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The importance of habitat-type for defining the reference conditions and the ecological quality status based on benthic invertebrates: the Ria Formosa coastal lagoon (southern Portugal) case study.

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Abstract

Coastal lagoons are complex systems, with considerable habitat heterogeneity and often subject to high temporal dynamics, which constitutes a great challenge for ecological assessment programs. For defining reference conditions for benthic invertebrates, under the EU Water Framework Directive objectives, historical data from the Ria Formosa leaky lagoon (wet surface area of about 105 km^2) located in Southern Portugal was used. The influence of habitat features, such as channel depth, sediment type and seagrass cover, on the expression of these biological communities was inferred by analysing subtidal data collected at stations with different environmental characteristics. Such heterogeneity effect was analysed at the community compositional and structural levels, and also for three indices included in a multimetric Benthic Assessment Tool (BAT). This tool for the assessment of ecological status includes the Margalef index, Shannon-Wiener diversity index, and AZTI's Marine Biotic Index (AMBI). Significant variations associated with environmental features were reflected on specific reference conditions at four habitats in the lagoon. After habitat calibration, the Benthic Assessment Tool (BAT) revealed that, in general and for the period of time covered by this historical data set, the status of the lagoon corresponded to a good ecological condition, which is mainly due to its high water renewal rate. Such classification is in accordance with the majority of studies at the lagoon. However, at punctual sites with human induced high water residence times, significantly lower BAT values were registered. Such community degradation can be associated with physical stress due to salinity increase and to a degradation of water quality, with occurrence of occasional dystrophic crisis, triggered by low water renewal. Habitat

differentiation was a crucial step for a correct evaluation of the ecological condition of invertebrate communities across the lagoonal system.

Keywords: Coastal lagoons, habitat heterogeneity, ecological assessment, multimetric indices, BAT – Benthic Assessment Tool, Water Framework Directive (WFD)

1. Introduction

Coastal lagoons are “inland water systems connected to the ocean by one or more restricted inlets that remain open at least intermittently, and have water depths which seldom exceed a few meters” (Kjerve, 1994). These shallow water systems have been classified as transitional waters (TW) by most of the European countries, especially in the Mediterranean basin and in some Baltic countries (McLusky and Elliott, 2007). However, other countries classified them as coastal waters (CW), namely Portugal (Bettencourt et al., 2004). Coastal lagoons may be regarded as singular water bodies within the Water Framework Directive (WFD) goals, since they usually do not present a clear salinity gradient and frequently are not substantially influenced by freshwater. Tagliapietra and Ghirardini (2006) preferred to use the term ‘transitional environments’ or ‘transitional habitats’ and Pérez-Ruzafa et al. (2010) denominated coastal lagoons as “transitional ecosystems” between transitional and coastal waters. The location of coastal lagoons between land and sea subjects them to strong anthropogenic pressures due to tourism and /or heavy shellfish/fish farming (Alliaume et al., 2007). Diffuse pollution is an addition threat, mainly through agricultural and/or industrial effluents and domestic sewage drainage from their catchment areas (Alliaume et al., 2007).

Under the WFD implementation several problems and constrains arose associated to the natural large environmental variability of aquatic systems. As explained above, the categorization of some water systems as TW or CW is sometimes dubious and difficult, particularly for coastal lagoons. Before ecological quality status (EQS) assessment, the water systems must have been classified not only into different categories, such as TW or CW, but also their typology and the different water bodies within each system must have been previously defined (Vincent et al., 2002). For the division of TW and shallow CW into relatively homogenous water bodies, Ferreira et al. (2006) proposed a methodology based on three aspects: salinity and morphology as natural component; a normalized pressure index and an eutrophication symptoms classification. Within these waterbodies there is however a mosaic of habitats (Gamito, 2008) and, while in the end the ecological status must be reported at the water body level (Vincent et al., 2002), reference conditions to determine that EQS need to be defined accounting for the type of habitat features that will influence biological communities (de Paz et al., 2008; Muxika et al., 2007; Teixeira et al., 2008a). Therefore, if within a water body different habitats are to be monitored, then reference conditions that reflect the expected natural biological communities at each habitat should be defined (Teixeira et al, 2008a).

By the imposition of the WFD, the ecological EQS of the main water bodies has to be defined. Several methodologies have been proposed for the different ecological components, and for benthic invertebrates one of the methodologies is the Benthic Assessment Tool (BAT) (Teixeira et al., 2009), a multivariate metric based on the Margalef (1958), the Shannon-Wiener diversity (Shannon and Weaver, 1963) and AMBI (AZTI Marine Biotic Index, Borja et al., 2000) indices. The results of the application of this tool were comparable to the results of the application of other multimetric indices adopted by different European countries, and therefore the methodology was approved in the last intercalibration exercise (Carletti and Heiskanen, 2009). BAT was adopted by Portugal to assess the ecological quality of coastal and transitional waters using macroinvertebrate communities.

The Ria Formosa is a mesotidal leaky lagoon, located in Southern Portugal. The lagoon has five sand barrier inlands and six inlets. The tidal amplitude ranges from 3.6 m on spring tides to 1.0 m on neap tides, which causes important semidiurnal and fortnightly tidal amplitude variations. The lagoon geomorphology and the tidal amplitude allow important diurnal water exchanges with the ocean. Consequently, the water residence time is short, with an estimated average time of 1.5 days (Saraiva et al., 2007). However, upstream locations present higher residence times due to irregular tidal flushing throughout the lagoon (Tett et al., 2003). In these locations, residence time can reach an average of 2.4 days (Mudge et al., 2008). The salinity in the main tidal channels varies between 32 and 36.5 throughout the year (Newton and Mudge, 2003), with occasional lower values due to run-off episodes, and higher values at the inner locations due to intense evaporation and lower water renewal rates. The lagoon has a wet area of approximately 105 km^2, which comprises the tidal channels with seagrass beds (26.7 km^2), extensive intertidal areas with salt marshes (35.7 km^2), intertidal bare sediments (28.5 km^2), salt-pans (11.5 km^2) and fish farms (2.6 km^2) (Meireles, 2004). The seagrasses *Zostera noltii*, *Zostera marina* and *Cymodocea nodosa*, dominate the intertidal mudflats and the shallow subtidal (Cunha and Santos, 2009; Cunha et al., 2009). The Ria Formosa, with this large wet area, together with the sand-barrier islands and the back terrestrial lands, covering a total area of 184 km^2, constitutes a National Park since 1987. The park is also a Ramsar site since 1980, and an important bird area, which denotes its environmental importance.

Over the last decades, the resident population around the lagoon and its hydrographic basin has increased by almost 60%, from 100 thousand inhabitants in 1970 to 160 thousand in 2001 (Rodrigues, 2004). Every summer, the population increases significantly due to tourism. Consequently, the anthropogenic pressures on the system have increased, mostly in the vicinity of the main cities. High levels of bacteria, nutrients, metals and organochlorine compounds were detected in several areas of the Ria Formosa, mainly in the surroundings of the main cities (Bebianno, 1995). The benthic invertebrate composition also reflected the degraded environment near the main cities (Austen et al., 1989). Recently, Redondo-Gómez et al. (2009), reported the presence of high concentrations of heavy metals near the vicinity of Faro airport, although in other areas of Ria Formosa the concentration of metals in the water column is low (Caetano et al., 2007).

Five waterbodies have been identified in the Ria Formosa coastal lagoon (Ferreira et al., 2006) resulting essentially from the morphology and drainage system patterns of the dendritic tidal channels; and also from the variation of chlorophyll a and dissolved oxygen concentrations, acting as indicators of state of nitrogen and phosphorus pressure. According to these authors, one of the waterbodies, located in the eastern side of Ria Formosa presented a lower water quality. Three of these water bodies, located in the center and in the western side of the lagoon, were covered by the present study.

During the last decades of the 20th century, several researchers carried out extensive sampling of benthic invertebrates in the Ria Formosa (Gamito, 2008 and references there in). The objective of this study was to use the historical data gathered to a) define significantly distinct habitats within the lagoon from a WFD assessment perspective; b) establish habitat specific reference conditions for the subtidal soft-bottom macroinvertebrate communities; and finally c) test the behaviour of a WFD compliant multimetric method - the BAT, using an additional dataset, including data on relevant pressures in the lagoon, such as a decrease on water renewal, to validate the method.

2. Methods

The data analyzed and discussed in detail in Gamito (2008) was used to select two datasets (Table 1): one dataset including reference sites (RC) to establish habitat specific reference conditions; and a validation dataset (VS) including both impacted and undisturbed sites to test the adequacy of uni and multiparametric indices to assess the ecological quality status of the Ria Formosa (Fig. 1). Two additional sites, sampled in 2006, were added to the VS dataset

(Table 1). Only subtidal soft-bottom stations were considered, namely among those sampled by Gamito (2006) and Calvário (1995). The criteria used in stations selections for each dataset are explained in section 2.2.

2.1. Dataset description

Different sampling areas and methods were applied. Gamito (2006) sampled four to six sediment replicates with a 0.011m^2 corer in four water reservoirs of salt-pans and a tidal mill, every two months between January 1985 and December 1986 (Station 1 to 4). Later on, using the same methodology, sampling was carried out on a fifth water reservoir (Station 5), monthly between 1996 and 1997. In this reservoir eight replicates were taken at each sampling occasion. These artificial habitats present similar conditions to those in the outside tidal channels, except when the water is not daily renewed (Gamito, 2006). In station 1 the water was partly renewed only once every fourteen days, while in stations 3 and 4 the water was renewed during the spring tides; in stations 2 and 5 the water was renewed every day. In station 1, large variations on salinity occurred, from 13 psu to more than 80 psu; the occasional occurrence of high BOD5 and chlorophyll a concentrations, mainly in station 3, indicated some environmental degradation (Gamito, 2006).

Calvário (1995) seasonally sampled five subtidal stations along the Faro channel (Station 6 to 10) in 1989, using a van Veen grab of 0.05 m^2. At each station, six replicates were taken, covering an area of 0.3 m^2. Later, in 1990, Calvário (1995) also sampled, with a corer of 0.015 m internal diameter, every month in spring tides, near the spring low water level: Seagrass bed (Station 11) (possibly *Zostera* spp. or *Cymodocea nodosa)*, sandy mud (Station 12), muddy sand (Station 13) and sand banks (Station 14). In each habitat and sampling occasion the total area sampled was of 0.3 m2.

More recently, in 2006, two subtidal stations (Stations 15 and 16) were sampled in two shallow channels near the area of the 1990s Calvário (1995) campaigns. Sampling took place in two occasions, winter and autumn, and at each station three replicates were collected using a van Veen grab (0.05 m^2), covering a total sample area of 0.15 m^2 per station.

All teams used a 1 mm mesh sieve. Taxa not belonging to invertebrate fauna were eliminated from the data matrix, as well as rapid moving invertebrates such as shrimps and mysids, which were collected by hazard, without the appropriate sampling methods. Truncation rules recommended by the Northeast Atlantic Geographical Intercalibration Group, within the WFD intercalibration exercise (Borja et al., 2007), were followed, such as removal of fauna characteristic of rocky substrates (fauna which was attached to small stones or shells), the agglomeration of all oligochaete taxa to subclass level, priapulida to class level, and Nemertea, Platyhelminthes, Echiura, Sipuncula and Phoronida to phylum level.

All teams analysed sediment granulometry to characterize the sampled stations, despite that at some stations sediment data was only collected in few sampling occasions. Sediment samples were washed in hydrogen peroxide solution to destroy organic matter, and then rinsed and dried. The dried residue was sieved into a column of several sieves of decreasing mesh size. The percentages of gravel, sand and mud were calculated as: >2 mm fraction, 63 µm - 2 mm and <63 µm, respectively (Holme and McIntyre, 1984). Sediment samples were classified according to Folk (1974) diagram. For the remaining occasions sediment type was assumed to be the equivalent to that previously registered in that site (Table1). In stations 11-14, since sediment information was available for all sampling occasions, the coefficient of variation (CV, defined as the ratio of the standard deviation to the mean) was assessed to understand the degree of grain size variability associated to those stations.

One special characteristic of Ria Formosa is the low residence time of the water in the main tidal channels. However, in the upstream locations the residence time is higher (Newton and Mudge, 2003; Tett et al. 2003) as well as in some of the water reservoirs studied by Gamito (2006). For this study the residence time in each station was not determined but an ordinal scale, varying from 0 to 4, based on previous knowledge on water dynamics in the lagoon was elaborated to stand as a proxy of water renewal. Station 1, where water was only partially renewed every 15 days (Gamito, 2006), received a 4. Station 3, with a manual tidal gate, received a 3, and station 4, with an automatic tidal gate a 2. Stations 2 and 5, where water was renewed every day, received a 1. All other stations (6 to 14), sampled in the tidal channels of the Ria Formosa, received a 0 (Table 1).

2.2. Approach to reference conditions definition

According to previous studies in the Ria Formosa, several benthic habitats may be described: tidal channels, seagrass beds, sandbanks and mudflats, to which distinct benthic assemblages are associated (Gamito, 2008 and references there in). In order to propose adequate habitat specific reference conditions, the differences between those benthic invertebrate communities were evaluated to verify if they clearly reflected on the biological parameters to be assessed under the WFD: species richness, patterns of abundance and taxonomical composition. To achieve that, and since no pristine conditions can be found in the lagoon, sites corresponding to low impact situations were used (Table 1: Reference sites RC dataset). These sites were representative of the most common subtidal biotopes, for which environmental data to characterize the habitat was available (sampling occasions with real data measurements). For the selected RC sites (Table 1), the most important environmental features identified as determinant for benthic communities' distribution and expression of WFD parameters were related with biological data as described below.

First, a multivariate approach was used in order to detect possible patterns of benthic invertebrates related with these environmental parameters and allow habitat definition. Since different data sources were used, to reduce problems of inaccurate identifications and also data matrix size, species were assembled into family level prior to data analysis. Abundances were square-root transformed to reduce the weight of dominant taxa. Non metric multidimensional scaling (nMDS) was applied to community data, at both species and family levels (Bray-Curtis similarity on abundance data previously transformed) and the RELATE analysis was used to test for hypothesis of no relation between multivariate pattern from the two resemblance matrices (species and family). Then a permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001; McArdle and Anderson, 2001) was applied to test for the significant effect of habitat features on the lagoon invertebrate communities, at the familiy level. Three factors were considered: depth (fixed, 2 levels: shallow; channel); seagrass coverage (fixed, 2 levels: seagrass; bare bottom); and type of sediment (fixed, 3 levels: sand, muddy sand; sandy mud). Significant terms and interactions were investigated using *a posteriori* pair-wise comparisons with the PERMANOVA *t*-statistic, using 9999 permutations of residuals under a reduced model, with an *a priori* chosen significance level of $\alpha = 0.05$. For a number of possible permutations under 150, the Monte Carlo p-values were considered.

Secondly, the variance of community structural parameters across the defined habitats was studied using the ecological indices Margalef, Shannon-Wiener and AMBI as metrics of the WFD required features. Data on absolute numbers of the different species per sampling period, were used to determine the Margalef diversity index (1958), following Gamito (2010) recommendations. Shannon-Wiener diversity (Shannon-Weaver, 1963) and AMBI (Borja et al., 2000) were also applied to the same data set; since these indices are based on relative proportions it is indifferent if data is in absolute numbers or in densities. Then, each ecological index variance was tested for significant effects of habitat related features. For these univariate analyses, the same experimental PERMANOVA design described previously for community multivariate analysis was used.

The effect of sample area was evaluated using a community structural parameter highly dependent on the sampling effort, species richness. For the effect a Spearman rank correlation was applied, after removing stations under higher physical pressure (stations 1, 3 and 4).

Finally, environmental features with significant effect on the variance of benthic invertebrate structural features communities guided the definition of habitat-specific reference conditions for each metric. Since the sites considered for establishing reference conditions are not pristine but already reflect some degree of anthropogenic influence, reference values for Margalef and Shannon-Wiener indices were settled considering the 95 percentile encountered in the dataset according to significant variations of indices across habitats. For the AMBI, even though it varies in a fixed scale between 0 and 7 (Borja et al., 2000), since invertebrate communities at the lagoon are subjected to potentially higher water retention times, leading to natural organically enriched conditions (Gamito, 2008), the reference values were adjusted to reflect the expectations regarding faunal composition of such biological communities. The Bad status reference was defined as the worst possible value to obtain with each index.

2.3. Ecological quality status (EQS) assessment

Previous to the ecological quality assessment, the new sites included in the VS dataset (stations 1, 3, 4, 15 and 16) were assigned to pre-defined habitats using environmental characteristics. The potential discriminating environmental variables (Table 1) describe the morphological characteristics of the system and were selected for being variables likely to influence invertebrate distribution. The significance of such environmental features for sound habitats' definition was evaluated using a stepwise forward discriminant analysis (DA) (Alpha-to-Tolerance = 0.05 and Alpha-to-Remove = 0.05). Four factors were considered: channel depth, mud content and sand:mud ratio, included as continuous variables; and seagrass presence or absence, treated as categorical variable. Continuous variables were standardized previous to analysis. The objective was to test the similarity of the habitat groups suggested by biological data according to the above environmental descriptors, and use the resultant discriminant functions to predict the probability of a new site belonging to one of the pre-defined habitats. Statistical analysis was performed using the software Statistica 7.

After having the habitats clearly defined, BAT methodology was applied (as described in Teixeira et al., 2009) to assess the EQS based on benthic macroinvertebrates at the Ria Formosa during the study period. For BAT determination, both reference sites (RC) and validation sites (VS) were used (Table 1). To calculate the Ecological Quality Ratio (EQR) *sensu* BAT, data on ecological indices was organized according to the relevant habitat types and using habitat-adjusted reference conditions. To establish a correspondence between the EQR and classes of EQS, the thresholds presented in Teixeira et al. (2009) were adopted: 0 ≤ Bad ≤ 0.27; 0.27 < Poor ≤ 0.44; 0.44< Moderate ≤ 0.58; 0.58 < Good ≤ 0.79; and 0.79 < High ≤ 1. Spearman rank correlation analysis was applied to evaluate the contribution of each index for the final EQR obtained across habitats. The final EQR was also tested for the influence of season effect using Kruskal-Wallis test, after removing stations under higher physical pressure (stations 1, 3 and 4). Sample occasions were attributed to the most adequate season resulting on the following data redistribution: winter n = 30; spring n = 23; summer n = 24; and autumn n = 24.

To extract overall patterns of samples distribution, a Detrended Canonical Correspondence analysis (DCCA) was carried out with community data (partial RC and VS sites, at family level), and environmental features data information (Table 1) (CANOCO version 4.5). Only some stations were used in this analysis since the environmental data was collected only for some sampling occasions.

3. Results

3.1. Invertebrate communities' structural distribution patterns

The global data matrix included a total of 241 taxa, after truncation, organized in 118 families. In the data subset of 32 stations with environmental information, 90 different families were registered. In the RC data subset of 21 stations, 85 families were registered. Stations differed on sediment type, percentage seagrass cover and depth (Table 1) and nMDS ordination of invertebrate community's abundances, at the family level, by station reflected also some of these differences (Figure 2). The deepest stations were projected in the right side of the diagram and shallower stations in the left side (Figure 2). Also, only shallow areas registered seagrass. Vegetation associated benthic invertebrate communities are slightly separated from bare bottom ones. Sediment type seems to take a secondary role in structuring communities after these first two parameters. The patterns exhibited did not differ whether invertebrates communities were treated at family or species level (RELATE analysis: Spearman Rho = 0.907, p = 0.01). While testing for the significant effect of these three habitat features in the invertebrate families distribution (Table 2), some interactions were not possible to test due to limitations on factors combination, e.g., when testing for *Depth* x *Vegetation* effect, since seagrass level of factor vegetation does not occur within deep depths level. Nevertheless, the PERMANOVA corroborates figure 2 displayed, pointing to a significant effect of depth in the families abundance distribution in the lagoon and also to a significant interaction between factors vegetation and sediment in their distribution. Channel depth seems to be the first determinant of the type of invertebrate communities that will settle, acting independently of other factors. Additionally, pair-wise comparisons revealed that, in similar sediment conditions, the presence of seagrass would contribute to have different benthic communities, but within seagrass beds, associated only with finer sediment bottoms, communities also differed depending on the presence of more or less muddier sediments (Table 2). At bare bottoms, either of deep or shallow channels, invertebrate communities also change significantly across the three sediment types considered.

To account for the effect of data collection heterogeneity on the results, the number of species was analyzed in function of sample area (Table 1), and no significant correlation was found (Spearman Rank r = 0.101, *p*-value = 0.314, n = 101 after removing most impacted stations: Stations 1, 3 and 4).

Similarly to community data, some significant variations were also observed in the ecological indices related with the three studied habitat features, depth, vegetation and sediment type (Fig. 3). The Shannon-Wiener index only presented significant differences between vegetated and bare bottom benthic communities (pseudo-F = 5.3, $p = 0.04$). Despite the maximum value was registered for bare bottom communities, the index presents however greater variability in these habitats, while seagrass beds tend to present less deviant and higher mean equitability values (Fig. 3a). According to the PERMANOVA, Margalef index showed no significant depth effect (pseudo-F = 0.35, $p = 0.543$), but showed a significant interaction between the other two factors (pseudo-F = 26.8, $p = 0.0004$), sediment type and vegetation. The presence or absence of vegetation interferes significantly with the species richness in muddier sediments, with much higher Margalef values within seagrass bottoms comparatively to bare bottoms (*t*-test = 11.2, *p* (MC) = 0.0007) (Fig. 3b). On the other hand, considering slightly coarser sediments (muddy sand) Margalef values are significantly higher in bare bottoms than in seagrass beds. If the sediment type is compared within each vegetation level, then at seagrass level, muddy sand bottoms are poorer than sandy mud ones (*t*-test = 7.6, *p* (MC) = 0.0018); while in bare bottom communities the opposite was observed, with sandy mud ones being significantly poorer than muddy sand bottoms. Within bare bottom coarser sediment types no significant differences regarding species richness were found ($p > 0.05$) (Fig. 3b). The AMBI was the only index that showed a significant effect of channel depth but dependent on the type of sediment (pseudo-F = 7.0, $p = 0.016$). A significant interaction between sediment type and vegetation seems also to influence this index values in the lagoon (pseudo-F = 8.7, $p = 0.011$). It was not possible to test for the three-way interaction significance since no vegetation is present at higher depths. Pair-wise tests showed that for sand sediments significantly lower AMBI values were registered at higher depths (t-test = 3.9, p (MC) = 0.012) (Fig. 3c). While at shallow habitats, lower values of AMBI seem to be associated with muddy sand environments comparatively to either sand (t-test = 2.6; p = 0.041) or sandy mud ones (t-test = 3.3, p = 0.009). If we consider additionally the effect of vegetation, the pair-wise tests showed that in muddy sand sediments the communities present higher AMBI values at seagrass beds than at bare bottoms (t-test = 3.1, p = 0.047). On the other hand, at bare bottoms, significantly higher AMBI values are found at sandy mud environments comparatively to either sand (t-test = 2.2; p = 0.040) or muddy sand ones (t-test = 3.4, p = 0.023).

Overall, as expected, the highest values of Margalef index were found in a seagrass bed (St 11), where an average number of 74 different taxa were registered *per* sampling occasion. The station with the second highest richness was the sandy station (St 14), with an average number of 47 different taxa. However, Shannon-Wiener values of station 11 were comparable to other seagrass beds or to stations at bare bottoms. In general, higher AMBI values tend to be enhanced by the combination of shallow depths with finer sediments and absence of vegetation.

In agreement with benthic invertebrates distribution patterns across habitats (Fig. 2) and accounting also for the significant variation of the selected ecological indices across them (Figure 3), reference conditions to assess ecological quality status of such communities in the lagoon are proposed as presented in Table 3. As described in the methodological approach, for significant groups the 95 percentile values were adopted regarding Margalef and Shannon-Wiener indices; while the AMBI reference values were adjusted to better reflect the expected community composition at shallow coastal lagoons habitats. In general, it was observed that an important proportion of the communities across all habitats at the lagoon was constituted by tolerant species (ecological group EG III) (Figure 4), which are more adapted to the natural environmental fluctuations in the lagoon (Gamito and Furtado, 2009). At muddier sediment habitats (H2 and H4) there was no clear dominance of sensitive species (EG I and EG II) over the remaining groups, and as expected, the presence of opportunistic species (EG IV and V) was of considerable importance; even at seagrass beds (H2) were EG I and II were very well represented. Accounting for these natural patterns, the values adopted as reference condition for AMBI were established to approximately reflect such distribution of ecological groups (Table 3).

3.2. Ecological quality assessment across habitats

Once invertebrate community's patterns of distribution in the lagoon have been evaluated and habitat-specific reference conditions have been adjusted, the new dataset was used to validate such reference conditions and test the BAT multimetric performance. To do so, the new sites were first allocated to one of the previously defined habitats according to their environmental characteristics. The results of the stepwise discriminant analysis (Table 4) revealed that the environmental descriptors selected could discriminate between three of the habitat groups revealed by biological data. The best subset of descriptors was the combination of factors: channel depth / mud content / sand:mud ratio (lowest Wilks $\lambda = 0.0236$); while the presence or absence of seagrass seems to be a poor discriminant variable, redundant with one of the previous variables (low tolerance value = 0.000). In fact the analysis indicated that using just these environmental features, shallow seagrass habitat (H2) did not differ from the other shallow habitats (H2/H3: Squared Mahalanobis distance = 3.18, F = 3.02, $p = 0.063$; and H2/H4: Squared Mahalanobis distance = 3.67, F = 2.16, $p = 0.136$). Nevertheless, and since biological data, from both community analysis and ecological indices, were significantly influenced by the presence of seagrass such aspect was maintained for habitat proposal. These environmental variables allow for a correct assignment of approximately 76% of the sites to a habitat and the new sites were distributed across habitats as indicated in Table 5.

The BAT method integrated the three indices with a factor analysis, using the pre-established High and Bad reference conditions (Table 2) to define the space distribution of the sampling stations values (Teixeira et al., 2009). After accounting for habitat heterogeneity, the EQR variation can be interpreted from Figure 5. Despite that not all three indices that constitute BAT were strongly correlated between each other (*d vs*.H': r = 0.73; *d vs*. AMBI: r = - 0.37; H' *vs*. AMBI: r = - 0.28), Pearson correlations showed that they were all significantly and strongly correlated with final BAT EQR (*d*: r = 0.85; H': r = 0.82; AMBI: r = - 0.58; all *p*-values = 0.000 for n = 137). The EQR results did not present significant differences between seasons (Kruskal-Wallis Test statistic = 3.7, *p*-value = 0.299).

Independently of habitat, benthic invertebrate communities of stations under low water renewal conditions presented the worse ecological status, and the higher the residence time the lower the EQR exhibited. At St 1 (Figure 5c), where extreme environmental variation and increased salinity stress were observed (Gamito, 2006), poor ecological condition was registered by the BAT at some sampling occasions. In seagrass beds, EQS indicates some degradation in stations 3 and 4 (Figure 5b). In these stations the slightly higher water retention time increases variation of some environmental parameters such as salinity and BOD5, and occasional dystrophic episodes were observed in station 3 (Gamito, 2006). In the two artificial water reservoirs with the least effect of low water renewal (Table 1) the indices did not detect signs of physical stress but still AMBI mean values pointed to slightly disturbed situations with unbalanced benthic communities (St 2 AMBI mean BC = 2.8; St 5 AMBI mean BC = 2.4). All remaining stations assessed in the lagoon oscillated between Good and High status, according to BAT.

For some of the stations it was observed a slight EQR oscillation through time, sometimes leading to EQS class change (Figure 5), despite that no particular change associated with anthropogenic disturbances was documented. When the coefficient of variation of sediment grain size (sand:mud ratio) at stations 11, 12, 13 and 14 was measured and compared with the coefficient of variation of these stations' EQR, it was observed that those with higher sediment variability through time were also those with higher EQR variability (Table 6).

The joint analysis of families and environmental data emphasized the importance of residence time in stations differentiation (Figure 6). The increasing residence time is related with physical stress imposed artificially to some locations through water renewal regulation by means of tidal gates. In fact, the first axis of DCCA analysis differentiated the stations with the highest residence time, in the left side of the ordination diagram, from the deepest stations, in the right side. The second axis differentiates the stations with seagrasses from the bare bottoms. A positive correlation is observed between depth and the sand:mud ratio, which means that deeper stations had higher sand content. A positive relation is also visible between seagrass cover and mud content.

The bivalve *Abra segmentum* was common in all habitats, being one of the dominant species in almost all of them (Table 7). In habitat 1 the density of organisms was low (average density of 443 ind.m−2) when compared with the other habitats, with no clear dominance of one or two species. In the perturbed seagrass habitats (stations 3 and 4), with a relatively lower water renewal and a tendency to organic matter accumulation (Gamito, 2006), the polychaete *Capitella capitata* was one of the dominant species. This species was also dominant in the sandy station, considered to be subjected to physical stress (Gamito, 2006, 2008), due to very low water renewal and high variation of the environmental parameters.

4. Discussion

The diversity of benthic invertebrates in the Ria Formosa is high when compared with other lagoonal systems or transitional waters, with the highest diversity found on seagrass beds (Gamito, 2008). As pointed out in this work, most of the benthic fauna is common to the Abra communities of estuarine and sheltered regions (Thorson, 1957) or of the "biocenose lagunaire euryhaline and eurytherme" (Pérès and Picard, 1964). In seagrass beds species diversity is higher, with approximately the double of the average number of species found in bare bottoms. The meadows create an above ground three dimensional structure that traps fine sediments and provide habitat for several faunal species; their bellow ground rhizome network stabilizes sediment and create favorable conditions for diverse infaunal organisms (Boström et al., 2006, Fredriksen et al., 2010). Nevertheless, the natural deposit-feeders dominance increases AMBI values, erroneously pointing out to a degraded habitat.

In sandy bare bottoms, the opposite occurs; there is a lower number of species but a dominance of suspension-feeders (Gamito, 2008; Gamito and Furtado, 2009). In this habitat, the diversity decreases but the AMBI values also decrease and consequently the reference values for EQS evaluation must be different. Muddy sediments should also be considered separately since, in these habitats, the benthic invertebrate communities are dominated by deposit feeders and the number of species is naturally lower than in sandy sediments, except if associated with seagrass beds, where high species richness occurs (Gamito, 2008; Gamito and Furtado, 2009).

Independently of habitat type, the BAT methodology was sensitive to some degradation of the environmental conditions due to increased water retention time. The location with extreme physical stress (St 1) exhibited high AMBI values as a response to the lower ecological condition observed under increased salinity stress due to very low water renewal, leading to an abundant presence of small opportunistic species. Likewise, at two other stations where water retention time was also quite high (St 3 and St 4), the lower BAT values denote some benthic community degradation triggered by poor water quality, where occasional dystrophic crisis occurred (Gamito, 2006). At the two artificial water reservoirs with the least effect of low water renewal (St 2 and St 5) the level of stress imposed to benthic communities might not have been strong enough to cause them severe impoverishment.

Results point out, in general, to a Good ecological status of the Ria Formosa, which is mainly due to its high water renewal rate. However, no locations near pollution sources were analyzed. The classification obtained was based on historical data, thus limiting the type of data available. A completely independent validation of the results was not possible since part of the validation samples belonged to new sampling occasions at some of the reference sites, and no sites representative of all type of pressures harassing the system were available. Using benthic invertebrates as environmental quality indicators, Austen et al. (1989) and Hubert et al. (2006) pointed out to localized degraded areas, close to sewage outflows and to semi-intensive fish farms, which exhibited low diversity values and were dominated by small opportunistic deposit feeders. Since the period when the main sampling campaigns in this study took place, several sewage treatment plants have been built in the Ria Formosa and, although an increase of human population has occurred, some of the pressures may have decreased, and general conditions may have improved in relation to total organic loads.

The short water residence time in the majority of Ria Formosa wet area allows a good water renewal and the prevention of degraded conditions. Nobre et al. (2005) classified Ria Formosa as being in good ecological status due to the short water residence time that did not allowed emergence of eutrophication symptoms in the water column. In a recent study based mainly on phytoplankton, macroalgae and seagrass of two sampling sites, Goela et al. (2009) classified the lagoon as being in good to high EQS. Martins et al. (2009) concluded that the physical limitation due to a short residence time is the main factor controlling primary production in the Ria Formosa. The authors state that a generalized eutrophication situation is improbable, and that only the shallow inner small channels present some risk of eutrophication. Overall, the EQS in Ria Formosa, during the period here analysed, was between Good or High, and this classification indicates the same trends as other classifications based on other WFD quality elements such as the phytoplankton.

The inclusion of a number of metrics allows modelling several community aspects based on theoretical expectations at specific conditions, and therefore, as our results show, the metrics are not necessarily expected to correlate well with each other; on the contrary, redundancy should be avoided and instead all metrics should contribute with useful information for the final assessment. In addition, the use of multimetric tools allows overcoming the sensitivity of single metrics by combining several indices (Buckland et al., 2005; Teixeira et al., 2008b). For example, in the BAT, the sensitivity of AMBI to an accumulation of organic matter due to natural causes, such as at seagrass bottoms habitat, can be balanced by the index that accounts for the species richness. Faunal composition of healthy benthic communities from naturally organic enriched sediments, and especially at stabilized seagrass beds such as *Zostera* spp., do not reflect the theoretical model for the expected distribution of ecological groups at unpolluted situations as described by Borja et al. (2000) after modification of Hily (1984), Hily et al. (1986) and Majeed (1987) models. At these habitats, the relative proportion of abundance of ecological groups is more evenly distributed, with no clear dominance of sensitive species (EG I) over the remaining groups, and also with a typical presence of opportunistic species (EG IV and V) (Fig. 4). In fact, the habitats defined at the Ria Formosa present different characteristics and the separation by habitats, for the purpose of defining reference conditions before BAT application, allowed for a more accurate definition of their ecological status. Different reference values have already been adopted or proposed by some countries for specific habitats, such as Bulgaria for Shannon-Wiener diversity index, several Mediterranean countries for BENTIX and M-AMBI application, and Germany and the Netherlands for Shannon-Wiener and AMBI (Carletti and Heiskanen, 2009). The establishment of reference conditions is a key process and should be habitat-specific in order to properly reflect natural benthic gradients (Blanchet et al., 2008, Dauvin et al. 2007, de Paz et al., 2008; Puente et al., 2008; Teixeira et al., 2008a).

5. Management considerations in the scope of WFD application

5.1. *Habitat stability*

At sites with generally healthy benthic invertebrate communities, the results revealed an oscillation of EQR through time, occasionally corresponding also to a shift on quality class classification (Fig. 5). Although caution is needed due to lack of representativeness, the analysis of sediment stability at those stations, near the Ancão outlet (Figure 1: St 11 to 14), revealed that benthic communities EQR oscillated more at stations with higher coefficient of variation regarding sediment grain size characteristics. Since no other source of punctual disturbance was determined, this might be partially related to natural habitat dynamics. The implications of this in the framework of an environmental monitoring and assessment plan are evident, especially if a specific classification determines whether or not action would have to be undertaken by managers. In naturally unstable habitats however it will become impracticable for any assessment method to cope with systems natural dynamics, as the responses of unbalanced communities often mimic those of natural variability. One way to overcome such frailties will be to conduct adequate monitoring of the events that might determine benthic communities' natural shifts. For example, in the case of coastal lagoons formed due to a highly dynamic barrier-island system, such as the Ria Formosa lagoon, a greater natural variability in the biotic communities at the most dynamic habitats will be expected. In fact, the spatial change of inlets and islands alters the hydrodynamics of the lagoon and induces a substrate disturbance that is likely to alter habitats characteristics, namely seagrass distribution (Cunha et al., 2005). Among other aspects, the distance to the disturbance was found to determine the level of impact on the studied habitats (Cunha et al., 2005) and hence, the same would be expected for the associated invertebrate communities.

5.2. *Habitat specific reference conditions*

The variability associated with habitat features such as depth, sediment type and seagrass cover requires that, within each water body, habitat heterogeneity is evaluated and accounted for. Therefore, when defining the reference conditions for the classification of water bodies based on benthic invertebrates, habitats should be considered as another level of assessment, which can even be established across water bodies. For the five water bodies proposed by Ferreira et al. (2006), the same set of reference conditions can be used for any given habitat type that appears at the pre-established water bodies of the lagoon.

When defining reference conditions, one usually reports to specific habitats monitored under specific conditions, since the values obtained will also be influenced by the survey techniques employed. This study, since it used historical data to propose reference conditions for Ria Formosa coastal lagoon, had to cope with different sampling methods, where differences in sampling devices, areas, seasons, periods, etc, were registered. Ideally this background noise should not be present in the establishment of reference values for assessment purposes. Despite that, on the final assessment, no apparent influence was found in any structural parameter highly dependent on sampling effort, such as species richness, neither on sampling season, further studies across the pre-defined habitats are necessary to confirm trends revealed by the present study.

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Table 1
Main environmental characteristics of Ria Formosa sampling stations and stations' codes.

Dataset	Station	Number of samples (codes for sampling occasions)	Sample area (m^2)	Environmental characterization					
				Depth (m)	Seagrass (%)	Sediment type	Organic matter (%)	Residence time	Reference
Reference sites (RC) (n= 21 samples)	2	3 (a – January; b – March; c – May 1985)	0.066	0.8	90	Muddy sand	[a]	1	Gamito (2006)
	5	1 (i – January 1997)	0.088	1.5	0	Muddy sand	4.2	1	Gamito (2006)
	6	1 (a – Spring 1989)	0.3	15	0	Sand	0.3	0	Calvário (1995)
	7	"	0.3	15	0	Sand	0.5	0	Calvário (1995)
	8	"	0.3	10	0	Sand	2.3	0	Calvário (1995)
	9	"	0.3	10	0	Sand	1.1	0	Calvário (1995)
	10	"	0.3	7	0	Muddy sand	3.9	0	Calvário (1995)
	11	3 (a – January, b – February; c – March 1990)	0.3	0.5	90	Sandy mud	2.8	0	Calvário (1995)
	12	3 (a – January, b – February; c – March 1990)	0.3	0.5	0	Sandy mud	6.6	0	Calvário (1995)
	13	"	0.3	0.5	0	Muddy sand	1.3	0	Calvário (1995)
	14	"	0.3	0.5	0	Sand	1.5	0	Calvário (1995)
Validation sites (VS) (n= 116 samples)	1	12 (a – January; b – March; c – May; d – July; e – September, f – November 1985; g – January; h – March; i – May; j – July; k – September, l – November 1986)	0.066	0.7	0	Muddy sand	[a]	4	Gamito (2006)
	3	"	0.044	0.8	60	Muddy sand	[a]	3	Gamito (2006)
	4	"	0.044	0.9	80	Muddy sand	[a]	2	Gamito (2006)
	2	9 (the same codes as above but without the first 3 sampling occasions)	0.066	0.8	90	Muddy sand	[a]	1	Gamito (2006)
	5	12 (a – May 1996; b – June 1996; . . .; m – May 1997; except i – January 1997)	0.088	1.5	0	Muddy sand	4.2	1	Gamito (2006)
	6	3 (b – Summer; c – Autumn; d – Winter 1989)	0.3	15	0	Sand	0.3	0	Calvário (1995)
	7		0.3	15	0	Sand	0.5	0	Calvário (1995)
	8		0.3	10	0	Sand	2.3	0	Calvário (1995)
	9		0.3	10	0	Sand	1.1	0	Calvário (1995)
	10		0.3	7	0	Muddy sand	3.9	0	Calvário (1995)
	11	10 (d –April, e – March; . . .; l – December 1990; m – January 1991)	0.3	0.5	90	Sandy mud	2.8	0	Calvário (1995)
	12		0.3	0.5	0	Sandy mud	6.6	0	Calvário (1995)
	13		0.3	0.5	0	Muddy sand	1.3	0	Calvário (1995)
	14		0.3	0.5	0	Sand	1.5	0	Calvário (1995)
	15	2 (a – Winter; b –Autumn 2006)	0.15	1.5	0	Sand	–	0	Project RECITAL INAG
	16		0.15	1	0	Sand	–	0	Project RECITAL INAG

[a] Organic content varied between 1.3 and 2.5%.

Table 2. PERMANOVA on Bray-Curtis distances for invertebrate community families at 21 subtidal sampling stations distributed along three types of sediment (*Sed*), on bare or seagrass bottoms (*Veg*), at two distinct depths (*Depth*) in the Ria Formosa coastal lagoon.

Source	*d.f.*	*SS*	*MS*	*Pseudo-F*
Depth	1	9432.2	9432.2	9.3*
Veg	1	4482.8	4482.8	4.4*
Sed	2	5293.1	2646.6	2.6*
Depth x Veg§	0	0		*no test*
Depth x Sed§	1	2006.8	2006.8	1.9
Veg x Sed§	1	2395.5	2395.5	2.4**
Depth x Veg x Sed§	0	0		*no test*
Residual	14	14181.0	1012.9	
Total	20	47378.0		

pair-wise *post-hoc* comparisons:

	Sandy mud	Muddy sand	Sand
Seagrass *vs* Bare bottom	2.2**	1.7**	§ *no test*

	Seagrass	Bare bottom
Sand *vs* Sandy mud	*no test*	1.3**
Sand *vs* Muddy sand	*no test*	1.4**
Sandy mud *vs* Muddy sand	2.4**	1.6**

§ Term has one or more empty cells.

* $p \leq 0.001$.

** $p \leq 0.05$.

Table 3. Reference conditions for the three ecological indices constituting BAT, at the most relevant subtidal habitats (H) in the Ria Formosa coastal lagoon. High reference values are indicated for each index for the 4 habitats proposed; the lower condition limits (Bad) are equal across habitats.

Habitats according to invertebrate community patterns (family level)			EQS	Margalef *d*	Shannon-Wiener H' (log2)	AMBI
Deep channels /	Bare bottom /	Sand or Muddy sand (H1)	High	7.1	4.1	1.0
Shallow subtidal /	Seagrass beds /	Sandy mud or Muddy sand (H2)	High	8.5	4.1	2.0
	Bare bottom	Sand or Muddy sand (H3)	High	7.1	4.1	1.0
		Sandy mud (H4)	High	4.3	4.1	2.5
All habitats			Bad	0.0	0.0	7.0

Table 4. Significant discriminant functions after forward stepwise analysis.

i) Chi-square tests with successive roots removed; sigma-restricted parameterization.

Removed	**Eigen-value**	**Canonical R**	**Wilk's λ**	**Chi-Sqr.**	**df**	***p*-level**
0	14.62	0.967	0.024	61.81	9	0.000
1	1.702	0.794	0.369	16.46	4	0.002

ii) Standardized canonical discriminant function coefficients; sigma-restricted parameterization.

	Level	**Function 1**	**Function 2**
Intercept		0.000	0.000
Mud content		-0.131	1.070
Depth		-1.468	0.152
Sand:Mud ratio		0.883	0.109
Vegetation	P	0.000	0.000
Eigen-value		14.616	1.701
Cumulative Probabilty		0.896	1.000

iii) Factor structure coefficients; sigma-restricted parameterization.

	Level	**Function 1**	**Function 2**
Intercept			0.000
Mud content		0.125	0.975
Depth		-0.777	-0.176
Sand:Mud ratio		-0.140	-0.148
Vegetation	P	0.000	0.000

Table 5. Classification statistics for new sites, prediction sample N = 11. Selected habitat (H) classifications are highlighted.

New sites	Probability of belonging to habitat				Habitat classification probability			
	H1	H2	H3	H4	Highest	Second	Third	Fourth
1a	0.000	0.565	0.421	0.014	H2	**H3**	H4	H1
1b	0.000	0.270	0.729	0.002	**H3**	H2	H4	H1
1c	0.000	0.253	0.746	0.001	**H3**	H2	H4	H1
3a	0.000	0.595	0.367	0.037	**H2**	H3	H4	H1
3b	0.000	0.576	0.394	0.030	**H2**	H3	H4	H1
3c	0.000	0.507	0.483	0.009	**H2**	H3	H4	H1
4a	0.000	0.763	0.184	0.053	**H2**	H3	H4	H1
4b	0.000	0.696	0.275	0.028	**H2**	H3	H4	H1
4c	0.000	0.557	0.432	0.011	**H2**	H3	H4	H1
15b	0.000	0.063	0.937	0.000	**H3**	H2	H4	H1
16b	0.000	0.022	0.978	0.000	**H3**	H2	H4	H1

Table 6. Coefficient of variation (CV) of the sediment grain size (sand:mud ratio) and of the EQR at four stations from 3 habitats in the coastal lagoon.

Stations	CV grain size	CV EQR
11	0,385	0,053
12	0,961	0,096
13	0,857	0,076
14	0,218	0,070

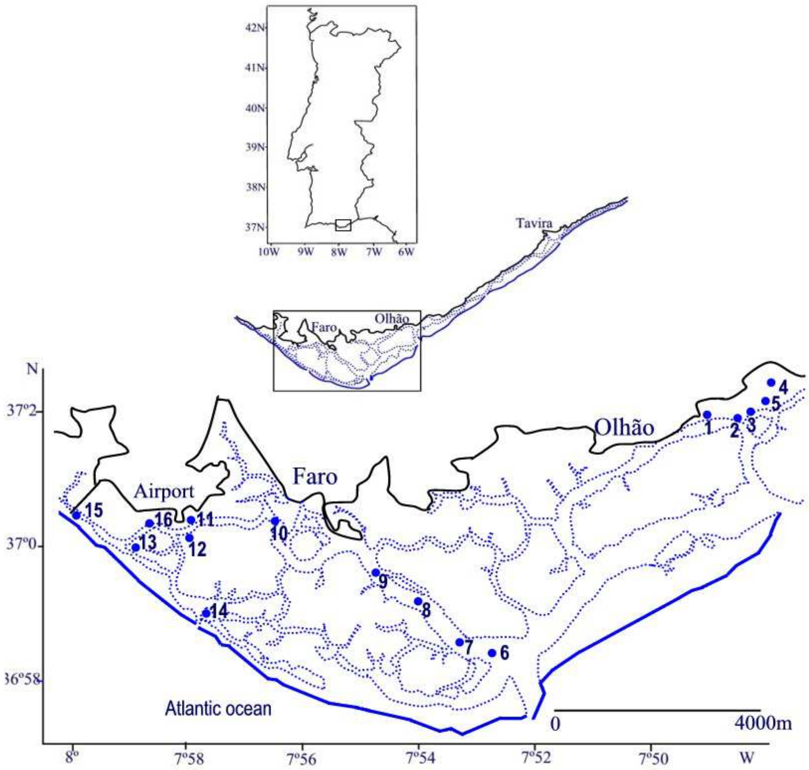

Figure 1. Ria Formosa and approximate location of subtidal sampling stations: 1 – 5 (Gamito, 2006); 6 - 14 (Calvário, 1995); 15 and 16 (Project RECITAL INAG).

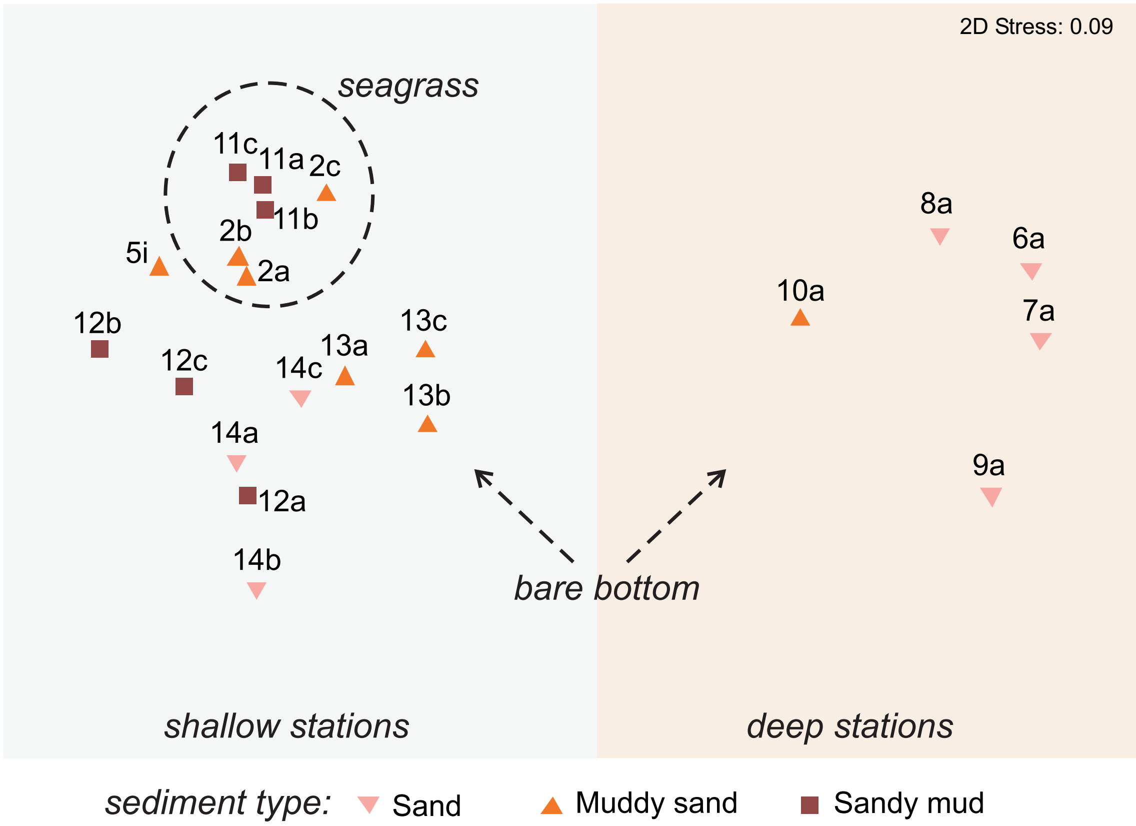

Figure 2. Diagram of non-metric multidimensional scaling analysis carried out with 85 benthic invertebrate families on Reference sites dataset (21 stations, for codes: see table 1).

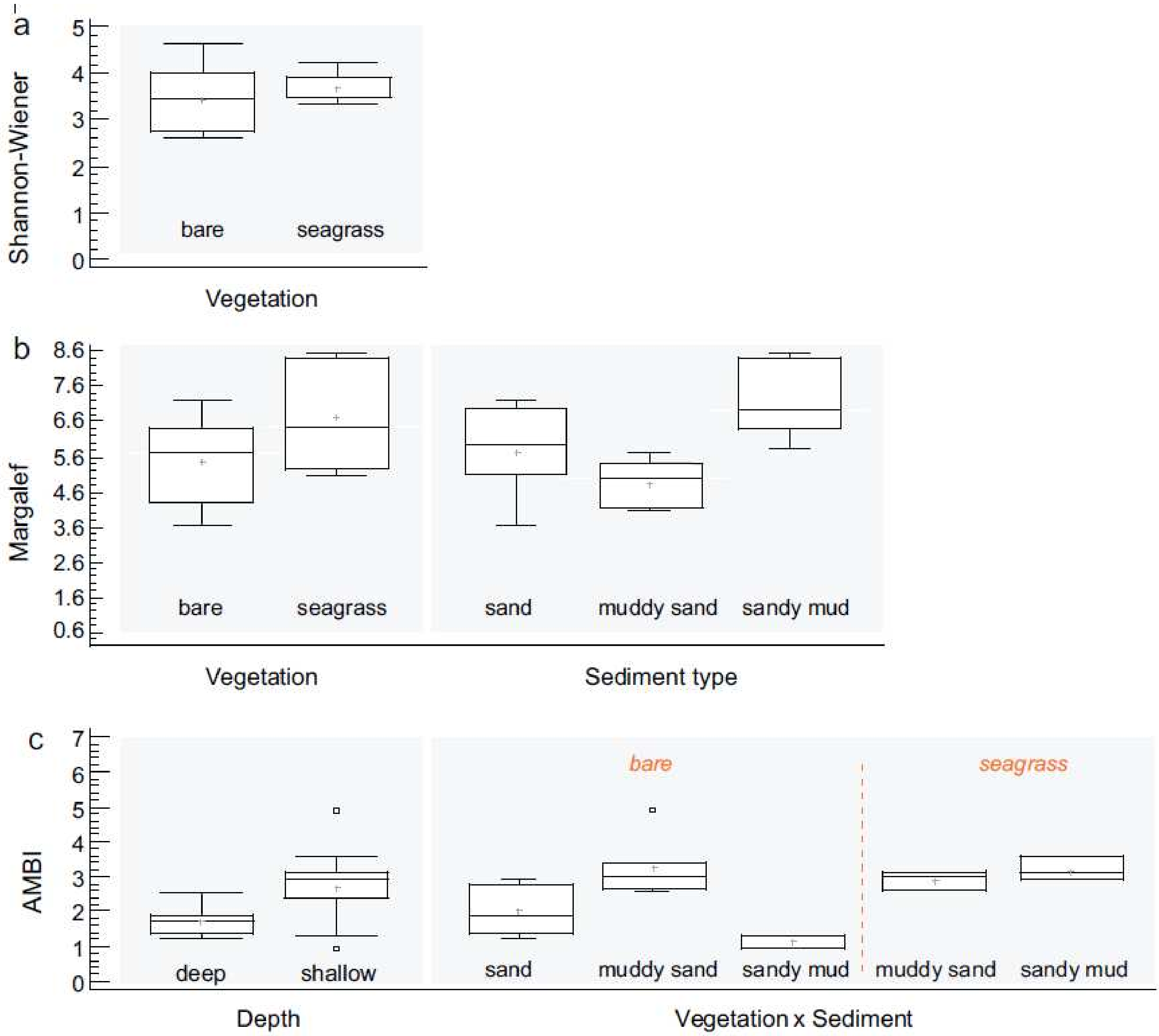

Figure 3. Variation of each of the three ecological indices (a) Margalef, (b) Shannon-Wiener and (C) AMBI, across significant habitat features (factors) according to PERMANOVA results (n = 21 stations RC dataset). The levels of each factor are indicated below the boxplots (mean - grey dots; median – black line within box; Q25 and Q75 – box lower and upper limits; standard deviation – branches out of box; outliers – empty dots).

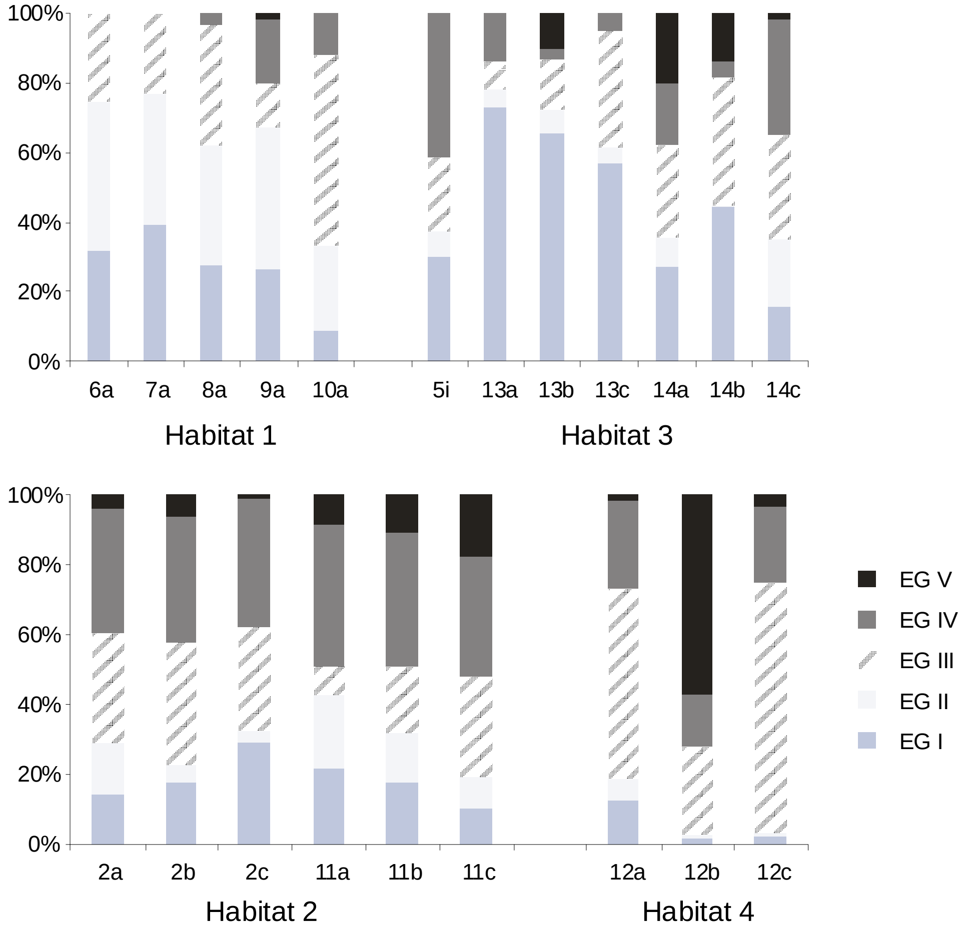

Figure 4. Distribution (%) of AMBI ecological groups (EG) across four habitats in the Ria Formosa lagoon (as described in Table 3: H1 to H4); EG I - species very sensitive; EG II: species indifferent; EG III: species tolerant; EG IV: second-order opportunistic species; EG V: first-order opportunistic species.

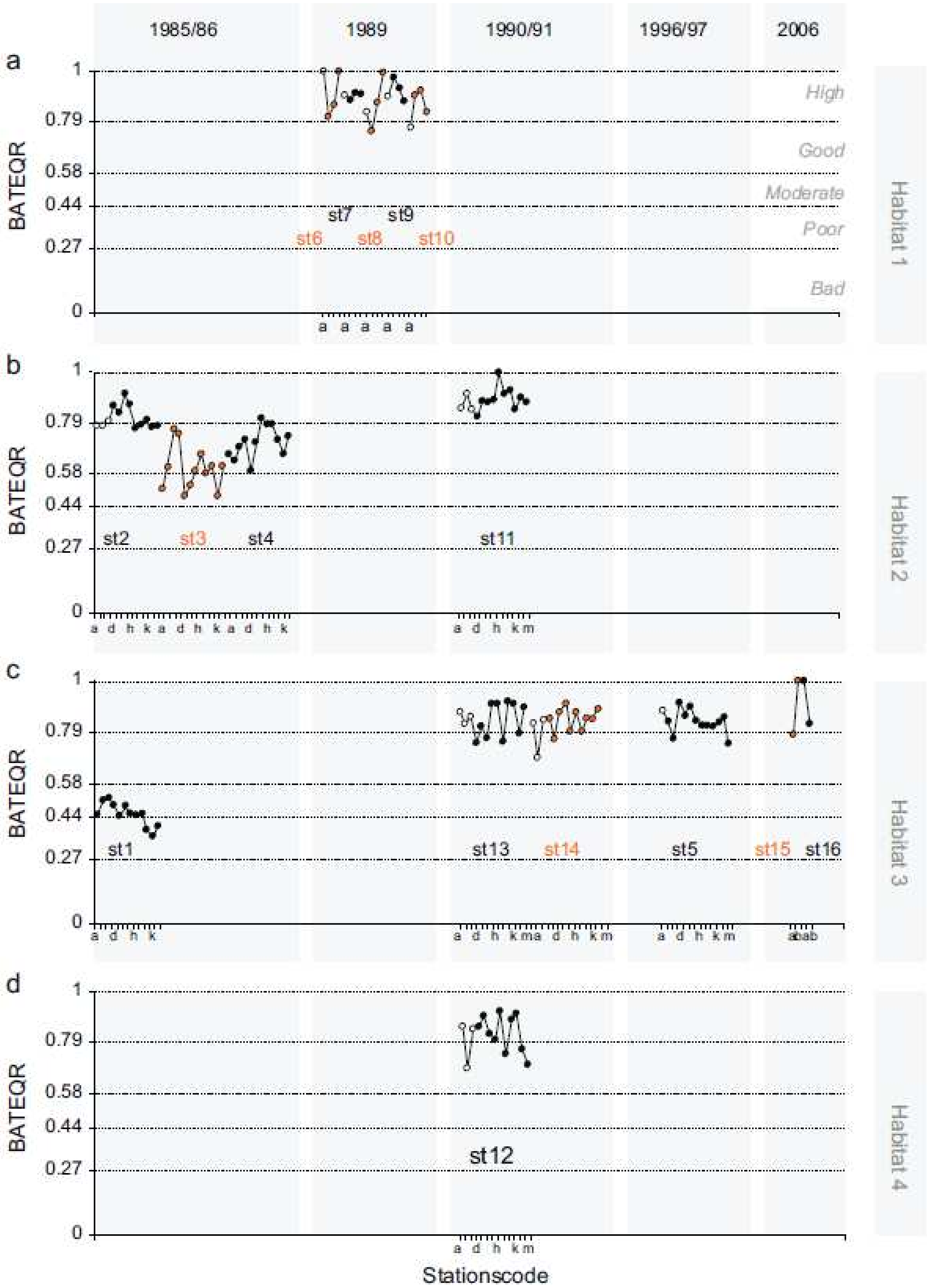

Figure 5. Variation of the Ecological Quality Ratio (EQR) estimated with the Benthic Assessment Tool (BAT) in the different stations and sampling periods, for a) deep channels; b) shallow seagrass beds; and c) shallow sandy and d) muddier bare bottoms. The correspondent classes of Ecological Quality Status (EQS) are indicated in graph a). Reference stations were represented as empty circles. Station codes follow those of Table 1.

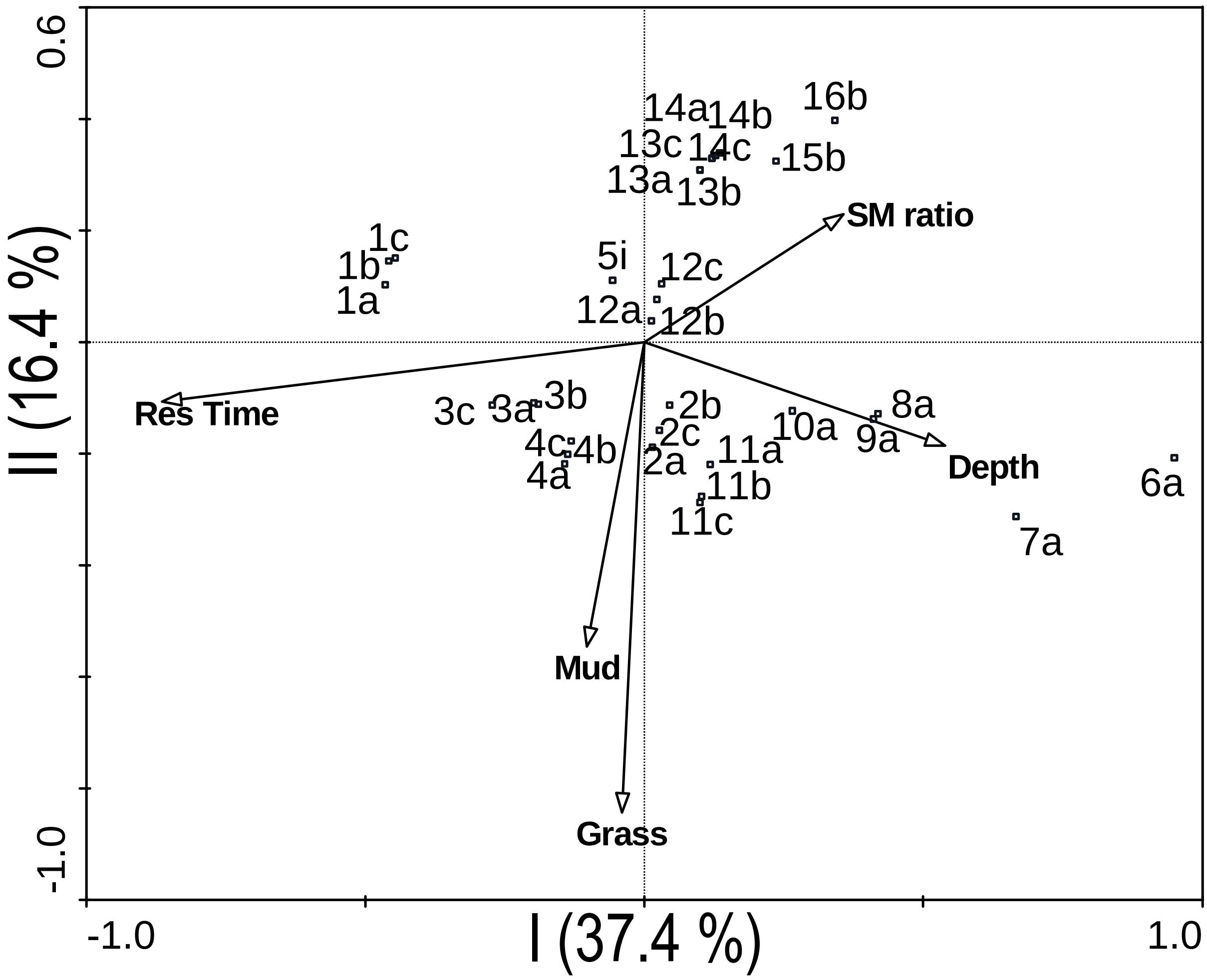

Figure 6. Stations and environmental variables projected on DCCA ordination diagram. SM ratio - sand:mud ratio, Res Time – residence time; Grass – seagrasses. Monte Carlo test: p=0.002.