Marine Pollution Bulletin 71 (2013) 129–138

Contents lists available at SciVerse ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Characterization of an estuarine environment by means of an index based on intertidal macrofauna

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ARTICLE INFO

Keywords:
Indicator species
Conditional probability
Vector norm
IndVal
Transformations
Estuarine limits

ABSTRACT

Macrobenthic intertidal assemblages from five Atlantic Iberian estuaries were analyzed to develop an estuarine index. An R-mode analysis revealed a close association between the isopod *Cyathura carinata*, the polychaete *Hediste diversicolor* and the bivalve *Scrobicularia plana*. Although these species are abundant in all the estuaries considered, they tend to be absent from sites at the marine and freshwater ends of the environmental gradient. Three different ways of calculating the estuarine index are proposed. The index is comprised in the interval [0,1] and was constructed using relative abundances rather than absolute abundances. Transformation of the raw data helped improve the performance of the index. A non-parametric statistical test is proposed for application to the estuarine index to find sites with the same values after a significant omnibus test. The index appears to be a good proxy for recognizing estuarine limits by use of indicator species.

1. Introduction

Estuaries may be defined, according to the European Water Framework Directive (WFD, EU Directive 2000/60/EC; European Community, 2000), as ‘transitional waters that are surface water bodies partly saline due to their proximity to coastal waters but significantly influenced by freshwater flows’.

The WFD established that ‘Member States shall identify the location and boundaries of bodies of surface water’. Further, surface waters are split into ‘water bodies’ that are classification and managerial units, in accordance to the Directive. The WFD is concerned with the quality and health status of the water bodies, and a number of benthic quality indices have been developed for this purpose. However, biotic indexes devoted to identify brackish environments are scarce (e.g., Wolf et al., 2009). In this sense, Elliott and McLusky (2002) raised the question whether it would be possible to differentiate the intertidal fauna between the estuarine zone and the marine and freshwater domains at the edges of estuaries. Moreover, the latter authors highlighted that the question on the estuarine limits is a matter of intense debate including the scientific, legal and managerial dimension of the problem. For instance, the identification of estuarine boundaries is not trivial because estuaries are subjected to fluctuations along time depending on factors such as changes in river discharges or the spring/neap tidal cycle (Mclusky, 1993). In addition to seasonal fluctuations in the estuarine boundaries, sea-level rise related to climate change is expected to cause further changes in the estuarine environment and its limits on a long term perspective (Fujii, 2012).

Among the mandatory features in defining transitional waters in the WFD, salinity is largely recognized in the scientific literature as determinant in the distribution of the estuarine macrobenthic assemblages (Barnes, 1989; Ysebaert et al., 1998). Biogeographically, dominant macrobenthic species of the estuarine assemblages are subjected to latitudinal variation on the European coast (e.g. Bocher et al., 2007). Species may even be replaced considering the latitudinal gradient. For instance, the geographical distribution of the bivalve *Macoma balthica* is restricted in Southern Europe because of its thermal tolerance (Jansen et al., 2007), although its ecological function is similar to that of the estuarine bivalve *Scrobicularia plana* that inhabits southern European estuaries. The latter species belongs to the *Scrobicularia plana–Cerastoderma edule* community that is commonly described in Atlantic Iberian estuaries and is composed by euryhaline organisms, such as the polychaetes *Hediste diversicolor*, *Streblospio shrubsolii*, *Oligochaeta* spp., the gastropod *Hydrobia ulvae* and the isopod *Cyathura carinata*, amongst other species (Borja et al., 2004; Silva et al., 2006; Viéitez, 1976).

Estuarine communities are not static entities in estuaries but represent smooth transition between assemblages, implying serial trends rather than a zonation (Ysebaert et al., 1998, 2003). In turn, the

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http://dx.doi.org/10.1016/j.marpolbul.2013.03.024

occurrence of a species along an ecological continuum or its confinement to a limited range in a gradient is commonly observed in ecosystems, as explored by ter Braak (1986) when developing canonical correspondence analysis. In the case of the estuarine gradient, some authors argue in favor of the existence of truly brackish species (Remane, 1934), while others support the marine or freshwater origin of these species able to colonize brackish environments (Barnes, 1989; Cognetti and Maltagliati, 2000). The development of an estuarine index based on key indicator macrobenthic species with a limited range of distribution within the estuarine gradient appears feasible for characterizing estuarine environments. In this sense, Simboura and Zenetos (2002), propose to define habitat types using indicator species that are dominant or exclusive of a specific habitat.

The aim of the present study was to identify a number of macrobenthic species for developing an estuarine index as a proxy for recognizing the estuarine environment. The index should rely on the relative occurrence and site-specific dominance of key indicator estuarine species. Several transformations of the raw species data set are considered in order to assess the performance of the index. It is hypothesized that the estuarine index is significantly different (i.e., not constant) along an estuarine gradient.

2. Methods

2.1. Study sites

Five estuaries on the Iberian Atlantic coast were sampled to find key indicator macrobenthic estuarine species. The following estuaries were considered (Fig. 1): the Guadiana estuary at the southernmost border between Portugal and Andalusia (Spain), the Tagus estuary (central Portugal), the Lima estuary (northern Portugal), the Minho estuary in the northernmost border between Portugal and Galicia (Spain) and the estuarine region of the Ria de Pontevedra (Galicia, NW Spain). The data related to these estuaries are characterized by different geographical locations, sample sizes and volumes, number of replicates, sampling spatial extent and times of sampling. The information regarding the sampling sites is summarized in Table 1.

2.1.1. The Guadiana estuary

The Guadiana estuary is approximately 70 km long, has a maximum width of 800 m and a mean depth of 6.5 m. The estuary is mesotidal, with mean tidal amplitude of 2 m (Chícharo et al., 2001). It is partially mixed estuary with a typical maximal turbidity zone (Garel et al., 2009). The estuary is surrounded by large unpopulated areas. Anthropogenic pressure is low, except at the mouth of the estuary where urban settlements are located (Chícharo et al., 2001). The beginning of the operation of the Alqueva dam in February 2002 introduced major environmental changes because of the influence of the Alqueva dam in the freshwater inflow into the Guadiana estuary (Chícharo et al., 2006). Additionally, the estuary was affected by a drought that lasted for 2 years (in accordance to the National Water Institute, INAG, http://snirh.inag.pt/), before year 2001.

2.1.2. The Tagus estuary

The Tagus estuary is located in the centre of the Portuguese coast. It is one of the largest estuaries in Europe (approximate length, 50 km, and maximum width, 15 km). The Tagus is a partially mixed mesotidal estuary with a tidal range varying from 1 m at neap tides to 4 m at spring tides. Most of the southern bank is dominated by intertidal mudflats and large salt marshes (França et al., 2009; Rodrigues et al., 2006). Fifteen sampling sites were considered in the southern bank of this estuary.

2.1.3. The Lima estuary

The Lima estuary is located on the northern coast of Portugal. It is part of the river Lima hydrological basin, which is mainly composed of a granitic basement. The river Lima originates in Galicia and extends for 108 km in a NE–SE direction. The tidal limit of the Lima estuary, which is a partially mixed mesotidal estuary, is located approximately 20 km upstream (Sousa et al., 2006). Sampling was carried out during ebb tide (the lowest low tide limit was approximately 1.2 m above the datum). Three sites were considered from an estuarine region at approximately 2.5 km away from the estuary mouth, in the southern bank. The sampling sites comprised one sampling station in the upper intertidal zone within a salt marsh located at 60 cm from the mean high water tidal level. Two additional intertidal sampling sites outside of the salt marsh were located at 135 cm and 195 cm under the mean high water tidal level respectively.

2.1.4. The Minho estuary

The estuarine part of the river lies between Portugal and Galicia (Spain). The Minho estuary has mesotidal features and is partially mixed, although it tends to be a salt wedge estuary when high flooding occur (Sousa et al., 2005). A number of studies have highlighted the low level of anthropogenic pressure on the Minho estuary. Thus, Reis et al. (2009) recommended using this estuary as a pristine reference site. Ten sites were sampled, including intertidal sites near the main axis of the estuary, a salt marsh and an estuarine island.

2.1.5. Ria de Pontevedra

The Ria de Pontevedra is located at the NW of the Iberian Peninsula. The estuarine area is located in the innermost part of the Ria, delimited upwards by the fresh water limit of the Lérez River outflow, flowing in a granitic basin (Cobelo-García and Prego, 2003). Sampling was conducted, in the southern banks of the estuary in the vicinity of the Lézez River mouth. The sampling sites were influenced by the river outflow, by an industrial freshwater effluent form a paper mill and by inflowing seawater.

2.2. Sampling methods and laboratorial procedures

The corer used for sampling, sampling depth and number of replicates per site were variable depending on the estuarine system (Table 1). All samples from the five estuaries were sieved through a 1 mm mesh. The retained material was preserved in 70% ethanol. Benthic animals were sorted under a dissecting microscope and identified to the lowest taxonomic level.

2.3. Statistical analysis and index calculation

The identification of indicator species along the estuarine gradient was a key part of the current study. In order to determine structural association between species along the estuarine gradient, the Jaccard coefficient (Jaccard, 1908) was used for R-mode analysis (association between species) of the entire set of species observed in the above mentioned five estuaries. Species only observed in one estuary were excluded from the analysis. The
R-mode analysis was carried out prior to transformation of the species presence–absence data (Legendre and Legendre, 1998).

After establishing the degree of association between species, complete linkage was considered in a hierarchical clustering, because space-dilating (or conserving) methods are preferred to single linkage along an ecological continuum (Legendre and Legendre, 1998). In cluster analysis, species were grouped in accordance with the values of the Jaccard coefficient. An arbitrary threshold of

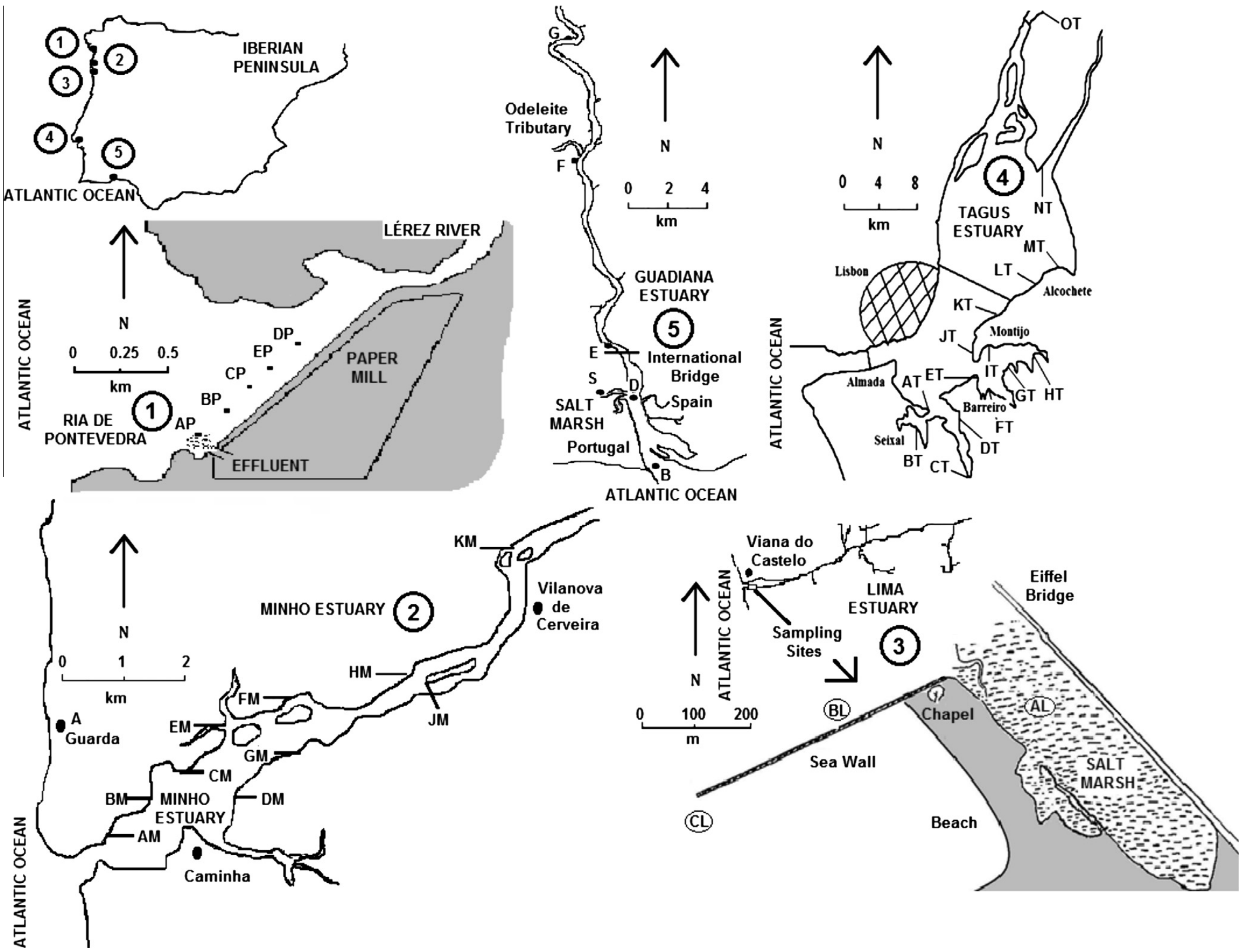

Fig. 1. Location of the estuaries considered in this study on the Atlantic coast of the Iberian Peninsula and sampling sites within each estuarine system.

Table 1
Summary of the characteristics involved in the sampling for macrofaunal species in the five estuarine systems considered for analysis. Note that salinity is a dimensionless variable (e.g., Stickney, 2009).

Estuary	Corer area (cm^{-2})	Corer depth (cm)	Sampling sites (n)	Replicates per site (n)	Sampling extension (km)	Sampling dates (mm/yy)	Salinity range (–)
Guadiana	400	30	6	6	30	10/00 and 02/01	30–0.5
Tagus	70	30	15	7	40	09/09	35–0.4
Lima	70	25	3	5	0.5	05/10	20–30
Minho	70	25	10	7	13	08/10	18–0.0
Ria de Pontevedra	480	15	5	5	1	02/97	31–25

association was chosen to form groups (Clarke and Warwick, 1994; Legendre and Legendre, 1998), with the precondition of having ecological meaning. NonMetric Multidimensional Scaling (NMDS) was used to indicate the association and grouping of the species under analysis. The scores of the first axis of the NMDS ordination were plotted against the relative abundance of the indicator species in each site to depict the relationship between the two variables. The computations were carried out with free R statistical software (R Development Core Team, 2009) and the Vegan package (Oksanen et al., 2006).

An estuarine index ranging in the interval [0,1] can be proposed after finding the macrobenthic indicator species associated with the estuarine environment. Three approaches are described below.

2.3.1. Estuarine index as a conditional probability

The probability that an individual occurs in a site in the estuary (event A), given the occurrence of an indicator species (event B), also called conditional probability $P(A|B)$, is equal to the probability of occurring in a given site and the occurrence of an indicator species occurs, or $P(A \cap B)$, divided by the probability of an indicator species occurring:

$$P(A|B) = \frac{P(A \cap B)}{P(B)} \quad (1)$$

The probability $P(A)$ is calculated for each site as the proportion of individuals found in that site in relation to the total number of individuals from the whole set of sites. The probability $P(A \cap B)$ is calculated by multiplying $P(A)$ by the proportion of indicator species in the site considered. The probability $P(B)$ is equal to the sum of the probability $P(A \cap B)$ over the whole set of sites. The estuarine index calculated as a conditional probability will be designated hereafter as CP.

2.3.2. Estuarine index as a ratio of vector norms

The concentration index of Simpson (1949) is equal to the probability that two randomly chosen organisms belong to the same species. For a large number of organisms and q species, the concentration index of Simpson may be expressed as follows (Legendre and Legendre, 1998):

$$\text{Concentration} = \sum_{i=1}^{q} p_i^2 \tag{2}$$

where p_i is the proportion of the ith species in a site. The formula of the concentration index resembles the mathematical expression used to calculate the norm of a vector (or vector length) under the Pythagorean Theorem:

$$\text{Norm} = \sqrt{\sum_{i=1}^{q} x_{ij}^2} \tag{3}$$

The estuarine index as a ratio of vector norm is calculated as the norm of the vector formed by the abundance of the indicator species divided by the norm of the vector associated with the abundance of all species observed in a site. The estuarine index calculated as a ratio of the vector norm will be designated hereafter as VN.

2.3.3. The estuarine index calculated analogously to IndVal

The indicator value index (IndVal, Dufrêne and Legendre, 1997) considers the relative abundance of a species (specificity, A_{ij}) and its relative frequency of occurrence (fidelity, B_{ij}) in a group of sites identified by cluster analysis, belonging to a particular habitat or to any experimental conditions. The indicator value index of Dufrêne and Legendre (1997) may be modified to calculate in the interval $[0, 1]$ an estuarine index where species i is the sum of the abundance of the indicator species in a site and cluster j is each of the replicates from a sampling site:

$$\text{Ind} = A_{ij} * B_{ij} \tag{4}$$

2.4. Index significance and data transformations

In order to avoid large discrepancies in the values of the estuarine index among the different calculations, the ranging method of Sneath and Sokal (1973) was used. This method adjusts the magnitude and the variability of the descriptors (Legendre and Legendre, 1998). The modified value x_i is calculated as the current value X of CP, VN or Ind with the minimum value ($x_{\min}$) subtracted and divided by the range ($x_{\max}$–$x_{\min}$):

$$x_i \frac{x - x_{\min}}{x_{\max} - x_{\min}} \tag{5}$$

After ranging the estuarine index, a statistical test can be applied in order to find significant differences (H_0: the estuarine index is a constant). The significance level was set at 5%. In order to overcome problems related to the test assumption, we opted to apply a non-parametric permutational multivariate analysis (PERMANOVA) (Anderson, 2001). In this context, PERMANOVA is applied to 2-dimensinal vector where the first component is the value of the index and the second component represents its complement value in the form $(x, 1-x)$, where x are the values of CP or VN after ranging in replicates of each site considered in the test. PERMANOVA also enables post hoc analysis based on an uncorrected permutational test for multiple testing (Anderson, 2001, 2005), if a significant difference is found by the omnibus test. The significance of the tests is based on Monte Carlo P-values (Anderson and Robinson, 2003).

The estuarine index based on the CP and VN approaches can be tested, because a value is produced for each replicate of a site. However, the estuarine index based on the Ind approach cannot be tested, because it has a unique value per site. The value of the estuarine index based on the CP and VN approaches for each site will be provided as the mean value of the index over the replicates for each site.

The calculations explained until now are computed on the raw data matrix. However, such data matrixes for species usually consist of pre-transformed data in ecological studies with different aims such as downweighting the values of the species with excessive abundance, stabilizing their variances or providing a linear relationship among the variables (Borcard et al., 2011; Clarke and Warwick, 1994; Legendre and Legendre, 1998; Quinn and Keough, 2002). Four different ways of pre-transforming the raw data matrix were considered prior to conducting the calculations, with the aim of studying the performance of the estuarine index. For this purpose, species abundances were pre-transformed by square root and logarithmic transformations, i.e. log $(x + 1)$. In addition, the Hellinger transformation was used as an alternative method of transforming the relative (rather than individual) species abundance by considering the abundance of the remaining species (Borcard et al., 2011; Legendre and Gallagher, 2001). Analysis of the same data under different transformations may reveal any influence of untransformed data on the results. This approach is analogous to that suggested by Quinn and Keough (2002) for comparing standardized and non-standardized data in ecological studies. Pearson's correlation was used to assess the degree of correlation between the raw and transformed values of the estuarine index.

2.5. Examples using artificial and real data sets

An artificial data set is presented to illustrate the performance of the estuarine index along an environmental gradient. The indicator species are represented in a unimodal distribution along the environmental gradient with an optimum centered in its middle. Additional species are added with different abundance values as a function of their location in the environmental gradient, although respecting the unimodal criteria.

Two sampling campaigns conducted in the Guadiana estuary in autumn 2000 (also used in the R-mode analysis) and in summer 2011, respectively, were used to assess the performance of the estuarine index in a real data set.

3. Results

3.1. The estuarine index applied to an artificial data set

The estuarine index based on CP, VN and Ind calculations was applied to an artificial data set (Table 2). The artificial data comprised 15 species and 15 sites (five replicates per site) resembling an environmental gradient, as for instance an estuary. The first two species in Table 2 had a unimodal distribution along the gradient with an optimum between sites 7 and 9. The remaining species were more abundant towards the ends of the gradient, except species 10 (Sp 10, Table 2). The estuarine index based on CP and VN was applied to the replicates of the sites that showed sensitivity to fluctuations in abundance (Fig. 2a). The unimodal distribution of the indicator species is also indicated by the shape of the curves, although their profiles are not exactly equal. The environmental gradient is accurately described. The estuarine index was also

Table 2
Artificial data set representing an environmental gradient in which the first two species in the table (Sp1 and Sp2), simulating indicator species, are present at maximal abundances in the middle of the gradient. The maximum abundances of the remaining species occurred towards the edges of the gradient, except for species that are present at maximal abundances in the middle of the gradient. Abundance is represented as the mean value of five replicates (see Fig. 2a).

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
Sp1	0.2	0.2	1	1.8	2.8	4.8	5.8	7	7.6	8.8	7	2.8	1.8	1	0.4
Sp2	1	1.8	2.8	4.8	5.8	7	7.6	8.8	7	2.8	1.8	1	0.4	0.4	0.4
Sp3	5.8	7	7.6	8.8	7	2.8	1.8	1	0.4	0.4	0.4				
Sp4	7	2.8	1.8	1	0.4	0.4									
Sp5	8.8	7	2.8	1.8	1	0.4									
Sp6	7	2.8	1.8	1	0.4	0.4									
Sp7	2.8	1.8	1	0.4											
Sp8	1.8	1	0.4	0.4	0.4										
Sp9	1	0.4	0.4												
Sp10					1.8	2.8	4.8	1.8	1						
Sp11							1	1.8	2.8	4.8	5.8	7	7.6	8.8	7
Sp12								1	1.8	2.8	4.8	5.8	7	7.6	8.8
Sp13									1	1.8	2.8	4.8	5.8	7	7.6
Sp14										1	1.8	2.8	4.8	5.8	7
Sp15											1	1.8	2.8	4.8	5.8

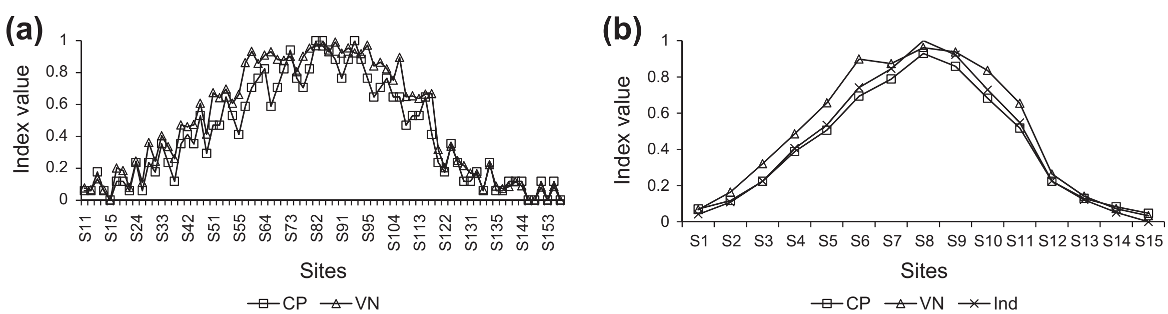

Fig. 2. Curves for the estuarine index applied to an artificial data set (a) over the replicates for each site (b) as a single value per site expressed as CP, VN and Ind calculations.

applied to render a unique value for each site, so that the index based in Ind calculations is also included (Fig. 2b). The curves accurately represent the artificial gradient, although they were of similar but not equal shapes. Interestingly, the VN curve showed a departure from the CP and Ind values of the index for most of the sites (Fig. 2b).

3.2. Indicator species

The ordination representing the association of species based on the Jaccard coefficient prior to cluster analysis is shown in Fig. 3. The NMDS ordination had a stress value of 0.17, representing an acceptable fit (Clarke and Warwick, 1994). Four groups of species associations are depicted, representing a gradient from oligohaline species (group at the left side of the plot) to marine-influenced species (the groups on the right of the plot). The central group comprises euryhaline species. From the latter group, the polychaete *H. diversicolor* and the isopod *C. carinata* formed an association at a lower level of dissimilarity in the cluster analysis (Jaccard coefficient = 0.33) as the association between the bivalve *S. plana* and the gastropod *H. ulvae* (Jaccard coefficient = 0.39). In addition, both associations were also linked together in cluster analysis at a low level of dissimilarity. This relationship is shown by the proximity among the four species in the ordination (Fig. 2). The four species were found in all estuaries considered in the analysis and they were observed in more than half of the overall sampling sites (minimum of 53% of occurrence for *S. plana*), with a maximum of 80% of occurrences in the case of *H. diversicolor*. Although the four species appear to be good candidates as indicator species of the estuarine environment, the gastropod *H. ulvae* was excluded, to balance the contribution of the indicator species between phylum, feeding habits and motility. Moreover, *H. ulvae* was extremely abundant in the Tagus estuary and Ria de Pontevedra but was much less abundant in the other estuaries. Therefore, the isopod *C. carinata* (a predator), the polychaete *H. diversicolor* (an omnivore) and the bivalve *S. plana* (a deposit-feeder; for the feeding type of these species, see for instance Ysebaert et al., 2003) were chosen to develop the estuarine index. The three species are referred to hereafter as CHS indicator species. The relationship between the relative abundance of the sum of the CHS indicator species and the values of the

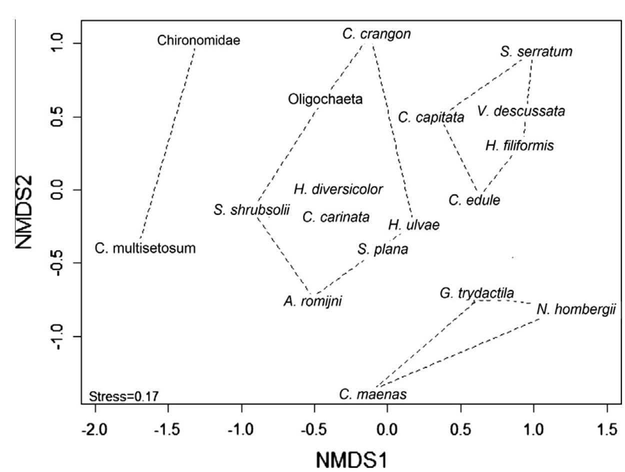

Fig. 3. NMDS ordination showing the association between species from the five estuaries considered in this study. The species association (R mode analysis) is based on the Jaccard coefficient. The grouping in the plot corresponds to those groups found by cluster analysis (complete linkage).

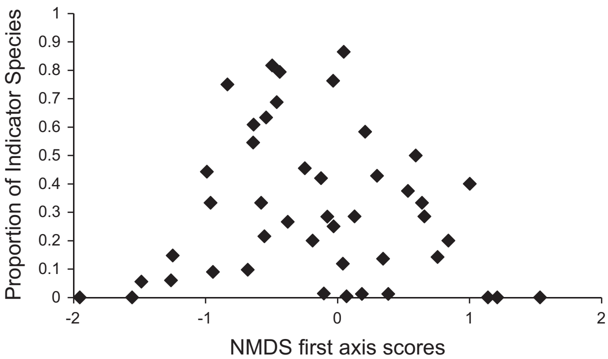

Fig. 4. Scatter plot of the relative abundance of the CHS indicator species at each site within the estuaries considered in the study against the NMDS sites scores of the first axis of the ordination for the association between the species (see Fig. 3).

NMDS first axis scores for each site is shown in Fig. 4. Considering that the NMDS ordination (based on binary data) grouped species in accordance with an estuarine gradient, the CHS indicator species tended to dominate in the assemblages belonging to the central part of the gradient (Fig. 4). The indicator species are progressively substituted by others towards the assemblages located in both marine and freshwater reaches of the gradient.

3.3. The estuarine index applied to a real data set

Data on intertidal macrofaunal assemblages from the Guadiana estuary were used to assess the performance of the estuarine index. In the sampling survey carried out in autumn 2000, 36 species were recorded, fewer than in the summer 2011, when 49 species were recorded, 21 of which were common to both sampling surveys. The CHS indicator species were observed on both sampling occasions (Table 3). The polychaete *H. diversicolor* occurred in the same sites in both years, the isopod *C. carinata* was observed in all sites, but was distributed differently depending on the year of sampling; the bivalve *S. plana,* which was mainly observed in autumn 2000, was absent from sampling sites B and G.

The values of the estuarine index based on CP, VN and Ind calculations applied to both the raw and transformed data from autumn 2000 are shown in Fig. 5. The values of the index suggest that the estuarine environment was located approximately between sites D and G. The shape of the curves based on the index is generally different for both the type of calculation and transformation used, although the curves tended to converge in the case of the Hellinger transformation (Fig. 5d). The log transformation of the raw data attributed a maximum for the estuarine index based on the CP and Ind calculation at the site S, while this maximum was found in site E in the other cases. Interestingly, the values of the index were the same for sites B, D and G, irrespectively of the calculation used. The results for the raw and transformed data of the summer 2011 campaign are shown in Fig. 6. The shape of the curves is very different in relation to the autumn 2000 sampling survey. The extremes of the estuarine environment are not as clearly indicated as in the former case, mainly in the upper reaches of the estuary (site G). The index no longer suggests an estuarine environment comprised between sites D and G as in the autumn 2000 sampling survey, but expanded beyond these sites. The maximum values for the estuarine index coincided at site D for all the calculations in the summer 2011. The minimum values were related to site F, where the Odeleite tributary flows into the estuary (Fig. 1). The curves based on the CP and Ind calculations rendered similar profiles, except in the case of the Hellinger transformation (Fig. 5d). The curve based on VN calculations were of different shapes, and in most cases were associated with higher values of

Table 3
Species observed in the sampling sites of the Guadiana estuary in 2000 (0), 2011 (1) or on both occasions (0|1), listed in alphabetic order. The total number of species observed in each site for each year is shown at the bottom of the table. The CHS indicator species are shown in bold type.

Species	B	D	S	E	F	G	Species	B	D	S	E	F	G
Abra sp.	0	0	0				*Limnodrilus hoffmeinsteri*						1
Alkmaria romijni			0|1	0	0		*Malacoceros fuliginosus*	1	1				
Anomia ephippium	0|1						*Melita palmata*	0	0|1	0			
Aricia foetida	1						*Mysella bidentata*	1					
Balanus sp.	1						*Nematoda* ind.	1		0|1			
Bittium reticulatum	0|1	0	0				*Nephthys hombergii*	0	0				
Bowerbankia sp.			1				*Obelia* sp.	0			1		
Braquiura ind.	1						*Oligochaeta* n.id			0			
Capitella capitata	0		0	1			*Owenia fusiformis*	0|1	0				
Caprella sp.			0				*Palaemon macrodactylus*					0	1
Cerastoderma edule	0|1	0|1	0	0			*Paragnathia formica*	1					
Chamelea gallina	0						*Pectinaria auricoma*	1	1				
Cirratulus cirratus	1	0	0				*Phronis psammophyla*	0	0				
Corbicula fluminea						1	*Phyllodoce mucosa*	1					
Corophium acherisicum	0	0	0	0			*Pinnotheres pisum*	0	0				
Corophium bonnellii	1						*Piscis* ind.				1		
Corophium orientale				1		1	*Planaria* ind.	1					
Corophium sp.						0	*Platyhelminthes* n.id				0		
Crangon crangon	1	0	0|1	0	0		*Retusa truncatula*	0	1	0			
Cyathura carinata	0|1	0|1	0|1	0|1	0	1	*Rissoa* sp.	0	0	0			
Diopatra neaplitana	0|1	0	0				*Ruditapes decussata*	0|1	0|1	0			
Glycera trydactila	0		0	0			***Scrobicularia plana***		0|1	0|1	0	0	
Gobio sp.					1		*Sigambra parva*	1					
Haminoe sp.			0|1				*Solem marginatus*	1					
Hedistes diversicolor		0|1	0|1	0|1	0|1	0|1	*Sponge* ind.					1	
Heteromastus filiformis	0|1	0|1	0	0			*Streblospio shurbsolii*		1	0|1		0	0
Hinia sp.	1						*Syllidia armata*	1					
Hydrobia ulvae	1	0|1	0|1	0|1		1	*Tanais dulongi*	1					
Kellia suborbicularis	0	0|1					*Tetrastema fozensis*		1				
Lekanesphera monodi	1	0|1					*Upogebia pusilla*				1		
Leptocheirus pilosus		1	1	1			*Venerupis aurea*	0	0	0			
Observed species 2000	22	22	25	11	7	3	Observed species 2011	28	16	11	9	3	7

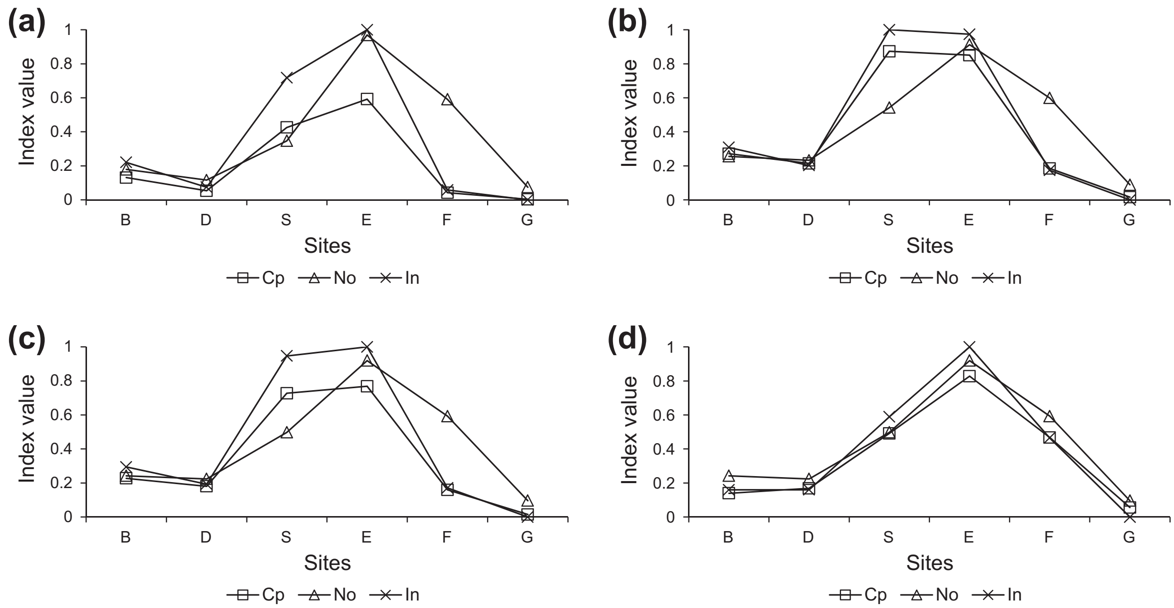

Fig. 5. Curves derived from the estuarine index calculated as CP, VN and Ind applied to the sampling sites of the Guadiana estuary, autumn 2000, showing the effects of the transformations on the shape of the curve: (a) raw data, (b) log transformed, (c) square root transformed, (d) Hellinger transformed data.

the estuarine index. In addition, the curve based on VN calculations decreased more smoothly than the other curves from the site D towards the upper estuary. The square root and Hellinger transformation applied to the VN calculations provided the same results for the estuarine index (Figs. 5 and 6). The correlation between the values of the estuarine index for each transformation in relation to value of the index calculated with the raw data is shown in Table 4. The square root and logarithmic transformations yielded the closest correlations with the index calculated on the raw data. The poorest correlation corresponded to the Hellinger transformation, especially with the CP and Ind calculations. The VN calculations were most closely correlated with the values of the estuarine index based on untransformed data.

The discrepancies in the curves profiles are reflected in the results of the PERMANOVA test applied to the estuarine index (Table 5). The test applied to the Hellinger transformation based on the CP calculations showed that the index provided equal results for more sites than other transformations. Moreover, the results for the Hellinger transformation based on the CP calculations showed inconsistencies in the pairwise comparisons for both years (Table 5, Figs. 5 and 6), mainly involving site F. The test for pairwise comparisons applied to the square root and logarithmic transformation based on the CP calculations was more consistent with the raw data. The test applied to the estuarine index based on the VN calculations produced the same results, regardless of the transformation used on the data. It is worth noting that the

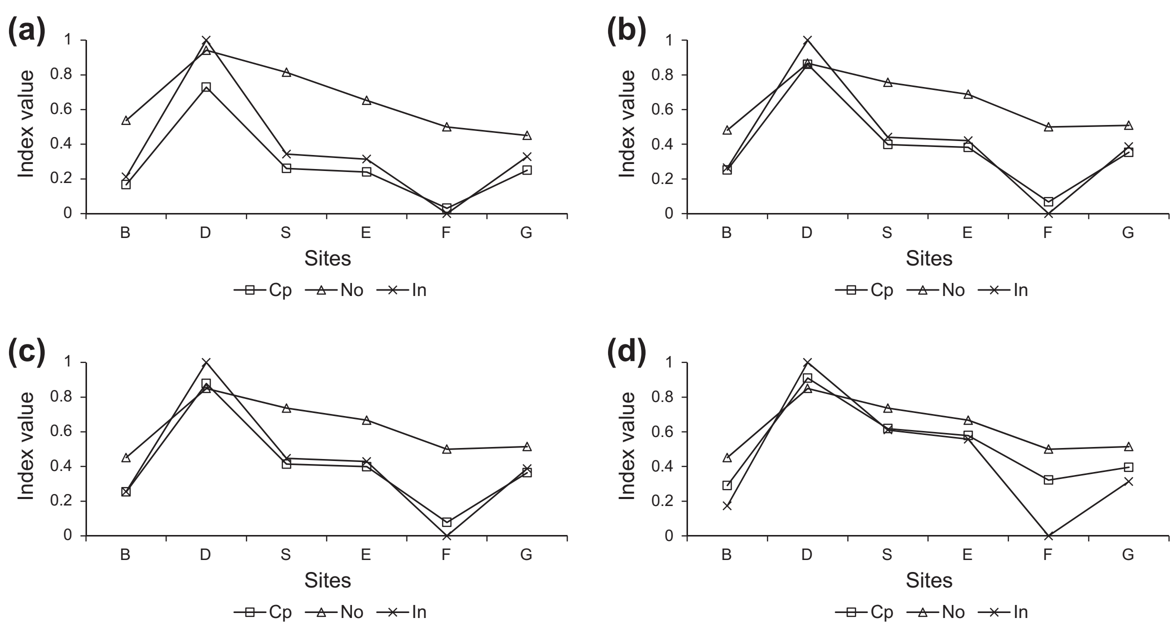

Fig. 6. Curves derived from the estuarine index calculated as CP, VN and Ind applied to the sampling sites in the Guadiana estuary, autumn 2011, showing the effects of the transformations on the shape of the curve: (a) raw data, (b) log transformed, (c) square root transformed, (d) Hellinger transformed data.

Table 4
Pearson correlation between the values of the estuarine index (expressed as CP, VN and Ind) for each transformation in relation to the values of the index calculated from the raw data. Transformations: L = Logarithmic, SR = square root, H = Hellinger.

Year	Index	L	SR	H
2000	CP	0.965045	0.977875	0.846023
2011	CP	0.992477	0.991374	0.905598
2000	VN	0.970277	0.983051	0.983051
2011	VN	0.968466	0.953386	0.953386
2000	Ind	0.967526	0.979777	0.890384
2011	Ind	0.990769	0.989242	0.920325

Table 5
Results of the PERMANOVA test applied to the estuarine index on CP and VN calculations for data collected in 2000 and 2011 (italic type) in the Guadiana estuary. Post hoc comparisons show sites with equal values of the index. Transformations (Transf): N = None, SR = Square root, L = Logarithmic, H = Hellinger. MS = Mean squares.

Year	Index	Transf	MS	$F_{5,35}$	*P*	Post hoc comparisons
2000	CP	N	3533.89	25.39	0.001	D = F, S = E
2000	CP	SR	6119.76	46.14	0.001	B = D = F, S = E
2000	CP	L	8068.93	58.24	0.001	B = D = F, S = E
2000	CP	H	5108.88	16.89	0.001	B = D = G, B = D = F, S = F
2011	*CP*	*N*	*3352.65*	*26.32*	*0.001*	*B = S = E = G*
2011	*CP*	*SR*	*4267.52*	*36.26*	*0.001*	*S = E = G*
2011	*CP*	*L*	*4155.02*	*36.49*	*0.001*	*B = G, S = E = G*
2011	*CP*	*H*	*3270.23*	*8.55*	*0.001*	*B = F = G, S = E = F, E = F = G*
2000	VN	N	7154.29	20.51	0.001	B = D = G, S = F
2000	VN	SR	5521.85	15.95	0.001	B = D = G, S = F
2000	VN	L	5517.09	16.49	0.001	B = D = G, S = F
2000	VN	H	5521.85	15.95	0.001	B = D = G, S = F
2011	*VN*	*N*	*2243.07*	*2.45*	*0.059*	–
2011	*VN*	*SR*	*1474.29*	*2.21*	*0.075*	–
2011	*VN*	*L*	*1549.6*	*2.33*	*0.069*	–
2011	*VN*	*H*	*1474.29*	*2.21*	*0.07*	–

omnibus test did not detect any differences in the values of the index based on the VN calculations for 2011, suggesting that the estuarine limits were not within the sampling area considered.

4. Discussion

The proposed estuarine index appears to be an adequate proxy for assessing the estuarine limits in Iberian estuaries. As such, the CHS indicator species were mainly distributed in the central part of the estuarine gradient, and they disappeared towards both ends of the estuaries considered in this study. The indicator species *C. carinata*, *H. diversicolor* and *S. plana* belong to the "*Scrobicularia plana–Cerastoderma edule* community", described on the Iberian Atlantic coast (Thorson, 1957). Other shallow shelf communities such as *Venus fasciata*, *Abra alba* and *Tellina tenuis* communities are better represented in subtidal areas under a higher marine influence (Muxika et al., 2007). By its turn, the "*Scrobicularia plana –Cerastoderma edule* community" is indeed extensively reported as typical from intertidal areas under estuarine influence on the Atlantic Iberian coast (Borja et al., 2004; Chainho et al., 2006; Silva et al., 2006; Muxika et al., 2007; Viéitez, 1976; this study).

Although the CHS indicator species were found in association with each other, they are not always found together in the transition from estuaries to both limnetic and full marine communities. For instance, Chainho et al. (2006) found the sequence *S. plana*, *H. diversicolor*, *C. carinata*, respectively, in a gradient of decreasing salinity in the Mondego estuary, Portugal. However, Rodrigues et al. (2011) described the sequence *C. carinata*, *S. plana*, *H. diversicolor*, respectively, from polyhaline to oligohaline zones of the Ria de Aveiro, Portugal. This study showed that *C. carinata* tended to occurs in assemblages with marine influence, *H. diversicolor* was found at the uppermost sampling station in both years and *S. plana* tended to occur in the central part of the estuary (Table 3). Additionally, the distribution of the HSC indicator species was not static in the Guadiana estuary when considering both years of sampling, except in the case of *H. diversicolor*, which was sampled at the same sites on both occasions.

Between-year variability in the index is expected because estuaries are markedly subjected to environmental fluctuation (Vernberg, 1983). However, a decreasing trend in the values of the index from its maximum toward the edges of the estuarine environment must be always observed. In this sense, the curve associated to the estuarine index is related to ecological models that describe changes in species composition along the estuarine gradient, such as the ecocline model proposed by Attrill and Rundle (2002) or the Attrill model (2002). Likely, the estuarine index may be correlated with abiotic parameters such as salinity. Attrill (2002) found a quadratic relation between the mean mid-tide salinity and the mean salinity range, resembling the shape of the curve of the estuarine index in the Guadiana estuary for 2000 (Fig. 5), also found for the artificial data set (Fig. 2). Nonetheless, this relation must be carefully explore in order to find the best correlation between the estuarine index and salinity because the latter may be measured and expressed as mean salinity, salinity range, mid-tide salinity or as interstitial salinity at a site. By its turn, the relation of the index with the ecocline model (Attrill and Rundle, 2002), implies a zero value in the marine and freshwater domains, while the estuarine index should show values in the interval $[0,1]$ within the estuary, where both ecoclines meet and vanish in opposite directions.

The sites chosen to apply the estuarine index should not include specific areas of the estuary under additional environmental pressure. For instance, in site F in 2011, the values of the index were minimal, probably because of the freshwater flow from the Odeleite tributary. Another example may be a site near a source of organic pollution where species that colonize organically enriched sediments, such as capitellids or spionid polychaetes (Pearson and Rosenberg, 1978), may artificially lower the index. Also important is that the curve associated with the estuarine index may be biased, providing additional ecological information. For instance, in 2011, the curve indicates a more pronounced gradient from the maximum value of the estuarine index (site D, Fig. 6) towards the mouth of the estuary and a smoother transition between the estuarine and freshwater communities in a landward direction.

Although the present study focused on Iberian estuaries, other sets of indicator species may be used to apply the index in estuaries of other latitudes or to a different gradient in the same latitude. For instance, *M. balthica* usually occurs in northern European estuaries (Bocher et al., 2007; Jansen et al., 2007), and therefore it may replace *S. plana* in the index. In fact, data from Ysebaert et al. (1998) suggest that the bivalve *M. balthica*, the amphipod *Corophium volutator* and the polychaete *H. diversicolor* might be potential indicator species for the Ems estuary. Moreover, *Corophium* spp. (Gamito et al., 2010; Queiroga, 1990) are potential indicator species between tidal freshwater and oligohaline sections of Iberian estuaries.

The estuarine index based on the different methods of calculation is able to detect the estuarine environment. However, the index based on the CP and Ind calculations was more similar than the values obtained by the VN calculations, although in some cases, similar curves were reached with all three kinds for calculations (Fig. 5d). The CP and Ind calculations take into consideration the CHS indicator species in relation to the whole set of sampling sites, while the values of the index based on the VN calculations only considered the relative contribution of the CHS indicator species in a single site. Therefore, in a site where only the CHS species are found in very low numbers, the CP and Ind calculations would yield low values of the index, while the index based on the VN calculations would yield much higher values (see for instance the case of site F in Figs. 5 and 6). Ecologically, this may be interpreted as the existence of an environmental constraint that reduces the occurrence of species due to environmental stress, but among the tolerant species a relative high number of the CHS indicator species are recorded. For instance, site F located near the Odeleite tributary may be under a higher saline stress (high salinity range, Attrill, 2002), thus explaining the low value of the index based in CP and Ind calculations. It must be stress that the value of the index for the three calculation methods converges to zero when the true limit of the CHS indicator species is reached at the extremes of the gradient (Figs. 2 and 4).

The transformations used prior to calculating the estuarine index rendered different results (Figs. 5 and 6). The Hellinger transformation (Legendre and Gallagher, 2001) provided the lowest correlation with the values of the index based in raw data (Table 4), and therefore squared root or logarithmic transformations are preferred. However, the transformation appeared to be less effective for the VN calculations, as similar results were obtained (Table 5). In ecological analysis, data are transformed to reduce the discrepancies of species abundances (Clarke and Warwick, 1994), thus balancing the abundance between common and rare species (Field et al., 1982).

Finally, the use of relative abundances to calculate the estuarine index may be important to minimize the potential effect introduced by different sampling sizes in studies conducted in the same area at different times, as in the case described above for the Guadiana estuary. Moreover, the ranging method (Sneath and Sokal, 1973) provides a common scale, thus facilitating comparisons. This advantage may allow the estuarine index to be used for historical data of different authors from a single estuary. A potential comparison between estuaries may be conducted if a variable correlated with the estuarine index, such as salinity or the relative distance from the estuary mouth, is found to be adequate for standardizing the estuarine index values. In such cases, the effect of sample size must be taken into consideration, as this would result in a higher standard error of the mean (Crawley, 2007) of the index, i.e. estimation of the index may be different from the population parameter in studies with small sample sizes (Quinn and Keough, 2002), therefore preventing comparisons being made.

5. Conclusions

The proposed estuarine index may be used as a proxy to identify the limits of Iberian estuaries. The index may assist in defining types of water bodies in accordance with the WFD guidelines. The identification of the CHS indicator species was essential to develop the index within the estuarine limits. The index considers the biological population structure and composition of estuarine macrobenthic assemblages, resulting in an alternative characterization of transitional waters that are also compatible with the mandatory features included in the WFD (annex II; European Community, 2000). Furthermore, the use of a minimal set of indicator species in the identification of the estuarine environment is 'practical and ecologically relevant' in accordance to the WFD. When physical criteria to define the estuarine boundaries are used, such as the tidal limit in the freshwater reaches, and they do not fit the limits found by the estuarine index, a surface water sub-division may be established, if necessary, between the latter boundaries because 'discrete and significant elements' are meaningful delineations of 'bodies of surface water' in agreement with the WDF (European Commission, 2002). The consistency between the CP and VN calculations and those provided by the Ind values based on a solid theoretical framework (Dufrêne and Legendre, 1997) increase the confidence in the usefulness of the index. The different calculations used to reach the values of the index based on CP or VN approaches render different results. While the CP calculation considers the relative abundance of the CHS indicator species in a site in relation to the whole set of sampling sites, the VN calculations only consider the relative abundance of the CHS indicator species in a single site. Therefore, the calculations used to determine the estuarine index may be carried out by both methods in order to complement the information that would be lost by using only one method of calculation. Transformation of the raw data helps to improve the index performance and overcomes the discrepancies related to species abundance. The simple square root and logarithmic transformations appear to be an adequate choice to improve the performance of the index. The estuarine index may be a versatile tool to be used in further research studies such as those devoted to establish the estuarine limits based on seasonal fluctuations, sea-level rise on a long term basis or comparisons in the profile of the index between estuaries standardized by the relative distance of the sampling sites from the mouth of the estuary or by using values of salinity.

Acknowledgements

This study was partly funded by the Project "Study of environmental conditions in the Guadiana estuary and adjacent coastal area", LNEC (subcontractor CCMAR), BIOTEJO (funded by the Lisbon Port Authority, APL) and by the University of Vigo. The first author was also funded by the Portuguese Foundation for Science and Technology (SFRH/BD/48928/2008).

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