



Intertidal macrofauna and environmental stress at a riverine–marine boundary



Anxo Conde^{a,b,*}, Júlio M. Novais^{a,1}, Jorge Domínguez^b

^aIBB-Institute for Biotechnology and Bioengineering, Center for Biological and Chemical Engineering, Instituto Superior Técnico (IST), Lisbon 1049-001, Portugal

^bDepartamento de Ecología e Biología Animal, Universidade de Vigo, Vigo E-36310, Spain

ARTICLE INFO

Article history:

Received 20 May 2013

Received in revised form

10 July 2013

Accepted 14 July 2013

Keywords:

Saline fluctuations

Fluid mud

Macrofaunal abundance

Species richness

Environmental harshness

Tagus estuary

ABSTRACT

A field experiment was carried out to test the effect of pore water salinity on the macrobenthic assemblages in an estuarine region of the Tagus estuary (Portugal) subjected to wide fluctuations in salinity. The conditions at the experimental site ranged from freshwater (minimum salinity 0.2) to mesohaline (maximum salinity 15.3). The experimental site was affected by an unexpected deposition of fluid mud during summer. Redundancy Analysis discriminated the experimental treatments along the first canonical ordination axis. The analysis also revealed an experimental gradient of increasing environmental stress, in which the minimal presence of organisms corresponded to treatments representing a high level of environmental stress. Sediment dynamics and saline fluctuations were the major factors that, together, determined the low macrofaunal abundance and species diversity at the experimental site. The most abundant macrofaunal species in this harsh environment were the polychaetes *Hediste diversicolor* and *Streblospio shrubsolei*.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Estuaries are natural ecologically important systems that provide a well-defined physical space in which freshwater and marine ecosystems meet. Estuaries are complex ecosystems where physico-chemical factors and processes limit the number of species adapted to live in adverse conditions (McLusky and Elliott, 2004). Among the environmental constraints, salinity (a dimensionless parameter; e.g. Stickney, 2009), has been identified as a structural factor that determines faunal distribution and diversity along estuaries (Sanders et al., 1965; Ysebaert et al., 1998).

The relationship between salinity and diversity is a matter of scientific interest (Telesh and Khlebovich, 2010; Whitfield et al., 2012). The change in diversity along a saline gradient was first addressed by Remane (1934) in a study carried out in the Baltic Sea. Although the model has often been cited since it was first proposed, it is also known to be deficient in some aspects. For example, it is not clear whether it is valid for all types of brackish water

organisms (Whitfield et al., 2012). Thus, the distribution pattern predicted by the model of Remane has been contradicted in some studies on planktonic organisms (e.g. Telesh et al., 2011), and it was also found to be only partly valid for macroalgal species (Schubert et al., 2011). Attrill (2002) developed a quantitative model as an alternative to Remane's qualitative model. Whilst the latter model identifies a minimum level of diversity that occurs at low salinity (between 5 and 7), Attrill's model establishes an inverse linear relationship between the range of salinity and alpha diversity for both meiofauna and macrofauna.

The critical saline zone characterized by minimal species richness was named *Artenminimum* by Remane (1934). This salinity range was later designated as *horohaliticum* by Kinne (1971) because "salinities between 5 and 8 constitute a significant ecophysiological boundary line, characterized by minimum number of species". Other authors recognized the existence of a saline range in brackish waters where only a small number of species are physiologically adapted to live (Khlebovich, 1968; Telesh and Khlebovich, 2010). As noted by the latter authors, in some cases the *horohaliticum* may shift to higher salinities and salinity ranges (as in the Caspian and Aral Seas; Plotnikov and Aladin, 2011). Subsequently, Deaton and Greenberg (1986) showed that drastic changes in water chemistry occur at salinity of 2 and not in the ranges attributed to the *horohaliticum* as defined by Kinne (1971). Thus, Deaton and Greenberg (1986) concluded that it is the ability

* Corresponding author. IBB-Institute for Biotechnology and Bioengineering, Center for Biological and Chemical Engineering, Instituto Superior Técnico (IST), Lisbon 1049-001, Portugal. Tel.: +351 218419124; fax: +351 218419062.

E-mail address: aconde@ist.utl.pt (A. Conde).

¹ Deceased.

of aquatic animals to regulate osmotic pressure (rather than changes in ionic composition) that explains the low species diversity in brackish waters.

Small numbers of marine and freshwater species occur simultaneously in the saline transition zone of estuaries. In European waters, these highly adaptable and widely distributed species include, for instance, the polychaetes *Hediste diversicolor*, *Streblospio shrubsolii* and the crustaceans *Corophium volutator*, *Lekanesphaera hookeri* and *Leptocheirus pilosus*, among species of marine origin, and the amphipod *Gammarus chevreuxi* and the tubifid oligochaetes *Limnodrilus hoffmeisteri*, *Tubifex tubifex* and *Psammoryctides barbatus*, among the freshwater tolerant species (Attrill et al., 1996; Cognetti and Maltagliati, 2000; Quintino et al., 2011). Furthermore, Attrill and Rundle (2002) proposed a model that explains the low numbers of species found at the boundaries of fresh and marine waters in the Thames estuary as the result of the convergence of two ecoclines of river and marine origin.

Other changes related to physical and chemical processes have been described in upper and inner locations of estuaries (Deaton and Greenberg, 1986; McLusky et al., 1993; Telesh and Khlebovich, 2010). Thus, changes in the ratios of the Ca^+/Na^+ and K^+/Na^+ ions, the electrical properties of particles and the chemistry of heavy metals are examples of physico-chemical processes that occur in the transition zone between freshwater and seawater. The most characteristic of the physical factors is the “turbidity maximum” (TM) (Eisma, 1993). High concentrations of particulate matter may lead to the formation of fluid mud over sediments (e.g. Kirby and Parker, 1983) in relation to the TM. The processes that occur towards the landward limit of the seawater intrusion are quite complex and are influenced by river inflow, bottom morphology, tidal currents and the tidal cycle (Attrill et al., 1996; Dolgoplova and Isupova, 2010; Schoellhamer, 2001; Uncles and

Stephens, 2010). This riverine–marine boundary is a challenging environment for carrying out ecological studies.

In this study we investigated how the environmental conditions affect macrobenthic assemblages in an estuarine saline boundary, by focussing on several different aspects. First, we carried out a manipulative experiment by modifying the pore water salinity in intertidal soft sediments in the upper reaches of the Tagus estuary. Second, because deposition of fluid mud occurred naturally during the experimental period, we compared the composition of the assemblages before and after erosion of the fluid mud. Finally, we compared the number of intertidal species and macrofaunal abundance in different seasons in the experimental site, and we also compared these variables in the experimental site and in another location in the estuary.

2. Material and methods

2.1. Study site

The Tagus estuary is one of the largest European estuarine systems and covers an area of approximately 325 km². The estuary is mesotidal and the tidal range is between 1 m at neap tides and 4 m at spring tides. The study site was located in an intertidal area of the northern bank of the estuary, 4 km seawards from the head of the estuary at Vila Franca de Xira (Fig. 1). This site was chosen for study because of its salinity characteristics and because access was granted to the shore through a private property. Additionally, the shoreline adjacent to this private property, partly surrounded by a fence, is generally avoided by local people and fishermen.

The experimental site is located at a saline border, ranging over time from freshwater to mesohaline (saline fluctuations of two orders of magnitude). The water column is very turbid at the site in summer. The maximum concentration of suspended sediment (up

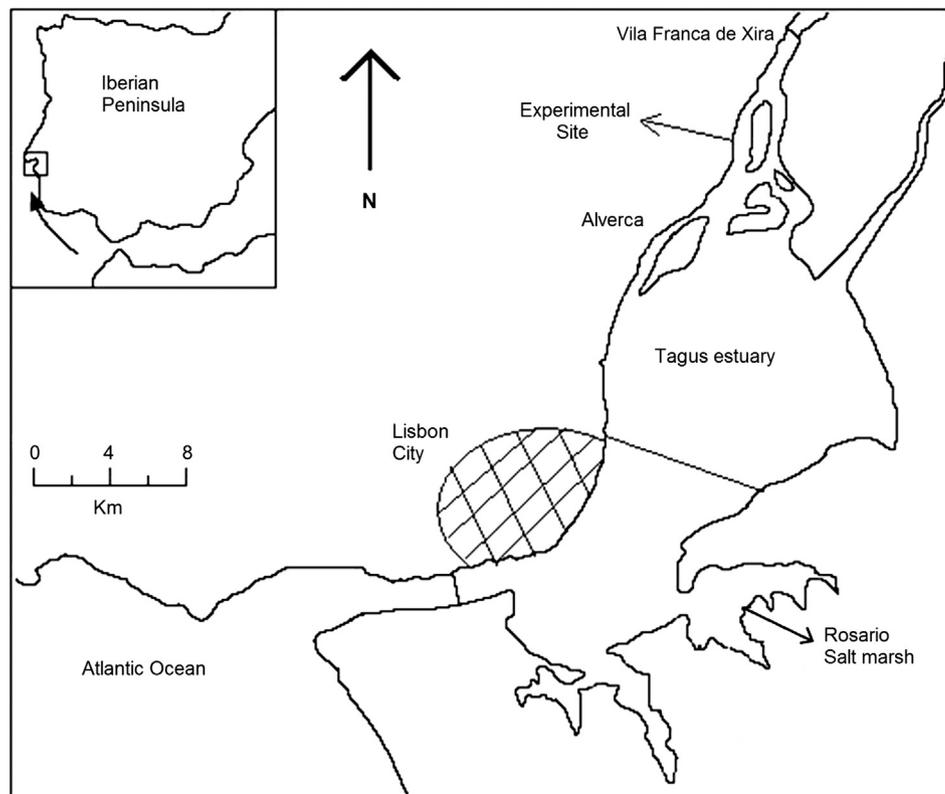


Fig. 1. Map of the Tagus estuary showing the location of the experimental site (Alverca) and the Rosario salt marsh.

to 250 mg l^{-1}) was measured in an estuarine area located about 5 km downstream of the study site (Portela and Neves, 1994). The concentration of dissolved oxygen is usually higher than 5 mg l^{-1} , but lower levels sometimes occur in the mixing zone close to the experimental site (Ferreira et al., 2007). The intertidal zone is approximately 30 m wide during spring tides. Around three quarters of the intertidal region is dominated by mud, and the remaining area (in the upper intertidal zone) is mainly constituted by coarse sand, stones and some ferric debris. The surrounding vegetation in the supratidal zone is mainly dominated by *Phragmites* sp. and *Juncus* sp.

2.2. Experimental design

Four experimental blocks were established in the intertidal zone above the low water mark defined by the low neap tide in June 2011. Location of the experimental units below this low water mark would prevent access to the experimental plots at some low tides (because the experimental units would be under water at low waters of neap tides). Each block comprised four plots (polyethylene rings) to be assigned one of four treatments plus an ambient level without polyethylene ring. Blocks were separated by a distance of 20 m from each other, and plots within blocks were separated by a distance of about 1 m from each other (Fig. 2). A polyethylene ring of diameter 52 cm and height 15 cm was inserted 8 cm deep in the sediment (thus 7 cm over the sediment) and anchored with two diametrically opposed stainless steel bars of diameter 2 mm. The presence of stones (rather than mud) made it impossible to establish the plots further upwards in the intertidal zone. The volume inside the plot from the sediment surface to the plastic ring top was approximately 15 l. The last block established seawards was approximately 100 m from a pier, which may have affected the local hydrology. It must be stress that no effluents are discharged or any industrial waste is dumped into the estuary from the pier.

It is known that the overlying water salinity determines the interstitial salinity, especially of silty sediments (Chapman, 1981). Three different amounts of dry salt (NaCl) were added to alter the salinity in each of the four plots (experimental units) within each

block: 446 g, 268 g, 74 g and no salt added, corresponding to salinities of 30, 18, 5 (when dissolved in 15 l of water) and ambient salinity respectively. These salinities represent at least the border between the euhaline–polyhaline (salinity 30), the polyhaline–mesohaline (salinity 18) and the mesohaline–oligohaline (salinity 5) estuarine divisions (McLusky et al., 1993; Venice system, 1959). An additional treatment level, which consisted of bare sediment within the block without a plastic ring, was used to control for any experimental artefacts (Underwood, 1997). It is important to note that treatment T1 (plastic ring with no salt added) was considered to control for experimental artefacts (Underwood, 1997). Treatments were randomly attributed within blocks and designated as follows: T0 for bare sediment, T1 for the experimental unit with ambient salinity and T2 to T4 for treatments of increasing manipulated salinity.

The experimental set up was functional between 1 July 2011 and 15 August 2011. Experimental conditions, i.e. the addition of salt to each treatment, were maintained daily until 20 July; from this date until the end of the experiment, the experimental conditions were maintained every two days. Daily restoration of the experimental conditions became increasingly difficult, especially at the weekends when access to the experimental site through the private property was closed.

2.3. Sampling

Sampling in the experimental units took place between 16 and 18 August 2011, at low tide. We initially planned to carry out the sampling during the first week of August, but the experimental units were temporarily buried under the newly deposited fluid mud (>7 cm thick). As the environmental conditions did not improve after one week, we decided to proceed with the sampling even under these adverse circumstances.

Sampling for macrofaunal organisms was carried out with a corer of inner diameter 6.5 cm, inserted to a depth of 25 cm. Five replicate samples were taken from each plot. A plastic ring was used to define an equivalent area for sampling macrofaunal species within the experimental block but outside any experimental plot (level T0). The same methodology was adopted for additional

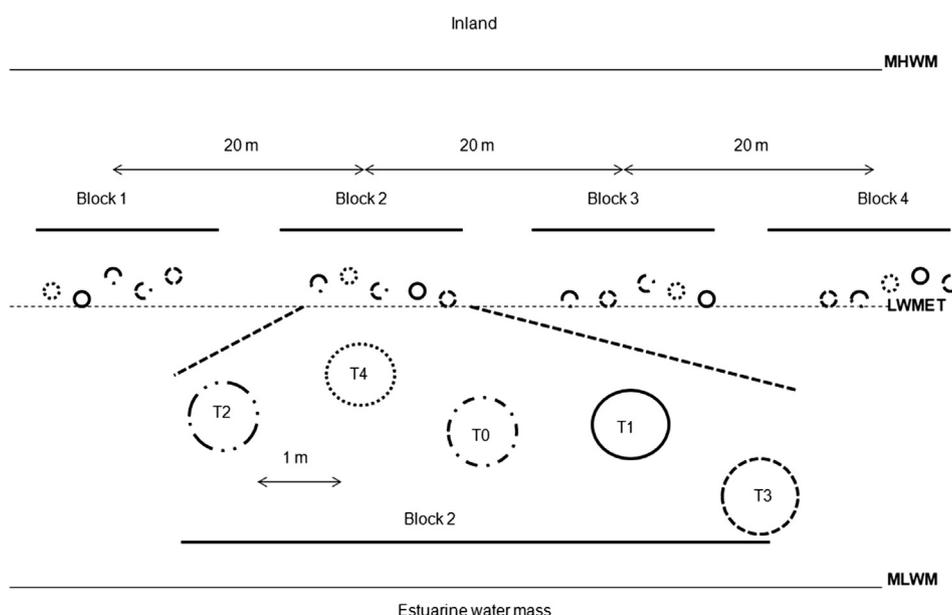


Fig. 2. Experimental set up showing the four block design with randomization of the treatment (from T0 to T4) in plots within each block located in the intertidal zone. Block 2 is shown in detail. MHWM: Mean High Water Mark; LWMET: Low Water Mark at Ebb Tide; MLWM: Mean Low Water Mark.

sampling (see below). All samples were sieved through a 1 mm mesh. The retained material was preserved in 70% ethanol.

After sampling for macrofaunal organisms, a sample of sediment was removed to assess the degree of hydration of the sediment and the organic matter content. Another sample was removed to determine the salinity of the sediment pore water in each treatment. A third sample was removed for determination of the concentration of chlorophyll-a in the surface sediment. Sampling for determination of sediment features was conducted within each experimental plot (one replicate per plot; i.e. four replicates per treatment).

Sampling was conducted on two further occasions. On the first occasion (28 August 2011), macrofaunal organisms were sampled outside the experimental units, but within each block in Alverca; we proceeded with the sampling because the unconsolidated sediment (fluid mud layer) was absent from the intertidal zone some days after the first sampling. On the second occasion, samples of macrofaunal organisms were collected in another estuarine site, the Rosario salt marsh, and in the experimental site in Alverca (Fig. 1) on 13 and 14 December 2012 respectively; this sampling (five replicates in each of four blocks) was carried out at low tide to enable comparison of the species richness and abundance in the experimental site between summer and winter and between the experimental site and another location in the estuary. The Rosario salt marsh is outside the estuarine zone with high fluctuations in salinity and the turbidity is far from the maximum (Portela and Neves, 1994), phenomena often described in central-to-upper estuarine reaches (e.g. Kirby and Parker, 1983). December was considered climatologically as a winter month.

Salinity was measured with a standard field probe (WTW 340i) in the water column at different times of the tidal cycle in Alverca, during six days (between 14 and 19 December 2012); the interstitial salinity was measured in the same way in the Rosario salt marsh (13 December 2012). The latter location is characterized by an extensive mud flat that prevented us from measuring the salinity at the low tide level.

2.4. Laboratory procedures

Benthic animals were sorted under a dissecting microscope and identified to the lowest taxonomic level. The porosity of the sediment was estimated from the weight loss after drying wet sediment to a constant weight at 100 °C. Organic matter was determined by loss of weight on ignition at 500 °C during 24 h in a muffle furnace. The pore water salinity within the experimental units was assessed after adding 50 ml of distilled water and mixing for 24 h in approximately 800 g of sediment. Salinity was measured in these samples with a standard field probe (WTW 340i). Photosynthetic pigments were extracted from the sediment with 5 ml of 90% acetone. Extracts were analysed spectrophotometrically, and chlorophyll-a was estimated by the trichromatic equation of Jeffrey and Humphrey (1975). Assessment of chlorophyll-a and phaeopigments by the latter method suffices in studies where reflectance is used as a comparative measure (Brotas et al., 2007).

2.5. Hypotheses and statistical analysis

The organisms that potentially responded to the experimental conditions were assumed to have moved from the nearby intertidal zone close to the experimental site rather than to have migrated from other estuarine farther locations. We tested three null hypotheses:

- i) No differences in the composition of the assemblages at different experimental levels of salinity.

- ii) No differences in the composition of the assemblages between eroded and deposited sediments.
- iii) No differences in the composition of the assemblages within the experimental area between summer and winter and relative to another site in the estuary.

The hypotheses were tested by multivariate analysis executed by the “adonis” function included in vegan package (Oksanen et al., 2006) and run in the R environment (R Development Core Team, 2009). The method resembles traditional analysis of variance and is based on summing square distances of any dissimilarity measure (Oksanen, 2011); in this case, we used the Euclidean distance between the chi-square transformed raw data. The method, which is similar to a PERMANOVA multivariate test (Anderson, 2001), has the advantage of testing the hypothesis in a non-parametric manner, through permutation, which enables relaxation of the model assumptions for a valid test. The permutation strategy for testing the null hypotheses enables an exact test or an approximate, asymptotically exact test, in relation to the a priori significance level (e.g. Anderson and Ter Braak, 2003). The approximated test is carried out when e.g. there are too few possible permutations for a valid test (Anderson and Ter Braak, 2003; Anderson and Robinson, 2001). The multivariate approach is recommended when there are several zero entries in the data matrix (Zuur et al., 2010). We fitted a model with treatment and block as factors, with no interaction between them. The model is therefore analogous to a univariate additive effect model described elsewhere (Quinn and Keough, 2002). It allows the residual variation to be reduced and is therefore a more powerful test for treatments (Quinn and Keough, 2002). The block factor was considered random and the permutation test was carried out within levels of the random factor for calculation of an exact test (Anderson and Ter Braak, 2003). The “F ratio” that we provide is a Pseudo-F ratio calculated by a permutation test (see e.g. Borcard et al., 2011). We tested hypotheses i) and ii) with this model, and we tested hypothesis iii) with a one factor model with two levels (summer and winter; site 1 and site 2). We used chi-square distance as a dissimilarity measure (Legendre and Legendre, 1998; ter Braak, 1987) in all cases. Additionally, because a t-test was not applicable, we used a Wilcoxon’s signed-rank test (Crawley, 2007) to test for differences in abundance between the two sites and seasons.

The model associated with the first hypothesis was submitted to ordination by Redundancy Analysis (RDA), after chi-square transformation of the raw data (Legendre and Gallagher, 2001). This method resembles Canonical Correspondence Analysis (CCA) in which chi-square distance is preserved among objects (ter Braak, 1987). CCA is recommended when “rare species are well sampled and are recognised as potential indicators of particular characteristics” (Borcard et al., 2011). The effect of the blocks in the experimental site was partialled out (Borcard et al., 2011; Oksanen, 2011) to focus the analysis on the treatment effects. RDA can also be used to test for the significance of the canonical axes (Borcard et al., 2011).

Nonmetric multidimensional scaling (NMDS, using Euclidean distance) was applied to the square root transformed data to distinguish between the assemblages in the experimental site in summer (with presence/absence of fluid mud), winter and in the Rosario salt marsh. Abundance and species richness were also analysed by NMDS ordination after square root transformation. This transformation was used because the different ecological parameters represented by species abundance and species richness would become meaningless the chi-square pre-transformation that standardizes data simultaneously considering both row and column sums (Legendre and Gallagher, 2001). NMDS represents the assemblages along a predetermined number of axes while

preserving the ordering relationships (Borcard et al., 2011), which was explored with the aim of indicating any environmental pattern.

Physico-chemical parameters were tested by one-way analysis of variance with one replicate per block. Models were tested for normality and heteroscedasticity by the Shapiro–Wilk normality test and the Fligner–Killeen test, respectively (Crawley, 2007). All statistical hypotheses were tested at the 0.05 significance level. Results are shown numerically and graphically as means \pm standard deviations (SD).

3. Results

3.1. Environmental characterization during the experimental period

The variation in the environmental parameters considered between treatment levels of plots in different blocks was not significant, with the exception of salinity (Table 1). Sediments contained large amounts of water and organic matter (mean of 94.39 ± 0.95 and 11.66 ± 0.32 percent respectively). The ANOVA revealed that maintenance of the experimental conditions (salinity levels) did not inhibit the activity of microphytobenthic algae or modify any of the estimated environmental parameters, which were similar to control values (T0). Salinity was successfully recreated at different experimental conditions within plots (Fig. 3). All treatment levels were significantly different (post-hoc test, $p < 0.0325$), except treatments T0 and T1 ($p = 0.968$).

The study site was subjected to large fluctuations in salinity. The salinity measurements made between 23 June and 15 August are shown in Fig. 4a. Two measurements taken at high tide (on 10 of both July and August) coincided with high values of salinity, especially in the first case (maximum in Fig. 4a). However, a value of 5.43 ± 0.21 was observed at low tide on 24 July. Daily fluctuations in salinity of up to one order of magnitude were recorded during the sampling period, between 16 and 18 August (Fig. 4b).

3.2. Response of the assemblages to experimental and ambient conditions

The total number of organisms observed in the experimental units was 14 and comprised 6 different species (Table 2). Treatment plots T1 and T2 each contained the maximum number of individuals and species (in both cases, 4) (Table 2). The polychaete *Hediste diversicolor* was the most abundant species, occurring at all treatments levels except T0, followed by *S. shrubsolii*, present at treatment levels T1, T2 and T3. The multivariate test did not reveal any significant differences between treatments (Table 3). However, the results of the RDA ordination (Fig. 5) clearly discriminated between treatments. Furthermore, the overall RDA model was not significant, but the first axis was significant ($F_{1,93} = 3.05$, $p = 0.01$), suggesting the existence of an ‘experimental gradient’ related to the environmental harshness (Fig. 5). Along this gradient, species were mainly observed in the least saline treatments within the plastic rings (T1–T2), whereas the control plots (T0) tended to harbour fewer species, similar to the numbers observed in treatments with higher pore water salinity (T3–T4). The first axis

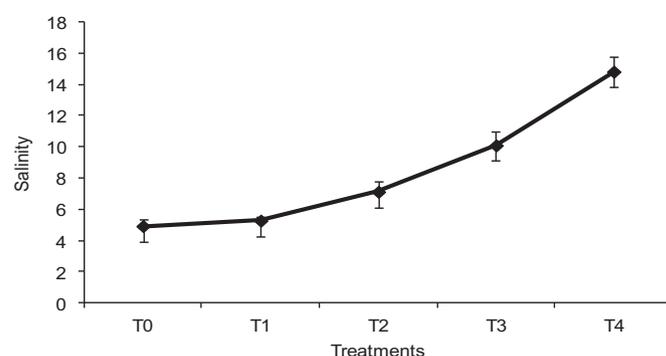


Fig. 3. Salinity measured in the sediment samples at representing each treatment level.

therefore distinguished between treatments with higher abundance and species richness and those plots with almost no fauna.

The multivariate test on the composition of the assemblages in the absence of fluid mud, compared against the species observed in presence of the fluid mud (T0 units) was significant ($F_{1,35} = 1.96$, $p = 0.048$) and attributable to differences in the composition of the assemblages (see columns ASP and ASA at Table 4). The mean number of individuals and species number per sample in the presence and absence of the fluid mud (0.1 ± 0.45 and 0.35 ± 0.59 , respectively) were almost significantly different (Wilcoxon rank test, $W = 247.5$, $p = 0.054$). The assemblage composition differed significantly (in the absence of fluid mud) in the experimental site between summer and winter ($F_{1,35} = 2.88$, $p = 0.004$), with significant differences in abundance (2.20 ± 2.2 mean number of individuals by sample in winter, $W = 346.5$, $p < 0.001$) and species richness (1.45 ± 0.89 mean number of species by sample in winter, $W = 63.5$, $p < 0.001$). Similarly, the composition of the assemblages in Alverca and in the Rosario salt marsh in the winter, pooled by blocks, differed significantly ($F_{1,6} = 10.16$, $p = 0.027$). The difference in the composition of the assemblages between Alverca and the Rosario salt marsh was significant, even when the samples were pooled over blocks. The mean abundance by block was significantly higher in the Rosario salt marsh (510.25 ± 124.06 , $W = 0$, $p = 0.029$) than in Alverca (11 ± 3.56) as was the species richness (mean by block of 8.75 ± 1.5 and 3.75 ± 1.71 in the Rosario salt marsh and in Alverca respectively, $W = 0$, $p = 0.029$).

The total values for species abundance in Alverca in summer (both in presence and absence of fluid mud) and in winter and also in the Rosario salt marsh are summarised in Table 4. Species abundance and richness increased from assemblages in the presence of fluid mud to its absence, from summer to winter in Alverca and from the experimental site relative to the Rosario salt marsh. As in the summer, high fluctuations in salinity were observed in Alverca at low and high tide during consecutive tidal cycles in winter (Fig. 6). No fluid mud was observed during the sampling period in winter. Furthermore, the water was obviously much more transparent in winter than in summer. The water was so turbid in summer that the bottom was not visible at any depth, even close to the water edge. The mean interstitial salinity in the Rosario salt marsh was 26.43 ± 0.29 .

Table 1

Results of the one-way ANOVA for the physico-chemical parameters measured at each treatment level. Mean values (\pm SD) are provided in the last column.

| Source of variation | Parameter units | Data transformation | Treatment MS | Residual MS | F ratio | Probability p | Mean value |
|---------------------|----------------------|---------------------|---------------------|----------------------|---------|---------------|------------------|
| Chlorophyll-a | $\mu\text{g g}^{-1}$ | None | 1.81 | 8.89 | 0.204 | 0.932 | 6.02 ± 2.72 |
| Organic matter | % | None | 35×10^{-6} | 105×10^{-6} | 0.336 | 0.849 | 11.66 ± 0.32 |
| Hydration | % | Arcsine | 34×10^{-5} | 99×10^{-5} | 0.671 | 0.842 | 94.39 ± 0.95 |
| Salinity | – | Log | 67.62 | 0.62 | 108.7 | <0.001 | 8.45 ± 3.84 |

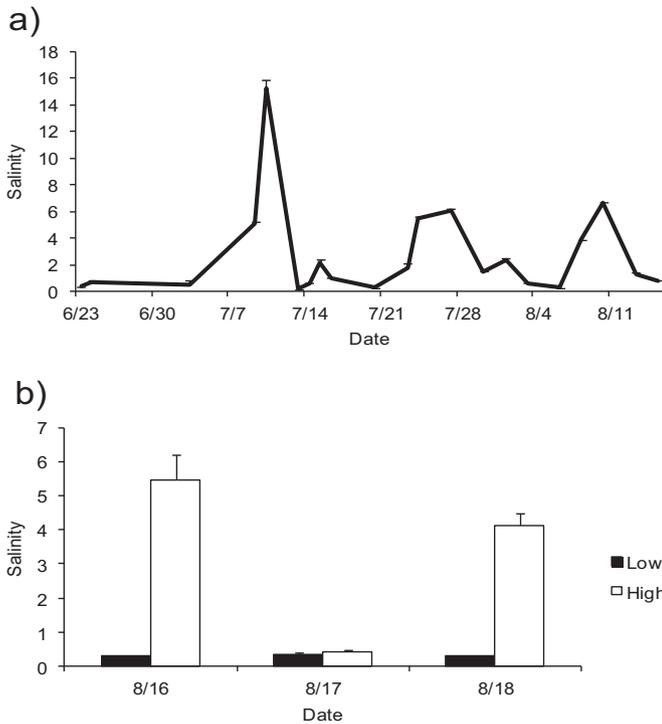


Fig. 4. Fluctuations in salinity measured at the experimental site a) over time b) at low and high tide during consecutive tidal cycles in summer 2011.

The ordination of the assemblages in Alverca and in the Rosario salt marsh by NMDS is shown in Fig. 7, in which the distance between the assemblages indicates their different compositions. A gradient related to the environmental harshness emerges when the first axis is considered. The same pattern was obtained when the NMDS was applied to the abundance and species richness as attributes describing the same assemblages (Fig. 7b; Table 4).

4. Discussion

The present data obtained at the riverine–marine interface in the Tagus estuary indicate that a small number of macrobenthic species are able to inhabit the extreme environment that characterized the study site in Alverca. These results are consistent with previous reports of minimal numbers of species at the oligohaline/mesohaline boundary (Fujii, 2007; Mouny et al., 1998; Nebra et al., 2011). Although salinity has been postulated to be the major physical stressor in relation to the osmoregulatory capabilities of the organisms that colonize the upper reaches of estuaries, we have identified another physical stressor for macrobenthic species related to sediment dynamics in this boundary zone of the Tagus estuary.

Table 2 Total number of species and total abundance by treatment and experimental blocks. Species codes: Hediste diversicolor (Hed); Streblospio shrubsolii (Str); Corophium multisetosum (Cor); Scrobicularia plana (Scr); Paragnathia formica (Par); Insects (Ins).

| Blocks | Treatments | | | | |
|----------------|------------|----------|----------|-----|-----|
| | T0 | T1 | T2 | T3 | T4 |
| B1 | Par, Ins | Str | Str | 0 | Par |
| B2 | 0 | 0 | Hed | Str | Hed |
| B3 | 0 | Scr | 0 | Hed | 0 |
| B4 | 0 | Hed, Cor | Hed, Cor | 0 | 0 |
| Species number | 2 | 4 | 3 | 2 | 2 |
| Abundance | 2 | 4 | 4 | 2 | 2 |

Table 3 Result of the multivariate test for the experimental data.

| Source of variation | Degrees of freedom | Mean squares | F ratio | Probability p |
|---------------------|--------------------|--------------|---------|---------------|
| Blocks | 3 | 0.0701 | 0.926 | 0.835 |
| Treatment | 4 | 0.0508 | 0.671 | 0.835 |
| Residuals | 92 | 0.0757 | | |
| Total | 99 | | | |

4.1. Experimental outcomes

The appearance of fluid mud in the experimental site conditioned the outcome of the experiment (Hypothesis 1). The presence of fluid mud increased the environmental stress on the macrofaunal species, which avoided this type of sediment (Hypothesis 2). The experimental site in Alverca has intrinsic low species richness and abundance expressed to a degree that depends on the pressure exerted by the environment (Hypothesis 3).

Although we succeeded in creating different salinities in the interstitial water of the experimental units, it was not possible to clearly demonstrate the effect of salinity on the faunal composition. However, the first axis of the RDA ordination (Fig. 5) discriminated between experimental conditions that favoured the occurrence of some species in relation to other experimental conditions in which the response was the occurrence of almost zero species. Experimental units with local salinity (T1) or with a slightly increased salinity (T2) contained more species and individuals than the higher salinity units (T3, T4) or control units (T0) (experimental gradient; Fig. 5). It is possible that an experimental artefact enhanced the occurrence of species within the microhabitat created by the experimental units in lower or ambient salinity treatments (in comparison with T0).

There are three possible reasons for the progressive absence of individuals from plots of lower to higher salinity in the experimental units: (1) the addition of increasingly larger amounts of salt at different treatment levels may have stressed the species until the dissolution of the salt within the plot, (2) the discrepancy between

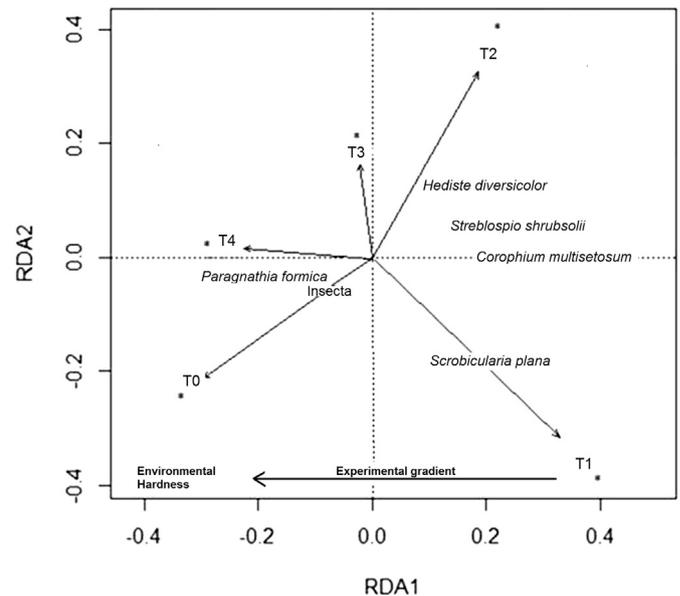


Fig. 5. RDA triplot displaying the ordinations of the species observed under the experimental conditions, constrained by treatment levels (arrows). Treatment responses are shown as centroids. Treatment levels were discriminated along the first RDA axis that recreates an experimental gradient attributable to an increase in the experimental (T1–T4) or natural environmental harshness (T0).

Table 4

Total abundance of each taxa belonging to the macrobenthic assemblage in Alverca (A) and in the Rosario salt marsh (R). The assemblages sampled in the summer (S) or in winter (W) with presence (P) or absence (A) of fluid mud are also shown for Alverca. Total abundance and species richness of each assemblage is also provided. Taxa are listed in accordance to their total abundance (last column).

| Taxa | ASP | ASA | AWA | RW | Totals |
|---------------------------------|-----|-----|-----|------|--------|
| <i>Hydrobia ulvae</i> | | | | 1539 | 1539 |
| <i>Scrobicularia plana</i> | | | 2 | 434 | 436 |
| <i>Streblospio shrubsolii</i> | | 3 | 32 | | 35 |
| <i>Cerastoderma edule</i> | | | | 30 | 30 |
| <i>Alkmaria romijni</i> | | | | 14 | 14 |
| <i>Cyathura carinata</i> | | | 4 | 4 | 8 |
| <i>Hediste diversicolor</i> | | 3 | 2 | | 5 |
| <i>Tharyx marioni</i> | | | | 5 | 5 |
| <i>Nephtys hombergii</i> | | | | 4 | 4 |
| <i>Limnodrilus hoffmeisteri</i> | | | 1 | 2 | 3 |
| <i>Corophium multisetosum</i> | | 1 | 2 | | 3 |
| Insecta und. | 1 | | 1 | | 2 |
| <i>Venerupis decussata</i> | | | | 2 | 2 |
| <i>Mya arenaria</i> | | | | 2 | 2 |
| <i>Mysta picta</i> | | | | 1 | 1 |
| <i>Pygospio elegans</i> | | | | 1 | 1 |
| <i>Tetrastema</i> sp. | | | | 1 | 1 |
| <i>Polydora ciliata</i> | | | | 1 | 1 |
| <i>Autolytus</i> sp. | | | | 1 | 1 |
| <i>Paragnathia formica</i> | 1 | | | | 1 |
| Abundance | 2 | 7 | 44 | 2041 | 2094 |
| Species richness | 2 | 3 | 8 | 15 | 20 |

the within plot pore water salinity and the salinity at the sediment-water column interface may have prevented the presence of species (Sanders et al., 1965), and (3) a combination of the last two explanations.

Apart from the effect of salinity, the presence of fluid mud was also a physical stressor that inhibited the occurrence of species in these unconsolidated sediments. The effect associated with the experimental plots suggests that sediment entrapped within the plastic rings did not interact with macrobenthic species in the same way as the fluid mud in ambient sediment (treatment level T0). Therefore, fluid mud appears to be highly unstable and mobile, preventing the presence of macrofaunal individuals. Instability at the sediment-water interface, generally related to the finest fraction of the sediment, may induce lower diversity (Rhoads, 1974). Thrush et al. (2003) also found that the highest contents of mud were generally associated with minimum values of macrofaunal density. Thus, the additional sampling conducted after erosion of the fluid mud revealed that the species previously found within experimental plots (mainly at T1–T2 units) were observed outside the plots and in higher numbers (Hypothesis 2; Tables 2 and 4).

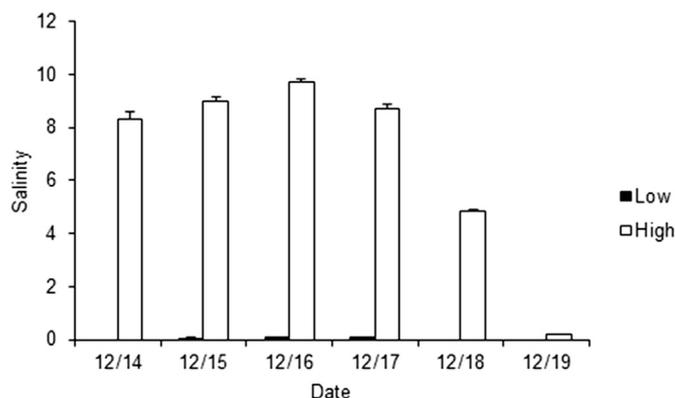


Fig. 6. Fluctuations in salinity measured in Alverca at low and high tide during consecutive tidal cycles in winter 2012.

Furthermore, comparison between the assemblages sampled in the absence of fluid mud in the summer and winter in Alverca reflected an improvement of the environmental conditions, which was probably caused by the seaward dislodgment of suspended particles by higher inflow of the rivers during winter (McLusky, 1993); dislodgment of particles would also explain the greater transparency of the water in Alverca during the winter sampling. Fluid mud occurs as the result of the deposition of suspended materials, and therefore there was a high concentration of suspended particulate matter in the water column near the TM (Eisma, 1993; Portela and Neves, 1994), which could have direct (abrasion, clogging of gills) or indirect effects (attenuation of light for microphytobenthic species) on the macrofaunal assemblages inhabiting the upper estuary. Attrill (2002) used the salinity range as a predictor for diversity, but suggested that although salinity is a good predictor of diversity, it may not be the only causative factor. Furthermore, comparison with the assemblages from the Rosario salt marsh, where there were no saline fluctuations or high turbidity (Portela and Neves, 1994), indicated that the assemblages in Alverca reflected the intrinsic environmental characteristics of the experimental site, which prevented higher species richness and macrofaunal abundance (Hypothesis 3; Fig. 7). The sediments in the Rosario salt marsh contain significantly higher levels of anthropogenic heavy metals than in the experimental site (Vale et al., 2008), and therefore the findings cannot be explained by pollution. The joint effect of both fluctuations in salinity and sediment dynamics cause low diversity and abundance of meiofaunal species in oligomesohaline areas in estuaries (Heip et al., 1985; Santos et al., 1996), causal factors that also affect the presence of macrofaunal species in the experimental site.

Remane's model displays 'species abundance–salinity relations' (Remane, 1934). The term 'species abundance', also used by Deaton and Greenberg (1986), was used as synonymous of 'species richness' by the latter authors. Few studies have highlighted the coincidence of the minimum of species with the minimum of species abundance in transitional oligohaline waters in estuaries (Peterson and Vyssieres, 2010; this study). It is usually accepted that natural stress in estuaries favours a low diversity of benthic faunal communities, which are often very abundant (Elliott and Quintino, 2007). However, in contrast to species richness or diversity, macrofaunal abundance has not been clearly documented or described in relation to transitional waters or the *Artenminimum* concept (Attrill, 2002; Attrill and Rundle, 2002; Whitfield et al., 2012), although it is implicit in some studies (e.g. Rundle et al., 1998). It would be interesting to clarify whether some species thrive in the *Artenminimum* zone or if the minimum number of species correspond to a minimum in macrofaunal abundance. Elliott and Whitfield (2011) included as part of the estuarine paradigm the idea that in oligohaline regions "tolerant organisms thrive but non-tolerant organisms are absent". However, our data suggest that highly natural stressful conditions in transitional waters may compromise both species richness and abundance. Analogously, the Habitat Harshness Hypothesis (Defeo et al., 2003) postulates that in reflective beaches, organisms expend more energy in maintenance because they inhabit a harsh environment, resulting in lower fecundity and higher mortality. The Habitat Harshness Hypothesis also predicts lower species diversity (community level) and abundance (population level) under increased physical stress (Defeo and McLachlan, 2005; Fig. 7).

4.2. Additional considerations

The low species richness is consistent with the model of Attrill (2002) in relation to the high fluctuations in salinity at the experimental site. Salinity fluctuations occurred both over time and

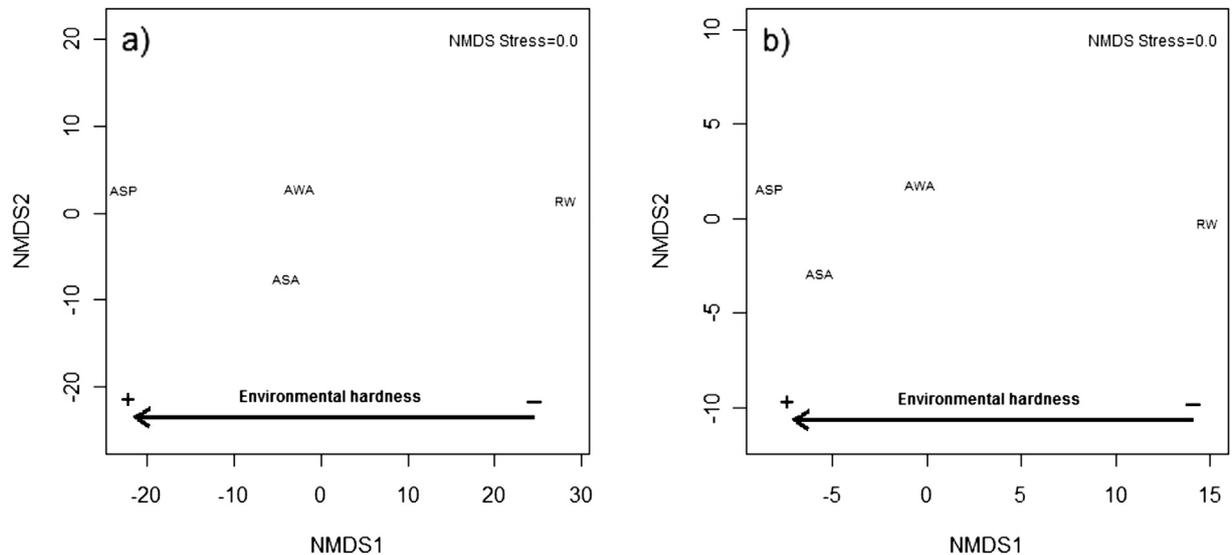


Fig. 7. NMDS plots showing the ordination of the assemblages, a) based on the species data matrix, b) considering the attributes abundance and species richness for each assemblage. The first NMDS axis indicates an increasing in the environmental harshness, displaying the following gradient: Alverca Summer Fluid Mud Present (ASP) > Alverca Summer Fluid Mud Absence (ASA) > Alverca Winter Fluid Mud Absence (AWA) > Rosario Salt Marsh Winter (RW).

within a daily tidal cycle (Figs. 4 and 6). The extreme environmental conditions imposed by salinity fluctuations and sediment dynamics suggest that the experimental site resembled an ecotone rather than an ecocline. An ecotone is an area of relatively rapid change, producing a narrow ecological zone between two different and relatively homogeneous community types, as set out by Attrill and Rundle (2002). Thus, an ecotonal region is compatible with the concept of *Artenminimum* proposed by Remane (1934). This narrow ecotonal region might be ecologically interpreted as the zone where there is a switch of dominance between the biological gradients considered in the two-ecocline model (Attrill and Rundle, 2002).

The ecotonal region in the experimental site was characterized by the polychaetes *Hediste diversicolor*, *S. shrubsolii* and to some extent by the amphipod *Corophium multisetosum*. It appears that these species or individuals of these genera are able to tolerate environmental conditions that other infaunal species are not adapted to cope with. For instance, *H. diversicolor* and *C. volutator* plus oligochaetes occurred towards the oligohaline zone of the Schelde and Ems estuaries (Ysebaert et al., 1998). Core samples revealed that *C. multisetosum* and *S. shrubsolii* dominated the assemblages in a zone of Ria de Aveiro (Portugal) with mean salinity of 2.4 psu (Quintino et al., 2011). The occurrence of *H. diversicolor*, *S. shrubsolii* and *Corophium orientale* was reported in a Mediterranean coastal lagoon of very low salinity (Mogias and Kevrekidis, 2005). Cognetti and Maltagliati (2000) reported that *H. diversicolor*, *S. shrubsolii* and *C. volutator* are among the species of marine origin usually found in transitional waters of salinity of about 5.

The occurrence of one specimen of Oligochaeta suggests that the species of these taxa are not as well adapted to cope with the environmental stress as that observed in Alverca. Accordingly, Seys et al. (1999) reported minimal density, biomass and species richness of oligochaetes in a section of the Schelde estuary (Belgium) with similar salinity fluctuation, very silty sediments and high environmental stress, as described here. McLusky et al. (1993) also reported minimal densities of oligochaetes at salinities of approximately 5 in the Forth estuary, Scotland. Oglesby (1969) suggested that, in intertidal worms, regular salinity fluctuations would be a major constraint to reaching osmotic equilibrium with the external medium.

Finally, sampling in the Rosario salt marsh was not necessary to reach the main conclusion. However, this additional sampling confirmed that the major results (low number of macrofauna and low species richness at the saline frontier) were not a sampling or methodological artefact, but intrinsic ecological features of the experimental site. Moreover, the use of a 0.5 mm mesh size for sampling (rather than the 1 mm used here) might have increased the number of species and abundance observed at the experimental site, thus masking the effect of the environmental stress observed in this estuarine zone.

5. Conclusions

The fluctuations in salinity at the freshwater/oligohaline/mesohaline boundary identified in the intertidal zone of the experimental site were accompanied by the presence/absence of fluid mud. The joint effect of salinity fluctuations and presence of fluid mud was more stressful to macrofaunal species than saline fluctuations alone. Only a few non abundant species were able to cope with the adverse environmental conditions observed in the experimental site (extreme situations observed in summer: saline fluctuations of two orders of magnitude; presence of a fluid mud with more than 7 cm in thickness). The minimum macrofaunal species richness expected in the estuarine freshwater/oligohaline zone (Attrill, 2002; Remane, 1934) requires additional clarification to determine whether it corresponds to a minimal macrofaunal abundance as a general common trend in this specific estuarine zone.

Acknowledgements

We are grateful to Dr. Manuel Aira for his valuable suggestions regarding the statistical analysis. We also thank Mr. Narciso Argibay for granting access to the experimental site through the Adarse industrial zone in Alverca. We gratefully acknowledge the comments and suggestions made by Dr Judi Hewitt and two anonymous reviewers on an earlier version of the manuscript. We also thank a native speaker for language revision and comments. The first author was funded by the Portuguese Foundation for Science and

Technology (FCT; SFRH/BD/48928/2008), which did not have any other role in this study.

References

- Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. *Austral Ecology* 26, 32–46.
- Anderson, M.J., Robinson, J., 2001. Permutation tests for linear models. *Australian and New Zealand Journal of Statistics* 43, 75–88.
- Anderson, M.J., Ter Braak, C.J.F., 2003. Permutation tests for multi-factorial analysis of variance. *Journal of Statistical Computation and Simulation* 73, 85–113.
- Attrill, M.J., 2002. A testable linear model for diversity trends in estuaries. *Journal of Animal Ecology* 71, 262–269.
- Attrill, M.J., Rundle, S.D., 2002. Ecotone or ecocline: ecological boundaries in estuaries. *Estuarine Coastal and Shelf Science* 55, 929–936.
- Attrill, M.J., Rundle, S.D., Thomas, R.M., 1996. The influence of drought-induced low freshwater flow on an upper-estuarine macroinvertebrate community. *Water Research* 30, 261–268.
- Borcard, D., Gillet, F., Legendre, P., 2011. *Numerical Ecology with R*. Springer, New York.
- Brotas, V., Mendes, C.R., Cartaxana, P., 2007. Microphytobenthic biomass assessment by pigment analysis: comparison of spectrophotometry and High Performance Liquid Chromatography methods. *Hydrobiologia* 587, 19–24.
- Chapman, P.M., 1981. Seasonal changes in the depth distributions of interstitial salinities in the Fraser River estuary, British Columbia. *Estuaries* 4, 226–228.
- Cognetti, G., Maltagliati, F., 2000. Biodiversity and adaptive mechanisms in brackish water fauna. *Marine Pollution Bulletin* 40, 7–14.
- Crawley, M.J., 2007. *The R Book*. Wiley, Chichester.
- Deaton, L.F., Greenberg, M.S., 1986. There is no Horohalimum. *Estuaries* 9, 20–30.
- Defeo, O., McLachlan, A., 2005. Patterns, processes and regulatory mechanisms in sandy beach macrofauna: a multi-scale analysis. *Marine Ecology Progress Series* 295, 1–20.
- Defeo, O., Lercari, D., Gómez, J., 2003. The role of morphodynamics in structuring sandy beach populations and communities: what should be expected? *Journal of Coastal Research*, 352–362.
- Dolgoplova, E.N., Isupova, M.V., 2010. Classification of estuaries by hydrodynamic processes. *Water Resources* 37, 268–284.
- Eisma, D., 1993. *Suspended Matter in the Aquatic Environment*. Springer-Verlag, Berlin.
- Elliott, M., Quintino, V., 2007. The estuarine quality paradox, environmental homeostasis and the difficulty of detecting anthropogenic stress in naturally stressed areas. *Marine Pollution Bulletin* 54, 640–645.
- Elliott, M., Whitfield, A.K., 2011. Challenging paradigms in estuarine ecology and management. *Estuarine Coastal and Shelf Science* 94, 306–314.
- Ferreira, J.G., Bricker, S.B., Simas, T.C., 2007. Application and sensitivity testing of an eutrophication assessment method on coastal systems in the United States and European Union. *Journal of Environmental Management* 82, 433–445.
- Fujii, T., 2007. Spatial patterns of benthic macrofauna in relation to environmental variables in an intertidal habitat in the Humber estuary, UK: developing a tool for estuarine shoreline management. *Estuarine Coastal and Shelf Science* 75, 101–119.
- Heip, C., Vincx, M., Vranken, G., 1985. The Ecology of Marine Nematodes. In: *Oceanography and Marine Biology: an Annual Review*, vol. 23, pp. 399–489.
- Jeffrey, S.W., Humphrey, G.F., 1975. New spectrophotometric equations for determining chlorophylls a, b, c_1 and c_2 in higher plants, algae and natural phytoplankton. *Biochemical Physiological Pflanzen* 167, 191–194.
- Khlebovich, V.V., 1968. Some peculiar features of the hydrochemical regime and the fauna of mesohaline waters. *Marine Biology* 2, 47–49.
- Kinne, O., 1971. *Marine Ecology*. Wiley Interscience, London.
- Kirby, R., Parker, W.R., 1983. Distribution and behaviour of fine sediment in the Severn Estuary and Inner Bristol Channel, UK. *Canadian Journal of Fisheries and Aquatic Sciences* 40, 83–95.
- Legendre, P., Gallagher, E.D., 2001. Ecologically meaningful transformations for ordination of species data. *Oecologia* 129, 271–280.
- Legendre, P., Legendre, L., 1998. *Numerical Ecology*. Elsevier, Amsterdam.
- McLusky, D.S., 1993. Marine and estuarine gradients – an overview. *Netherlands Journal of Aquatic Ecology* 27, 489–493.
- McLusky, D.S., Elliott, M., 2004. *The Estuarine Ecosystem*. John Wiley and Sons, New York.
- McLusky, D.S., Hull, S.C., Elliott, M., 1993. Variations in the intertidal and subtidal macrofauna and sediments along a salinity gradient in the upper forth estuary. *Netherlands Journal of Aquatic Ecology* 27, 101–109.
- Mogias, A., Kevrekidis, T., 2005. Macrozoobenthic community structure in a poikilohaline Mediterranean lagoon (Laki Lagoon, northern Aegean). *Helgolander Marine Research* 59, 167–176.
- Mouny, P., Dauvin, J.C., Bessineton, C., Elkaim, B., 1998. Biological components from the Seine estuary: first results. *Hydrobiologia* 373/374, 333–347.
- Nebra, A., Caiola, N., Ibáñez, C., 2011. Community structure of benthic macroinvertebrates inhabiting a highly stratified Mediterranean estuary. *Scientia Marina* 75, 577–584.
- Oglesby, L.C., 1969. Salinity stress and desiccation in intertidal worms. *American Zoologist* 9, 319–331.
- Oksanen, J., 2011. *Multivariate Analysis of Ecological Communities in R: Vegan Tutorial*. <http://cc.oulu.fi/~jarioksa/opetus/metodi/vegantutor.pdf> (last accessed 08.06.12.).
- Oksanen, J., Kindt, R., Legendre, P., O'Hara, R.B., 2006. *VEGAN: Community Ecology Package Version 1.8-2*. <http://cran.r-project.org> (last accessed 15.07.12.).
- Peterson, H.A., Vayssières, M., 2010. Benthic assemblage variability in the upper San Francisco estuary: a 27-year retrospective. *San Francisco Estuary Watershed Science* 8, 1–27.
- Plotnikov, I.S., Aladin, N.V., 2011. An overview of hybrid marine and lacustrine seas and saline lakes of the world. *Lakes and Reservoirs: Research and Management* 16, 97–108.
- Portela, L.I., Neves, R., 1994. Numerical modelling of suspended sediment transport in tidal estuaries: a comparison between the Tagus (Portugal) and the Scheldt (Belgium-The Netherlands). *Netherlands Journal of Aquatic Ecology* 28, 329–335.
- Quinn, G.P., Keough, M.J., 2002. *Experimental Design and Data Analysis for Biologists*. Cambridge University Press, New York.
- Quintino, V., Sangiorgio, F., Mamede, R., Ricardo, F., Sampaio, L., Martins, R., Freitas, R., Rodrigues, A.M., Basset, A., 2011. The leaf-bag and the sediment sample: two sides of the same ecological quality story? *Estuarine Coastal and Shelf Science* 95, 326–337.
- R Development Core Team, 2009. *R: a Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. www.Rproject.org (last accessed 28.10.11.).
- Remane, A., 1934. Die Brackwasserfauna. *Verhandlungen der Deutschen Zoologischen Gesellschaft, Greifswald* 36, 34–74.
- Rhoads, D.C., 1974. Organism-sediment Relations on the Muddy Sea Floor. In: *Oceanography and Marine Biology: an Annual Review*, vol. 12, pp. 263–300.
- Rundle, S.D., Attrill, M.J., Arshad, A., 1998. Seasonality in macroinvertebrate community composition across a neglected ecological boundary, the freshwater-estuarine transition zone. *Aquatic Ecology* 32, 211–216.
- Sanders, H.L., Mangelsdorf, P.C.J., Hampson, G.R., 1965. Salinity and faunal distribution in the Pocasset River, Massachusetts. *Limnology and Oceanography* 10, 216–229.
- Santos, P.J.P., Castel, J., Souza-Santos, L.P., 1996. Seasonal variability of meiofaunal abundance in the oligo-mesohaline area of the Gironde estuary, France. *Estuarine Coastal and Shelf Science* 43, 549–563.
- Schoellhamer, D.H., 2001. Influence of salinity, bottom topography, and tides on locations of estuarine turbidity maxima in northern San Francisco Bay. In: McAnally, W.H., Mehta, A.J. (Eds.), *Coastal and Estuarine Fine Sediment Transport Processes*. Elsevier Science, Vicksburg, pp. 343–357.
- Schubert, H., Feuerpeil, P., Marquardt, R., Telesh, I., Skarlato, S., 2011. Macroalgal diversity along the Baltic Sea salinity gradient challenges Remane's species-minimum concept. *Marine Pollution Bulletin* 62, 1948–1956.
- Seys, J., Vincx, M., Meire, P., 1999. Spatial distribution of oligochaetes (Clitellata) in the tidal freshwater and brackish parts of the Schelde estuary (Belgium). *Hydrobiologia* 406, 119–132.
- Stickney, R.R., 2009. *Aquaculture: an Introductory Text*. CAB International, Wallingford.
- Telesh, I.V., Khlebovich, V.V., 2010. Principal processes within the estuarine salinity gradient: a review. *Marine Pollution Bulletin* 61, 149–155.
- Telesh, I.V., Schubert, H., Skarlato, S.O., 2011. Revisiting Remane's concept: evidence for high plankton diversity and a protistan species maximum in the horohalimum of the Baltic Sea. *Marine Ecology Progress Series* 421, 1–11.
- ter Braak, C.J.F., 1987. The analysis of vegetation-environment relationships by canonical correspondence analysis. *Vegetatio* 69, 69–77.
- Thrush, S.F., Hewitt, J.E., Norkko, A., Nicholls, P.E., Funnell, G.A., Ellis, J.L., 2003. Habitat change in estuaries: predicting broad-scale responses of intertidal macrofauna to sediment mud content. *Marine Ecology Progress Series* 263, 101–112.
- Uncles, R.J., Stephens, J.A., 2010. Turbidity and sediment transport in a muddy sub-estuary. *Estuarine Coastal and Shelf Science* 87, 213–224.
- Underwood, A.J., 1997. *Experiments in Ecology: their Logical Design an Interpretation Using Analysis of Variance*. Cambridge University Press, Cambridge.
- Vale, C., Canário, J., Caetano, M., Lavrado, J., Brito, P., 2008. Estimation of the anthropogenic fraction of elements in surface sediments of the Tagus Estuary (Portugal). *Marine Pollution Bulletin* 56, 1353–1376.
- Venice System, 1959. Symposium on the Classification of Brackish Waters, Venice, April 8–14, 1958. In: *Archivio di Oceanografia e Limnologia*, vol. 11, pp. 1–248.
- Whitfield, A.K., Elliott, M., Basset, A., Blaber, S.J.M., West, R.J., 2012. Paradigms in estuarine ecology – a review of the Remane diagram with a suggested revised model for estuaries. *Estuarine Coastal and Shelf Science* 97, 733–739.
- Ysebaert, T., Meire, P., Coosen, J., Essink, K., 1998. Zonation of intertidal macrobenthos in the estuaries of Schelde and Ems. *Aquatic Ecology* 32, 53–71.
- Zuur, A.F., Ieno, E.N., Elphick, C.S., 2010. A protocol for data exploration to avoid common statistical problems. *Methods in Ecology and Evolution* 1, 3–14.