 'reproductive technique and 'reproductive frequency' separated mainly st 23 from the other stations, because of the dominance of *C. fluminea* at this station, which has a hermaphroditic 'reproductive technique' and breeds in a distinctive period of the year. According to Neumann & Kröncke (2010), there is a relation between water temperature and reproductive frequency: the higher the temperature, the higher the reproductive frequency. This relation remained unclear in this study, since reproductive frequency hardly changed between summer and winter.

BTA as an indicator of environmental stress

The trait 'life span' mainly separated st 16 from the other stations because of the high percentage of individuals with short life spans. The dominance of *S. shrubsolii* and *C. multisetosum* in this station contributed the most to this trait display. These species also happen to be small 'infaunal' species with a 'detrivorous' feeding behavior. According to several authors (Pearson and Rosenberg, 1978; Norkko & Bonsdorff, 1996; Mouillot *et al*., 2006; MacLeod *et al*., 2008; Papageorgiou *et al*., 2009; Villnäs *et al*., 2011) these are typical traits of opportunistic species in nutrient enriched or stressed habitats. Indeed, nutrient levels measured at st 16 exceeded those of most other stations (except st 23). Thus, the dominance of opportunistic traits observed in this part of the estuary might be a response to both natural (*e.g.* low salinity) and human-induced (high nutrient input) stress. However, from our observation, it is not possible to quantify the extent to which single factors affect the community.

BTA allowed to highlight broad patterns in trait-environment relationships. However when it came to the details, it proved to be not easy to distinguish if traits responded to "natural" or to "human induced" stress, or a combination of both.

Functional and taxonomic composition

Apparently, the uppermost parts of the estuary, is showing a distinct pattern from the rest of the estuary. At this habitat, fewer species, occupied the available functional niche space, which might indicate that they are likely to have fewer traits in common. It potentially means that this community exhibits lower functional redundancy than the downstream assemblages. Functional redundancy is an insurance policy against the loss of function in the event that species are lost. The larger the number of

functionally similar species in a community, the greater the probability that at least some of these species will survive changes in the environment and maintain ecosystem functioning (Díaz & Cabido, 2001). Our results suggest that the ratio (FD / H) might be a helpful tool to visualize this system functional attribute: when the ratio increased, functional redundancy decreased and *vice versa*.

Nevertheless, it is important to have in mind that the "quality" of FD is very much dependent on the ability of the traits in explaining the distribution of the community (Botta-Dukát, 2005; Petchey & Gaston, 2006). Since most of our traits proved to be not relevant, a certain caution is needed when interpreting our results.

The selection of biological traits

The co-inertia analysis between the 'species' array and the 'traits' array showed that there was no significant co-structure between the two arrays for both seasons. Therefore, the hypothesis that species traits are not randomly distributed along the Mondego estuary cannot by fully accepted. However, the FCA explained to some degree subtle differences in the distribution of species within the estuary and between seasons.

In Biological Traits Analysis, the selection of traits and categories has to be made *a priori*, on the basis of (i) evidence of their importance in displaying the functioning of the ecosystem from the literature; (ii) personal observations/knowledge on the investigated benthic community; (iii) available literature information required to fulfill the traits data for all the collected species.

(i) Most of the BTA studies done in marine environments were successful in displaying the functioning of the ecosystem using similar traits as in the present study

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(Villnäs *et al*., 2010; Papageorgiou *et al*., 2009; Marchini *et al*., 2008; MacLeod *et al*., 2008; Bremner *et al*., 2003; Norkko and Bonsdorff, 1996). However, our study showed that not all of these traits have resulted similarly relevant in displaying the functional structure of estuarine environments. (ii) Conversely, our most relevant trait, 'salinity preference' , to the best of our knowledge, had never been included before in a study of marine/transitional communities. This is probably because, in marine and freshwater habitats, salinity is constant (either ± 35 or 0); therefore, it would be a trait without different categories, which will be useless when explaining the distribution of species. We cannot exclude that other traits could have explained the distribution of species more significantly. (iii) Unfortunately, for marine and estuarine species there is still a huge lack of trait information. Most of the available information is still spread out in the scientific literature (Statzner & Bêche, 2010; Marchini *et al*., 2008). This lack of information is probably one of the reasons why there are only a few studies concerning BTA in transitional and marine environment. Online traits databases such as MarLIN (MarLIN BIOTIC, 2011), where traits data from marine and estuarine species have been collected and made available for free download, can make BTA in these environments a lot easier by synthesizing traits information. Moreover, there has been no common agreement in which traits to use for BTA, which makes it difficult to perform comparisons among studies. The BTA approach is in its infancy and the following years it is expected to have an increase of published lists with relevant traits.

To perform an effective BTA, it is recommended to choose traits, which are related to ecosystem services, since ecosystem services are directly linked to the overall functioning of ecosystems (Díaz & Cabido, 2001). These trait-service associations

will be a crucial step in ecosystem service monitoring and in balancing the delivery of multiple, and sometimes conflicting, services in ecosystem management (de Bello *et al.*, 2010).

5. Concluding remarks

Traditional taxonomic analysis highlighted spatial and seasonal variations of community composition (*i.e.*, a general reduction of species richness, Shannon-Wiener diversity, and an increase of species density towards the upper parts of the estuary). BTA analysis highlighted the functional characteristics of the benthic community within the estuary and common features across species that have appeared or disappeared among seasons. Furthermore, BTA allowed to investigate interactions among traits (*i.e.*, the strong influence of the trait 'salinity preference') and was used to calculate functional diversity (FD). Indices of taxonomic diversity (Shannon-Wiener index) and functional diversity (RQE index) allowed exploring the relation between taxonomic and functional composition. This relation might potentially have indicated that the community at the upper most reaches of the estuary exhibits lower functional redundancy than the downstream assemblages.

For these reasons, it is recommended to include BTA when investigating the ecological functioning of estuarine/transitional environments. This study has been one of the first attempts to: (1) investigate multiple biological traits, (2) calculate functional diversity and (3) explore the relation between functional and taxonomic composition of the benthic invertebrate community in an estuarine environment by means of BTA. The next step would be to link biological traits with the services these environments provide. These trait-service associations will become a crucial step in ecosystem service monitoring and ecosystem management. In the next future, development of researches on BTA in transitional/estuarine environments are expected to deal with the following issues: (1) to include the trait 'salinity preference', which strongly affects estuarine species distribution as well as functional structure;

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however, for the optimization of BTA, also other relevant traits are needed, therefore,

(2) expanding more (detailed) knowledge on estuarine invertebrate traits is required.

(3) Finally, a selection of "standard traits", including those who have proven (and will

prove) to be the most relevant traits in these environments, should be proposed and

used for future comparative studies.

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