Universidade de Aveiro Departamento de Biologia
2014

MARTA FILIPA LOBÃO LOPES

AVALIAÇÃO DO ESTADO DE QUALIDADE ECOLÓGICA EM SISTEMAS DE TRANSIÇÃO

ECOLOGICAL QUALITY ASSESSMENT IN TRANSITIONAL SYSTEMS

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ECOLOGICAL QUALITY ASSESSMENT IN TRANSITIONAL SYSTEMS

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Professor Doutor Victor Quintino e da Professora Doutora Ana Maria Rodrigues, Professores do Departamento de Biologia da Universidade de Aveiro.

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário, através da atribuição da bolsa de Doutoramento com referência SFRH/BD/47865/2008.

o júri

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palavras-chave Qualidade ecológica; Índices taxonómicos; Índices não-taxonómicos; Indicadores funcionais; Sistemas de transição; Ria de Aveiro.

resumo

Os estuários são pólos de atração para a instalação de aglomerados humanos, constituindo uma fonte de pressão para as massas de água superficiais. Com a implementação da Diretiva Europeia Quadro da Água (DQA, 2000/60/CE) tem aumentado a investigação no sentido de desenvolver metodologias para avaliar o estado de qualidade ecológica (EQE) dos ecossistemas aquáticos. Os sistemas de transição são caracterizados por condições físico-químicas e hidromorfológicas extremamente dinâmicas e por espécies com uma maior tolerância à mudança, sendo difícil desenvolver indicadores de qualidade adequados para estes ecossistemas. O objetivo deste estudo é testar a capacidade de descritores de síntese, tais como a riqueza em espécies (*S*) e a diversidade de Shannon-Wiener (*H'*), índices de base taxonómica (AMBI e M-AMBI) e não taxonómica (ISS), as propriedades do tamanho corporal (distribuição de abundância por classes de tamanho corporal, comprimento, peso e relações comprimento-peso), bem como indicadores funcionais (taxas de decomposição de uma macrófita (*Phragmites australis*) e uma alga (*Fucus vesiculosus*)), para avaliar a qualidade ambiental dos sistemas de transição. Este estudo foi realizado ao longo de um gradiente completo de salinidade num dos canais com menor impacto antropogénico da Ria de Aveiro, o Canal de Mira, numa área com contaminação sedimentar por metais e metaloides, o Canal de Estarreja, e dois canais de referência (Canelas e Salreu). Neste estudo foram utilizadas diferentes técnicas de amostragem, a técnica dos sacos de folha e corers.

No Canal de Mira, a alga e a macrófita apresentaram uma tendência oposta na taxa de decomposição ao longo do gradiente de salinidade, com as taxas de decomposição da alga sempre superiores. As taxas de decomposição da macrófita e da alga foram mais elevadas a meio do estuário e em áreas de maior salinidade, respetivamente, correspondendo às preferenciais áreas de distribuição de cada espécie. A fauna bentónica associada aos substratos orgânicos e a um substrato artificial (controlo) mostrou a sucessão bentónica ao longo do gradiente estuarino e, apesar das grandes diferenças nas taxas de decomposição, não foram encontradas diferenças nas comunidades bentónicas entre ambos os substratos. As propriedades do tamanho corporal da fauna bentónica dos sacos de folhas de *P. australis* (1mm e 5mm) e corers foram estudadas ao longo do gradiente estuarino. As espécies dominantes do sub-conjunto de espécimes medidos não são as mesmas da fauna bentónica original mas, apesar disso, foram capazes de mostrar a sucessão bentónica ao longo do gradiente salino. A distribuição da abundância pelas classes de tamanho estabelecidas para o cálculo do índice ISS não mostrou nenhuma tendência ao longo do gradiente de salinidade em nenhum dos amostradores. Foram encontradas diferenças significativas no comprimento, peso e na relação comprimento-peso dos Anelídeos, Artrópodes, Moluscos e de algumas espécies ao longo do gradiente de salinidade. Não foram encontradas diferenças significativas nos valores AMBI, M-AMBI e ISS ao longo do gradiente estuarino. O EQE das amostras dos corer foi inferior ao dos sacos de folhas. O EQE obtido com o ISS foi mais elevado do que o obtido com o M-AMBI nos sacos de folha, mas não nas amostras do corer.

Os efeitos ecológicos associados à contaminação dos sedimentos por efluentes químicos lançados no Canal de Estarreja foram estudados uma década após a sua cessação, recorrendo à Tríade de Qualidade Sedimentar e dois canais de referência. Os resultados mostraram que o sedimento permanece contaminado com elevados níveis de metais e metaloide, disponíveis para serem bioacumulados e com graves consequências ao nível da comunidade. A contaminação do sedimento foi também estudada utilizando a técnica dos sacos de folhas com a macrófita, a alga e um substrato de controlo, tendo-se verificado que as taxas de decomposição, a macrofauna associada e a aplicação dos índices AMBI, M-AMBI e ISS aos sacos de folhas não foram capazes de identificar o problema. Ao contrário do AMBI, o M-AMBI e o ISS apresentaram diferenças significativas entre o canal contaminado e os canais de referência para as amostras do corer. No entanto, a utilização de índices tão complexos é questionável, na medida em que índices mais simples, tais como a *S* e a *H'*, permitem chegar às mesmas conclusões.

keywords Ecological quality, Taxonomic indices, Non-taxonomic indices; Functional indicators; Transitional systems, Ria de Aveiro.

abstract

Estuaries are poles of attraction for human settlement which is a source of pressures to surface water bodies. The implementation of the European Water Framework Directive (WDF, 2000/60/EC) has increased the investigation in order to develop methodologies to assess the Ecological Quality Status (EQS) of aquatic ecosystems. Transitional systems are naturally stressed and characterized by highly dynamic physical, chemical and hydro-morphologic conditions and by species with a higher level of tolerance to change, being more difficult to develop suitable quality indicators for these systems. The general purpose of this study is to test the ability of synthesis descriptors, including primary (*S*, taxa richness) and derived biological variable (*H'*, Shannon-Wiener diversity), biotic indices (AMBI and M-AMBI), body size properties (abundance distribution by body size classes, length, weight and length-weight relationships) and non-taxonomic indices (ISS), as well as functional indicators related to the decomposition rates of various experimental substrates, a macrophyte (*Phragmites australis*) and an alga (*Fucus vesiculosus*), to evaluate the environmental quality in transitional systems. This study was carried out in one of the most pristine channels of the Ria the Aveiro, Mira Channel, along a full salinity gradient and in a metals and metalloid sediment contamination area, the Estarreja Channel, and two reference channels (Canelas and Salreu). In this study were used different sampling techniques, the leaf-bag technique and a hand-held corer.

In Mira Channel, the alga and the macrophyte presented an opposite trend in the decomposition rate along the salinity gradient, with the decomposition rates of the alga always higher than those of the macrophyte. The decomposition rates of the macrophyte and the alga were higher in the mid estuary and in higher salinity areas, respectively, corresponding to the preferencial distribution areas of each species. The macrobenthic fauna associated with the decaying and an artificial substrate (control) showed equally well the benthic succession from the marine to the freshwater areas and, despite the strong differences in the decay rates, no significant differences were found between the benthic communities associated with the alga and the macrophyte. The body size properties of the macrobenthic fauna associated with the *P. australis* leaf-bag (1mm and 5mm) and corer samples were studied along the full salinity gradient. The dominant species of the sub-set of measured specimens were not the same of the original macrobenthic fauna sampled but, despite that, the sub-set of measured specimens was also able to show the benthic succession from the marine to the freshwater areas. The body size abundance distribution of the benthic macroinvertebrates according to the ISS size classes did not show a particular trend in any sampler along the salinity gradient. Significant differences were found in the length, weight and length-weight relationships of Annelids, , Molluscs and even some species along the salinity gradient. No significant differences were found in the AMBI, M-AMBI and ISS values along the salinity gradient for all the samplers. The EQS of the corer samples obtained using the M-AMBI was lower than that of the leaf-bags. The EQS obtained with the ISS was higher than that obtained with the M-AMBI in the leaf-bags but not in the corer samples.

The ecological effects of contaminated sediments associated with the industrial chemical effluents discharged in the Estarreja Channel were studied a decade after ceasing the emissions, using the Sediment Quality Triad approach and two reference channels. The results showed that despite the emissions ceased in 2004, the sediment remains polluted with high levels of metals and metalloid, available to bioaccumulation and with severe consequences at the community level. The sediment contamination problem was also studied using the leaf-bag technique with a macrophyte, an alga and a control substrate. The results showed that the decay rates, the associated macrofauna and the application of the AMBI, M-AMBI and ISS indices to the mesh-bag samples were not able to identify the sediment contamination. Contrarily to the AMBI, the M-AMBI and the ISS showed significant differences between the contaminated and the reference channels for the corer samples. Although such statistical significance, the interest of using these complex biotic indices could be questioned, when much simple ones, like the *S* and *H'* allow to reach the same conclusions.

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Chapter 1. Introdution

1.1 Transitional waters and biomonitoring

Transitional waters were defined by the European Community (2000/60/EC) as 'bodies of surface water in the vicinity of river mouths which are partially saline in character as a result of their proximity to coastal waters but which are substantially influenced by freshwater flows”. Transitional systems are characterized by highly dynamic physical, chemical and hydro-morphologic conditions, resulting in a mosaic of habitats in which species are particularly well adapted to variability. Such species are essentially more tolerant to change than their marine counterparts.

Transitional systems are very productive ecosystems with an important role in the life history of many species, being the preferential areas for the reproduction and growth of several aquatic species, some with high economic value (among others, McLusky and Elliott, 2004). Due to their natural characteristics, estuaries have been poles of attraction for human settlement and some present large urban and industrial settlements and intensive agriculture, constituting sources of pressures to surface water bodies (Ferreira et al., 2004; Chapman et al., 2013). Due to the important role of these ecosystems, the environmental impact assessment of human activities in these systems is of primordial importance.

Attempts to decode ecosystem properties based on species composition and sensitivity to disturbances date back to more than 100 years ago (Kolkwitz and Marsson, 1908) and have received increasing attention since the implementation of the European Water Framework Directive (WDF, 2000/60/EC). Thus, over the last years, the approach to water issues has changed significantly in order to fulfil the WFD requirements. The main objectives of the WFD are to prevent water ecosystem degradation, to protect and to enhance the status of water resources and, most of all, to achieve a ‘Good Ecological Status’ (GES) for all waters, by 2015. The ecological status of a water body is determined using a range of hydro-morphological and physicochemical quality elements, as well as, biological quality elements (phytoplankton, other aquatic flora, benthic macroinvertebrates and fish) (Salas et al., 2006). Besides the central functioning role that benthic macrofauna has in transitional ecosystems, several studies have frequently demonstrate that it responds relatively rapid to anthropogenic and natural stresses (Pearson and Rosenberg, 1978; Dauer, 1993). Due to their limited mobility, benthic communities are quite sensitive to local disturbance and, due to their permanence over seasonal time scales, they integrate the recent history of disturbance which may not be detected in the water column (Bettencourt et al., 2004). In benthic communities we can also find different species exhibiting different tolerance to stress (Dauer, 1993), which covers the WFD demand of integrating sensitive species. The WFD requires a water body to be compared against a reference condition and then its ecological status designated. If the water body does not meet good or high ecological status, then remedial measures have to be taken.

According to the "paradox of brackish water" standard for estuarine ecology, proposed by Remane (1934) and then reviewed by Whitfield et al. (2012), transitional waters have fewer number of taxa than fresh or marine waters (Figure 1.1). This paradigm suggests that brackish water fauna can survive and thrive in a wide range of salinity conditions. Thus, the three key concepts of the model for estuarine biodiversity related to salinity are: more marine than freshwater taxa; less penetration by freshwater taxa into more saline regions than by saltwater taxa into less saline regions; and, transitional water taxa (neither fresh nor marine) are less abundant than marine taxa (Figure 1.1). Benthic infaunal distributions will vary with season or other shifts in salinity (Chapman and Brinkhurst, 1981). Besides salinity, other factors, such as sediment type and hypoxia can influence taxa presence, abundance and distribution.

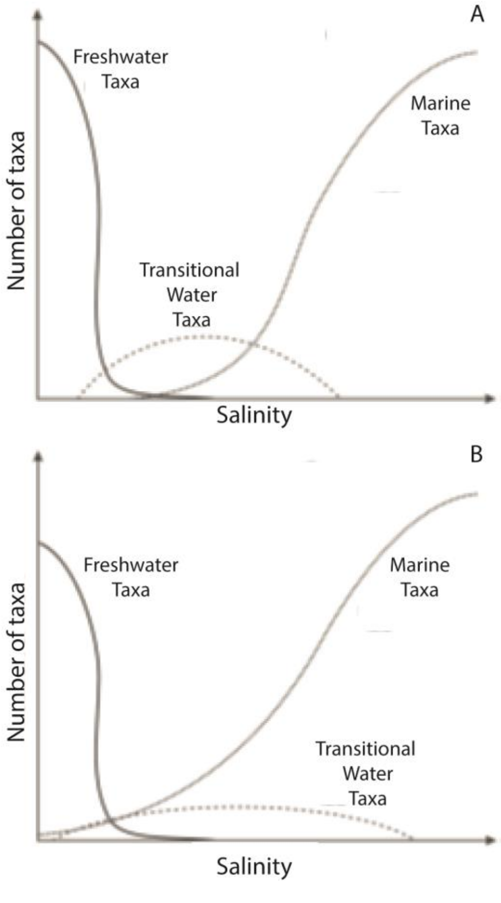

Figure 1.1. Paradigms of transitional waters taxa distributions. A) Remane's "paradox of brackish water". B.) Whitfield et al.'s new conceptual model (in Chapman et al., 2013).

Thus, considering the complexity and heterogeneity of transitional systems, it should be necessary to define stretches within these systems where specific reference conditions should rule. This is to ensure that the natural biological impoverishment that is observed along the salinity gradient towards inner parts of transitional waters systems is reflected and accounted for the ecological evaluation of multimetric methodologies (Quintino and Rodrigues, 1989; Rodrigues and Quintino, 1993; Uwadiae, 2009; Rodrigues et al., 2006; 2011; Whitfield et al., 2012). Other aspects such as morphological, hydrological or habitat type might also help to the definition of specific stretches among transitional systems in evaluation (Ferreira et al., 2006).

According to Pearson and Rosenberg (1978), the basic quantitative parameters in almost all benthic ecological investigations are the number of species, their abundance and biomass. These authors developed the 'Species, Abundance, Biomass model' (SAB), widely used to describe the successive changes in number of species, number of individuals and biomass along a gradient of organic enrichment (Figure 1.2). This conceptual model can be associated with a stressor-response relationship where the organic enrichment of sediments is the stressor component and the measures of macrobenthic attributes are the response variables. The SAB model or its underlying concepts, have been the basis for many indices developed under the European Water Framework Directive requirements. Several biotic indices based in the species tolerance/sensitivity to stress have been proposed to be used as ecological quality indicators for marine and estuarine systems (AMBI, Borja et al., 2000, BENTIX, Simboura and Zenetos, 2002, BQI, Rosenberg et al., 2004, M-AMBI, Borja et al., 2003; Muxika et al., 2007; BOPA, Dauvin and Ruellet, 2007). In this study were applied the AMBI and M-AMBI indices and, for this reason, both indices are described with more detail.

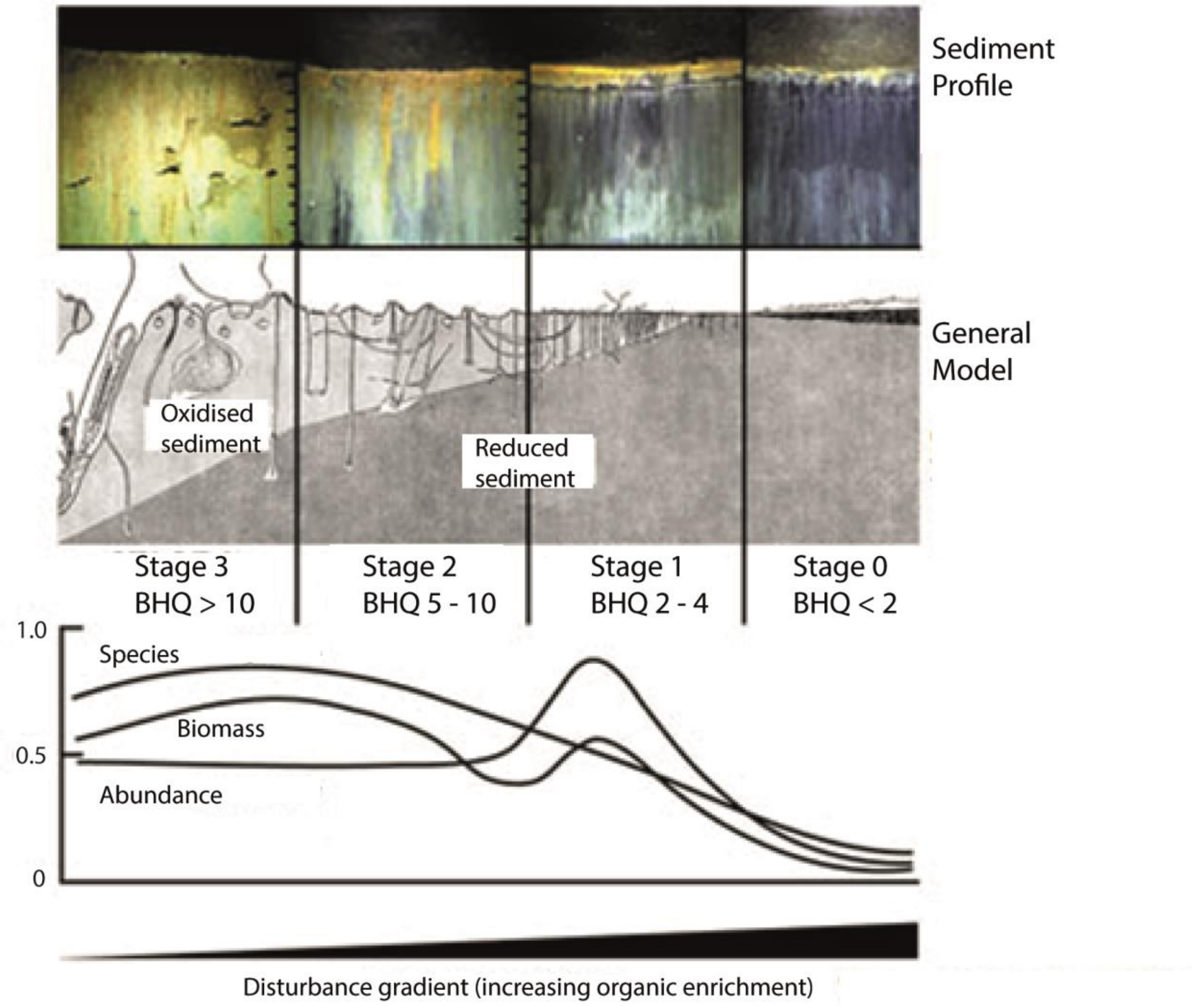

Figure 1.2 Distribution of benthic infaunal successional stages along a gradient of increasing environmental disturbance (Pearson and Rosenberg, 1978) and associated benthic-habitat quality (BHQ) index (Nilsson and Rosenberg, 1997). Sediment profile images assigned to successional stage are shown above the general model: oxidized sediment is rust-brown and reduced sediment grey or black. Bottom graph illustrates generalized changes in species, abundance, and biomass (Pearson and Rosenberg, 1978) (in Nilsson and Rosenberg, 2000).

The AMBI index is based on the tolerance/sensitivity of species to organic enrichment and classifies the soft-bottom macrofauna in five groups, according to their sensitivity to an increasing organic matter enrichment (Borja et al., 2000):

Group I. Species very sensitive to organic enrichment and present under unpolluted conditions (initial state). They include the specialist carnivores and some deposit-feeding tubicolous polychaetes.

Group II. Species indifferent to enrichment, always present in low densities with no significant variations with time (from initial state, to slight unbalance). These include suspension feeders, less selective carnivores and scavengers.

Group III. Species tolerant to excess organic matter enrichment. These species may occur under normal conditions, but their populations are stimulated by organic enrichment (slight unbalance situations). They are surface deposit-feeding species, as tubicolous spionids.

Group IV. Second-order opportunistic species (slight to pronounced unbalanced situations). Mainly small sized polychaetes: subsurface deposit-feeders, such as cirratulids.

Group V. First-order opportunistic species (pronounced unbalanced situations). These are deposit-feeders, which proliferate in reduced sediments.

The proportions of the five ecological groups (EG) provided by the AZTI laboratory's list (www.azti.es) were used to calculate the AMBI index according to the equation (Borja et al., 2000; Borja and Muxika, 2005):

$$\text{AMBI} = 0\text{EG}_{\text{I}} + 1.5\text{EG}_{\text{II}} + 3\text{EG}_{\text{III}} + 4.5\text{EG}_{\text{IV}} + 6\text{EG}_{\text{V}}$$

The results of the AMBI calculation can vary between 0 (high ecological status) and 6 (bad ecological status) (Borja et al., 2003).

The multimetric M-AMBI index combines the species richness (*S*), the Shannon-Wiener diversity (*H'*) and the biotic index AMBI (Borja et al., 2003; Muxika et al., 2007). The minimum reference values ('bad reference conditions') are represented by the metrics $S = 0$, $H' = 0$ and AMBI = 6 whereas maximum values ('high reference conditions') correspond to the highest values in the dataset (or the lowest in the case of AMBI). However, alternative reference values can be set. The results of the M-AMBI calculation can vary between 0 (bad ecological status) and 1 (high ecological status) (Borja et al., 2003).

Both indices were calculated using the software AMBI v5.0 freely available from http://ambi.azti.es and their calculation is based on a list of macroinvertebrate taxa, mostly at the species level, regularly updated (updated March 2012). This list is now composed of about 6500 taxa from all seas, representing the most important soft-bottom communities from estuarine and coastal systems, from the North Sea to the Mediterranean, North and

South America, Asia, etc.. In this study, the threshold values used to translate the M-AMBI values into ecological quality statements (EQS) were based upon the European intercalibration exercise (Borja et al., 2007; 2009): $0 \leq$ Bad $\leq 0.20 <$ Poor $\leq 0.38 <$ Moderate $\leq 0.53 <$ Good $\leq 0.77 <$ High > 1.

A significant challenge in assessing stressor-response relationship in transitional waters, such as estuaries and coastal lagoons, is that they are under the influence of multiple factors and have a great internal patchiness and heterogeneity, which can often bias the application of the most common indicators and indices of environmental quality and health status (Elliott and Quintino, 2007; Ruellet and Dauvin 2007). The estuarine quality paradox however, lead authors to suggest the importance to use functional as well as structural indicators of change in transitional waters (Elliott and Quintino, 2007). In the freshwater environment, several bio-monitoring studies have included both functional and structural approaches, namely leaf litter decomposition rates and structural characteristics of the associated benthic invertebrate community (Bergfur et al., 2007; Castela et al., 2008; Pascoal et al., 2003). In estuarine ecosystems, decomposition studies are far less common (Twilley et al., 1986; Rossi and Costantini, 2000; Menéndez and Sanmartì, 2007; Sangiorgio et al., 2008; Quintino et al., 2009). In studies using decomposition rates as functional indicator, the decomposition rate is modelled as a negative exponential decay function. This model, proposed by Jenny et al. (1949), has been frequently used to describe decomposition (Olson, 1963; Petersen and Cummins, 1974):

$$Mt = M_0 . e^{-kt}$$

where Mt is the percent mass remaining at time (t), M_0 is the initial percent mass, and k is the decomposition rate.

In order to overcome the difficulties to assess the ecological status of transitional and coastal systems using taxonomic based indices, non-taxonomic indices were also developed, including indices based on overall biomass distribution (Gray and Mirza, 1979; Warwick, 1986; Clarke, 1990; De Eyto and Irvine, 2007) as well as indices based on size distribution metrics, such as skewness (ISD, Reizopoulou and Nicolaidou, 2007), size diversity (Quintana et al., 2008), the proportion of large individuals or size-classes (Basset et al., 2008) and the measure of macroinvertebrate size classes sensitivity to stress (ISS, Basset et al., 2012). These metrics are based on the fact that large species are more sensitive to disturbance than small ones (Pearson and Rosenberg, 1978) and on the relationship between size class distribution and energy availability (Brown et al., 2004; Basset et al., 2008) and anthropogenic impact (Queiros et al., 2006; Reizopoulou and Nicolaidou, 2007). Individual metabolism, life cycle parameters, population growth and interactions are affected by individual body size (Basset, 1997; Brown et al., 2004; Brucet et al., 2005; Basset and De Angelis, 2007), functionally linking size spectra to ecosystem properties such as vigour, organization and resilience (Basset, 2010). Therefore, size spectra are particularly suitable structural community components for translating ecological status into measurable metrics (Basset et al., 2008; Trayanova, 2008; Gascón et al., 2009; Brucet et al., 2010; de-la-Ossa-Carretero et al., 2010).

In this study was explored the use of the non-taxonomic Index of Size Spectra Sensitivity (ISS) (Basset et al., 2012). The ISS involves the abundance distribution of the benthic fauna by the body size classes, the sensitivity/tolerance of macroinvertebrate body size classes to stress and a correction factor associated with the taxa richness. The ISS is calculated according to the formula (Basset el al., 2012):

$$ISS = \sum p(CL_i) * \omega i * s$$

where $p(CL_i)$ is the proportion of individuals in the ith size class, ωi is the sensitivity value assigned to the ith size class and s is a discrete correction factor for the number of taxa. The correction factors used were those suggested by Basset el al. (2012): 1 for all samples with more than 5 taxa, 0.8 for 4 and 5 taxa, 0.4 for 2 and 3 taxa, and 0.2 for samples with 1 taxon. The ecological quality statement from the ISS values was determined considering the boundaries established by Basset et al. (2013). The Ria the Aveiro was characterized as a large ecosystem, with an average tidal > 0.5 m and a salinity ≥ 30 in the euhaline and polyhaline areas and a salinity < 30 in the mesohaline, oligohaline and limnetic areas. Thus, for the euhaline and polyhaline areas the Ecological Quality Boundaries were Azoic = 0 < Bad < 0.8 ≤ Poor < 1.4 ≤ Moderate < 1.8 ≤ Good < 2.5 ≤ High ≤ 6.0 and for the mesohaline, oligohaline and limnetic areas the Ecological Quality Boundaries were Azoic = 0 < Bad < 0.8 ≤ Poor < 1.5 ≤ Moderate < 1.9 ≤ Good < 2.8 ≤ High ≤ 6.0.

1.2 Study area

Ria de Aveiro is located on the Northwestern coast of Portugal, between 40°38`N and 40°57`N, with a maximum width and length of 10 and 45 km, respectively. It has an irregular geometry, and its only connection with the Atlantic Ocean is through an artificial inlet constructed in 1808 (Dias and Mariano, 2011). This system includes four main Channels, Mira, Ílhavo, Espinheiro and São Jacinto, characterized by extensive intertidal mud and sand flats, salt marshes and islands (Figure 1.3).

In spring tides the lagoon area reaches a maximum area of 83 km^2 at high tide, which decreases to a minimum of 66 km^2 at low tide. The average depth in Ria de Aveiro is about 1 m, although the inlet channel can exceed 30 m deep in order to allow the navigation (Dias and Lopes, 2006). The main forcing agent driving water circulation in Ria de Aveiro is the tide, which is mesotidal, presenting an average tidal amplitude at the inlet of 2 m, and amplitudes of 0.6 m in neap tides and 3.2 m in spring tides (Araújo et al., 2008). Ria de Aveiro has five main rivers, discharging in all of the main channels: Vouga, Antuã, Caster, Boco and Ribeira dos Moinhos.

The lagoon is vertically homogenous during most of the time, although during strong freshwater flows, is classified as weakly stratified (Dias et al., 1999).

Mira Channel is a narrow 20 Km long channel, running south from the entrance (Figure 1.3). This channel presents a full salinity gradient, ranging from fully marine at the mouth to freshwater at the head, receiving continuous freshwater input (Moreira et al., 1993). It is also one of the most pristine channels in Ria de Aveiro (Castro et al., 2006). In this work, Mira Channel was used to study functional, body-size descriptors and benthic indicators along the full salinity gradient without the influence of anthropogenic stresses.

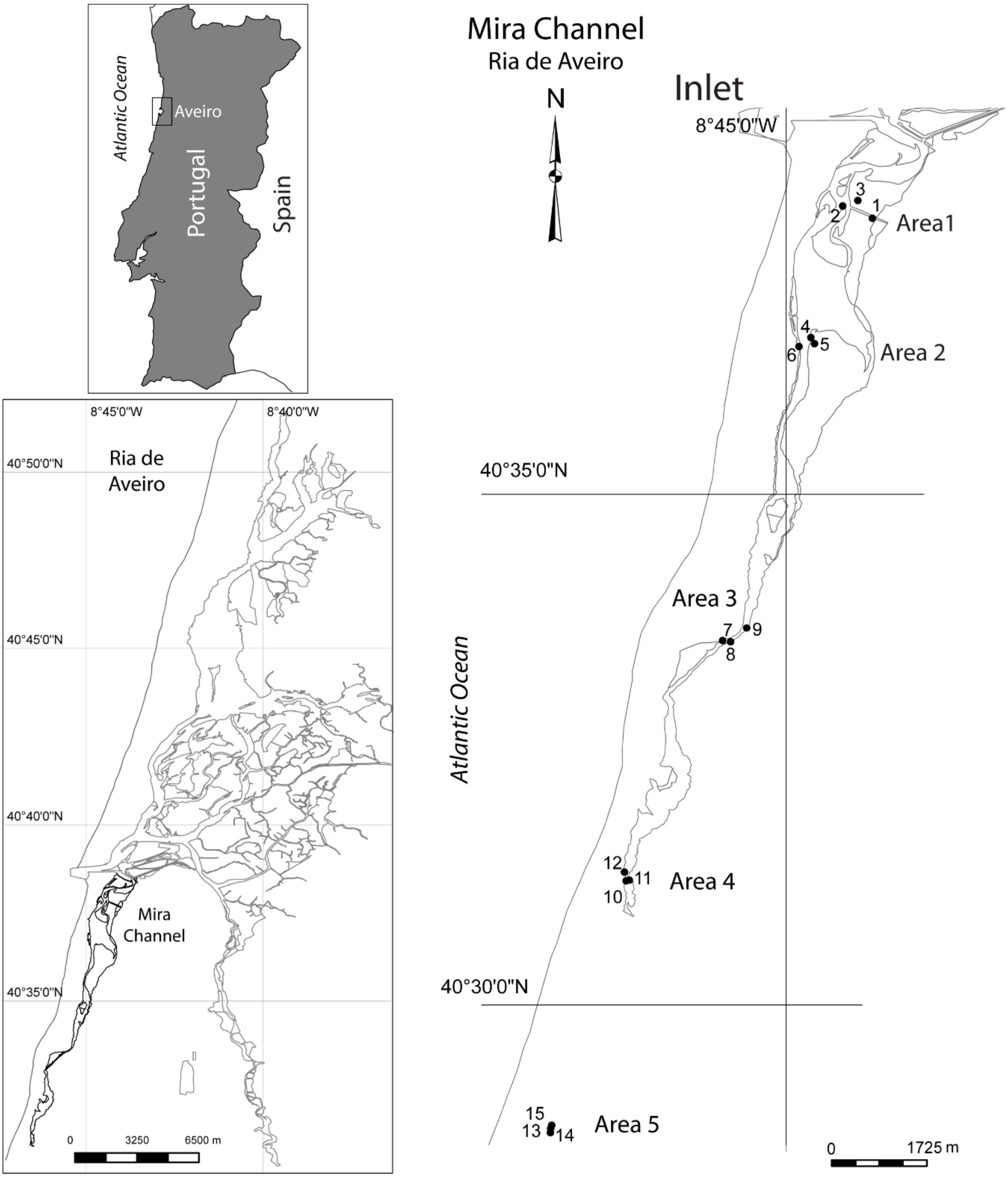

Figure 1.3 Positioning of the study sites in the Mira Channel, Ria de Aveiro, Western Portugal.

All the knowledge acquired in the Mira Channel was applied in an area affected by anthropogenic pressures, namely metals and metalloid sediment contamination. In this stage, the study was conducted in three small channels, Estarreja, Canelas and Salreu, discharging to the Laranjo Bay, in the central area of the Ria (Figure 1.4). The Estarreja Channel received from more than 70 years continuous industrial discharges from the Estarreja Chemical Complex (CQE), which began operating in the 1930s with the production of fertilizers, mineral acids, chlorine and soda, plastics, aromatics and other products. Until 1975, industrial effluents containing organic compound, metals and metalloid, such as mercury, arsenic, zinc, lead, aluminum, cadmium, copper, vanadium and iron were discharged into artificial channels and then into the Estarreja Channel. After 1975, the effluents were discharged directly by pipes into the Estarreja Channel. In 1994, mercury emissions were reduced to regulatory levels (50 µg L^{-1}, the limit value for discharges from chlor-alkali electrolysis industry in accordance with the European Directive 82/176/EEC, 1982) and then emissions ceased in 2002. In 2004 the effluents discharged into the Estarreja Channel were channelled to a multi-municipality sanitation system (SIMRIA) and ocean outfall, thus removing all sewage and industrial effluent discharges from the Ria de Aveiro.

The Canelas and Salreu Channels were used as reference sites, representing largely uncontaminated channels.

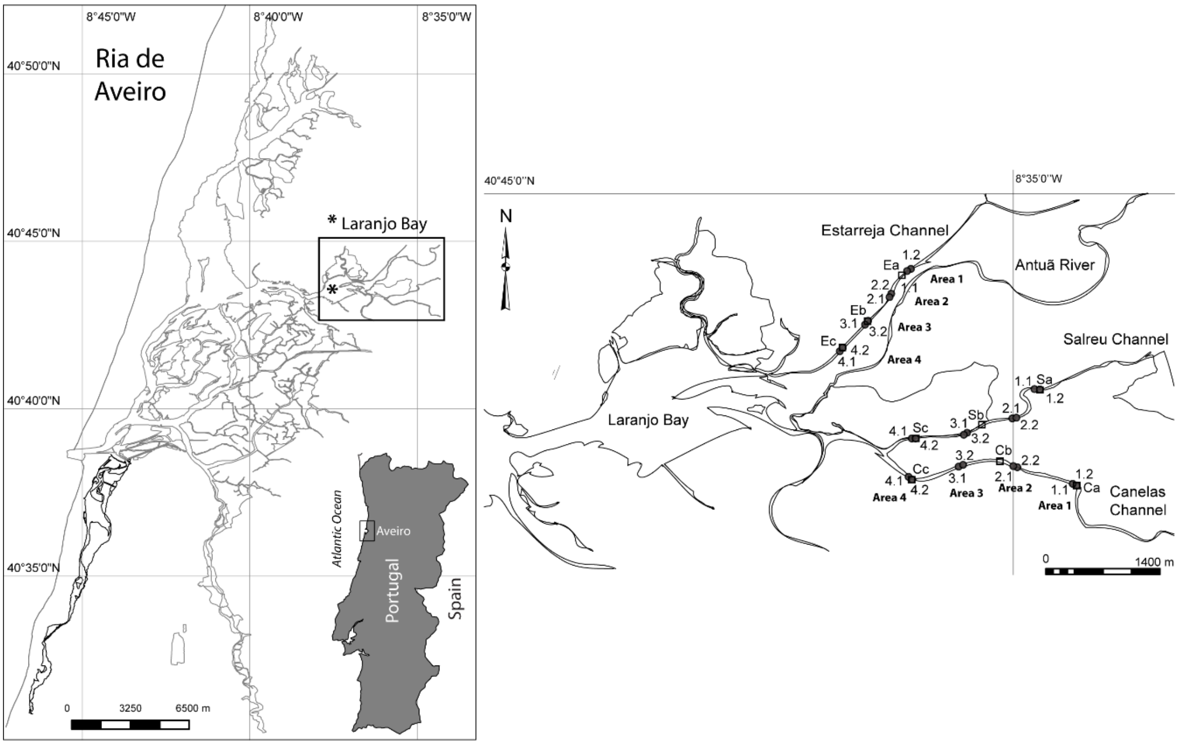

Figure 1.4 Positioning of the study sites in the Estarreja, Salreu and Canelas Channels, Ria de Aveiro, Western Portugal.

1.3 Objectives and thesis outline

The general purpose of this study is to test the ability of synthesis descriptors, including primary (taxa richness) and derived biological variable (Shannon-Wienner diversity), taxonomic indices (AMBI and M-AMBI), body size properties (abundance distribution by body size classes, length, weight and length-weight relationships) and non-taxonomic indices (ISS), as well as functional indicators related to the decomposition rates of various experimental substrates, a macrophyte (*Phragmites australis*) and an alga (*Fucus vesiculosus*), to evaluate the environmental quality in transitional systems.

At the beginning of this work, several studies were carried out in Mira Channel. This channel presents a full salinity gradient and is not affected by anthropogenic stress, being considered the most pristine channel in Ria the Aveiro (Castro et al., 2006). These characteristics make from this channel a favourable area to:

- ✓ compare the decomposition of a macrophyte (*Phragmites australis*) and an alga (*Fucus vesiculosus*) along a full salinity gradient using the leaf-bag technique (Chapter 2);

- ✓ analyze the macroinvertebrate communities associated with the decomposition of *Phragmites australis* and *Fucus vesiculosus* along the full salinity gradient (Chapter 3);

- ✓ analyze body size descriptors and taxonomic and non-taxonomic indices along a full salinity gradient using the leaf-bag technique and corer samples (Chapters 4 and 5).

After these studies in a channel not affected by anthropogenic pressures, the knowledge acquired was applied in a metals and metalloid sediment contamination area, the Estarreja Channel, and two reference channels (Canelas and Salreu), representing largely uncontaminated channels. The first approach to this part of the study was to identify the sediment contamination using the Sediment Quality Triad approach (Chapter 6). Then, the benthic communities sampled using a hand-held corer and mesh-bags with *Phragmites australis*, *Fucus vesiculosus* and a control substrate were compared between the contaminated and the reference channels in order to test their ability to perceive the problems associated with the contaminated sediments (Chapter 7).

A more detailed description of each chapter is outlined below:

Chapter 2 - *In situ* experimental decomposition studies in estuaries: A comparison of *Phragmites australis* and *Fucus vesiculosus*

In this chapter was investigated the hypothesis that the use of a test species from the upper reaches of the estuary may be inadequate to study decomposition in areas located close to the sea and vice-versa. Thus, the decomposition rates of a macrophyte (*Phragmites australis*) and an alga (*Fucus vesiculosus*) were tested along a full estuarine gradient, from marine to freshwater, in the most pristine channel in Ria de Aveiro, the Mira Channel, using the leaf-bag technique and five sampling times (days 3, 7, 15, 30 and 60). In this study, the null hypotheses of no significant differences in the decay rates of *P. australis* and *F. vesiculosus* throughout a full salinity gradient and between both species in the same salinity area at different time intervals (0-15, 0-30 and 0-60 days) were investigated. The material of this chapter was published in Lopes et al., 2011.

Chapter 3 - Macroinvertebrates communities associated with the decomposition of *Phragmites australis* and *Fucus vesiculosus* in transitional systems

In this chapter was studied the macrofauna associated with the decomposition of *P. australis* and *F. vesiculosus* using the leaf-bag technique and four sampling times (days 3, 7, 15 and 30). This experiment was conducted in Mira Channel, covering all levels of the Venice System (1959) classification of brackish waters, and in two other channels representing the mesohaline region, Canelas and Salreu Channels. The study used a control substrate, consisting of an artificial substrate made up of plastic pieces, and a procedure control, consisting of empty mesh bags. With this study we aimed to test the null hypotheses that, from the euhaline to the limnetic area, no significant different macrofauna communities were associated with the experimental substrates at a given sampling time as well as along the study period and that no significant differences exist between the macrofauna community captured in the leaf-bags with the decaying organic substrates, with the artificial control substrate, and in the empty mesh bags. The material of this chapter was published in Lopes et al., 2013.

Chapter 4 - Variability of body size descriptors in benthic invertebrates along a full salinity gradient sampled by leaf-bags and corers

The abundance distribution of macrobenthic community by the ISS index body size classes and the variability of length, weight and length-weight relationships of Arthropods, Molluscs and Annelids were studied in a system without anthropogenic stresses, the Mira Channel, using different sampling techniques, the leaf-bag technique with *Phragmites australis* and two mesh-sizes (1 mm and 5 mm) and a hand-held corer. The same study was carried out considering individual species with at least three distribution areas along the full salinity gradient.

The length, weight and length-weight relationships of Annelids, Arthropods and Molluscs were tested along the salinity gradient under the null hypothesis of no significant differences in the length, weight and length-weight relationships of each group along the salinity gradient. The same statistical analyses was made for species with at least three distribution areas and five individuals per area.

The body length and weight of the benthic macroinvertebrates were only determined for complete specimens. The loss of information were considered and studied in order to insure the representativeness of the sub-set of specimens and taxa measured. Thus, the dominant species of the original sampled macrofauna and the sub-set of taxa and specimens measured were compared and both were tested under the null hypothesis of no significant differences in the benthic community along the salinity gradient. The ability of the benthic fauna measured to show the natural replacement of species along the salinity gradient was also evaluated. A manuscript is under preparation.

Chapter 5 - Variability of taxonomic and non-taxonomic biotic indices applied to macroinvertebrates sampled by leaf-bags and corers

In this chapter, taxonomic (AMBI and M-AMBI) and non-taxonomic (ISS) indices were calculated considering the macrobenthic fauna studied in Chapter 4, in order to evaluate the variability of these indices along the full salinity gradient in Mira Channel. The

AMBI and M-AMBI were calculated considering all the benthic fauna sampled and the sub-set of measured specimens and the data was tested under the null hypothesis of no significant differences in the AMBI, M-AMBI and ISS values along the salinity gradient. The AMBI and M-AMBI values calculated with both data sets were also compared in each salinity area under the null hypothesis of no significant differences in the values between the original macrobenthic data sampled and the sub-set of measured specimens. The ecological quality was assessed considering the values from the taxonomic index M-AMBI (using all the fauna sampled and the sub-set of measured specimens) and the non-taxonomic index ISS. A manuscript with the results of this chapter is under preparation.

Chapter 6 - Ecological effects of contaminated sediments following a decade of no industrial effluents emissions: the Sediment Quality Triad approach

This chapter analyzed the ecological effects of contaminated sediments associated with the industrial chemical effluents discharged in the Estarreja Channel, a decade after ceasing the emissions, using the Sediment Quality Triad (SQT) approach. This methodology has been used in marine, estuarine and freshwater environments (e.g., Chapman et al., 1987; Quintino et al., 1995; 2001; Canfield et al.; 1996; Delvalls et al., 1998; Carr et al., 2000; Hollert et al., 2002; Khim and Hong, 2014) and integrates namely the study of sediment chemistry, sediment toxicity or bioaccumulation assays and resident benthic communities (Chapman, 1986; 1989; 1990). The SQT descriptors here studied included sediment metals and metalloid concentrations (Hg, Cr, Ni, Cu, As, Cd, Zn and Pb), the survival and metals bioaccumulation in the bivalve *Scrobicularia plana* exposed to the test sediments under controlled laboratory conditions and the resident macrofauna benthic communities' species composition and abundance. The descriptors were tested under the null hypothesis of no significant differences between the impacted (Estarreja) and the reference channels (Salreu and Canelas). The material of this chapter was published in Lopes et al., 2014.

Chapter 7 - Functional and structural benthic indicators in the assessment of estuarine contaminated sediments

Despite of industrial effluents discharges in the Estarreja Channel stopped a decade ago, the study using the Sediment Quality Triad approach showed that their negative ecological effects still associated with the contaminated sediments (Lopes et al., 2014). In this chapter were compared the benthic communities sampled by a hand-held corer, but also those captured in leaf-bags used in an experimental decomposition study with *Phragmites australis* and *Fucus vesiculosus*. An artificial substrate was also used as control. Synthesis indicators, including primary (*S*) and derived (*H'*) biological variables, as well as taxonomic (AMBI, M-AMBI) and non-taxonomic indices (ISS) and functional indicators (decomposition rates) were statistically tested under the null hypothesis of no significant difference between the impacted (Estarreja) and reference channels (Canelas and Salreu). The data from this chapter is under appreciation for publishing.

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Chapter 2. *In situ* experimental decomposition studies in estuaries: A comparison of *Phragmites australis* and *Fucus vesiculosus*

2.1 Abstract

The decomposition rates of *Phragmites australis* and *Fucus vesiculosus* were experimentally determined in an estuarine system using the leaf-bag technique. The study was conducted in fifteen sites arranged in five areas, extending from freshwater, outside the tidal range, to the marine environment, near the mouth of the estuary. The leaf-bags (5 mm mesh), were set up with 3.0 g of dried substrate, submerged in the experimental sites at day 0 and collected at days 3, 7, 15, 30 and 60, to follow biomass loss. The biomass loss through the leaching phase (day 3) was about 16% for *Phragmites australis* and 33% for *Fucus vesiculosus* and was independent of salinity for both substrates. The difference in the remaining biomass between the two species increased with time and the decomposition rates differed along the salinity gradient. For *F. vesiculosus*, the decomposition rate was highest near the mouth of the estuary, corresponding to the preferential distribution area of the algae, and decreased towards freshwater. For *Phragmites australis*, the fastest decay was observed in the mid estuary, where *Phragmites australis* occurs naturally, confirming previous studies. The decomposition rates measured at different time intervals (0 - 15, 0 - 30 and 0 - 60 days) were always higher for the algae and decreased with time for both species. These results indicate that the use of decomposition rates as a measure of ecosystem integrity or quality status in transitional waters will not be straight forward and must take into account, among others, the test species, the study area positioning along the estuarine gradient, and the time interval for the calculation of the decomposition rate.

Keywords: *Phragmites australis; Fucus vesiculosus*; Decomposition rates; Functional indicators; Salinity gradient; Ria de Aveiro.

2.2 Introduction

Estuaries are extremely productive systems, with large quantities of organic matter available to decomposition (among others, McLusky and Elliott, 2004), which enables the recycling of nutrients and chemical elements (Takeda and Abe, 2001; Cebrian and Lartigue, 2004). After physical, chemical and biological processes, detritus is reduced to elements which are released to the system and become available for uptake by organisms (Gessner et al., 1999). In aquatic ecosystems the decomposition of organic matter proceeds in three stages (Petersen and Cummins, 1974; Webster and Benfield, 1986): (i) leaching, during which a rapid weight loss is seen due to the washing out of soluble constituents; (ii) conditioning, which consists in the modification of the leaf matrix by microorganisms as a result of enzymatic activities; (iii) fragmentation, corresponding to the physical breakup of the coarse detritus, mostly mediated by invertebrates. The decomposition of organic matter, namely leaves, is affected by internal factors, such as the leaf species and its physico-chemical characteristics (Kok et al., 1990; Akanil and Middleton, 1997) and by external factors, which include abiotic and biotic factors. Biotic factors include the role of microfungi and invertebrates (Hieber and Gessner, 2002), and abiotic factors include water temperature (Carpenter and Adams, 1979; Reice and Herbst, 1982), salinity (Dang et al., 2009), pH (Thompson and Bärlocher, 1989), nutrients (Elwood et al., 1981; Bärlocher and Corkum, 2003), oxygen concentration (Chauvet, 1997) and regional characteristics (Denward et al., 1999; Lissner et al., 1999a, b). Abiotic factors can have a direct effect upon decomposition, affecting the leaching and fragmentation phases, but also an indirect effect, determining the conditions of the environmental niche and, consequently, filtering the traits of potential colonizers and affecting their metabolism (Suberkropp and Chauvet, 1995).

The study of decomposition using the experimental leaf-bag technique (Petersen and Cummins, 1974) has been widely used in the freshwater environment (van Dokkum et al., 2002; Sangiorgio et al., 2006), including a means of assessing ecosystem integrity of riverine systems (Bergfur et al., 2007; Castela et al., 2008). Decomposition studies in transitional waters are far less common and studies have shown an inverse relationship between water salinity and leaf breakdown, which can be related to the fact that salinity influences microbial diversity, biomass and activity (Tanaka, 1991; van Ryckegem and Verbeken, 2005; Roache et al., 2006). The use of decomposition studies to assess ecosystem integrity and functioning in transition waters is hindered by additional difficulties, when compared to riverine systems, arising from the fact that natural conditions vary along the estuarine gradient and these in turn affect decomposition. In a recent study of *Phragmites australis* decomposition along a full salinity gradient, Quintino et al. (2009) showed that the highest decomposition rates were located close to the species preferential distribution areas in the estuary but also mediated by time. That study showed that the differences between the decomposition rates obtained in estuarine areas with differing mean salinity were greater when the rates were calculated after an experimental 30 days decay time, than after 60 days. Even so, there was almost no reed biomass decay near the sea, following the initial leaching phase (Quintino et al., 2009). Those results suggest that the organic matter decomposition rates measured in estuarine systems will, among other factors, also depend on the type of experimental substrate.

The present study is part of a broader research programme aiming to compare structural indices and functional indicators, as a means to assess ecosystem integrity and classify ecological quality status in transitional waters, in the sense of the European Water Framework Directive. This has been mostly addressed through the use of taxonomic based indices and our aim is to ultimately compare these to functional indicators, the decomposition studies appearing as a tool for that ultimate goal. Here we investigate the hypothesis that the use of a test species from the upper reaches of the estuary may be inadequate to study decomposition in areas located close to the sea and vice-versa. This was done by comparing macrophyte and algal decomposition rates along a full estuarine gradient. The null hypotheses of no significant differences in the decay rates of *Phragmites australis* and *Fucus vesiculosus* throughout a full salinity gradient and between both species in the same salinity area at different time intervals (0-15, 0-30 and 0-60 days) were investigated. The experiments were carried out in Mira Channel, Ria de Aveiro, Western Portugal, at fifteen sites arranged in five areas extending from freshwater to the marine environment.

2.3 Materials and methods

2.3.1 Study area

Ria de Aveiro is located on the Northwestern coast of Portugal, between 40°38`N and 40°57`N, with a maximum width and length of 10 and 45 km, respectively. This system includes four main Channels, Mira, Ílhavo, Espinheiro and S. Jacinto, characterized by extensive intertidal mud and sand flats, salt marshes and islands. All the channels receive freshwater inputs and connect with the sea by an artificial inlet (Figure 2.1). This study was conducted in the Mira Channel, a narrow 20 Km long channel, running south from the entrance. This channel presents a full salinity gradient, ranging from fully marine at the mouth to freshwater at the head, receiving continuous freshwater input (Moreira et al., 1993). It is also one of the most pristine channels in Ria de Aveiro (Castro et al., 2006). These characteristics favor the comparative study of the decomposition rates of different substrates, in this case an alga (*Fucus vesiculosus*) and a macrophyte (*Phragmites australis*), along the full salinity gradient.

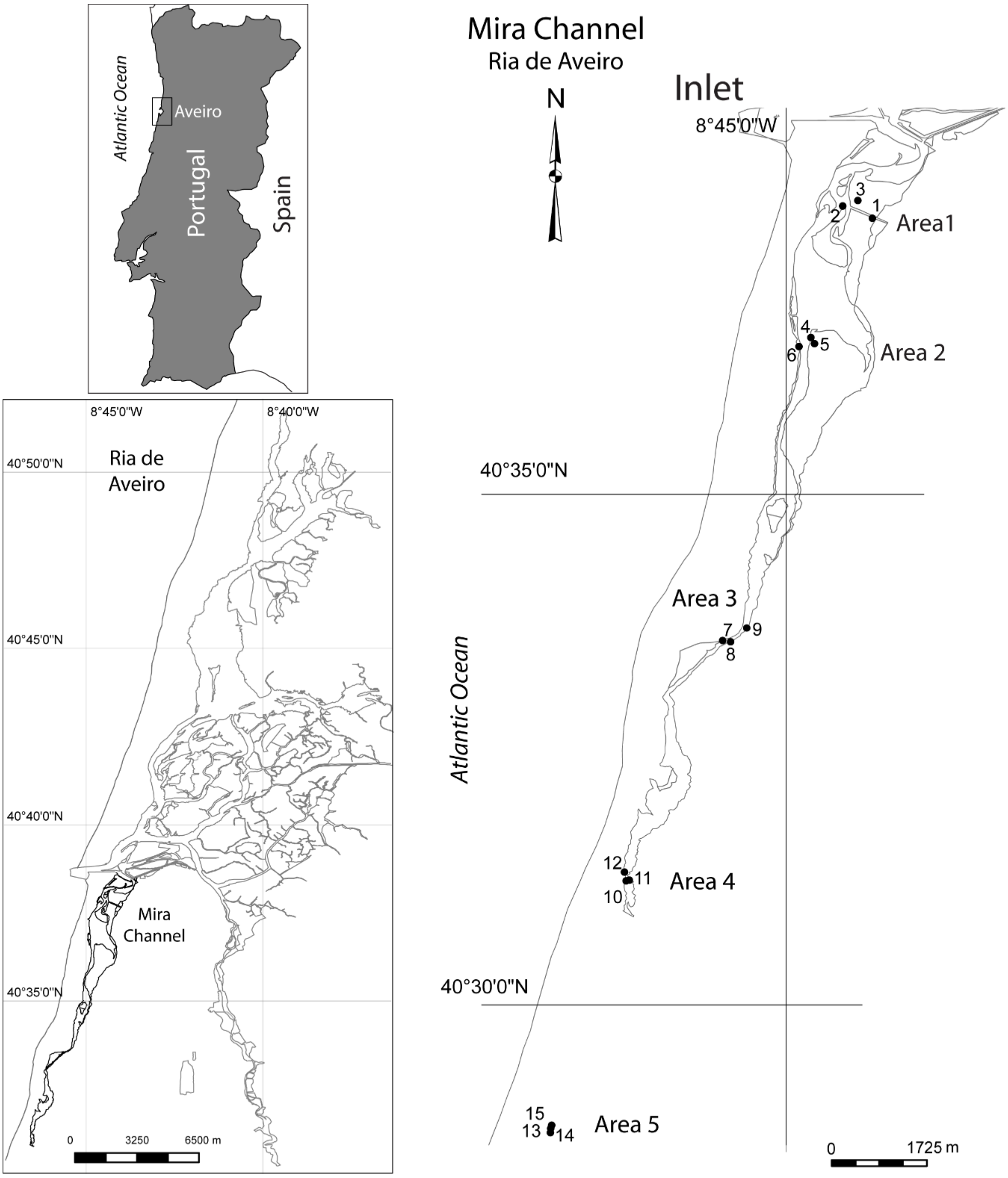

Figure 2.1 Positioning of the study sites in the Mira Channel, Ria de Aveiro, Western Portugal, for the leaf-bag decomposition experiments (sites 1 to 15, nested in areas 1 to 5).

2.3.2 Field and laboratory procedures

The experimental field work consisted of following the biomass decay of *Fucus vesiculosus* (L.) and *Phragmites australis* (Cav) Trin. Ex Steudel using the leaf-bag technique (Petersen and Cummins, 1974). Leaves of *P. australis* used in the experiment were collected simultaneously, from the same area, at the end of the 2008 growing season and before the natural senescence. The algae were collected at the same time. Both species were air dried and later oven-dried to constant weight (60 °C for 72 h). Aliquots of 3.000 ± 0.005 g dry weight were placed in 5 mm mesh bags. Leaves were cut into 8 cm long fragments excluding the basal and apical parts. The study was performed during winter 2009 (January-March) at 15 sampling sites distributed in 5 areas (area 1 to area 5), with 3 sites per area, along the Mira Channel (Figure 2.1). The 5 areas were defined considering the Venice System (1959) for the Classification of Estuarine Waters: euhaline (area 1), polyhaline (area 2), mesohaline (area 3), oligohaline (area 4) and limnetic (area 5) (Quintino et al., 2009). At the beginning of the experiment (day 0), all the bags with the leaves and the algae were placed in the field sites, at the bottom, and replicates were collected over time, at days 3 (leaching), 7, 15, 30 and 60. At each sampling time, four replicates of each decaying substrate were collected per site, placed in separate plastic containers and rapidly returned to the laboratory. Here, the leaves and algae were gently washed to remove sediments and macroinvertebrate colonizers. Leaves and algae from each bag were dried in an oven at 60 °C for 72 h and weighed.

2.3.3 Data analysis

The weight loss of *Phragmites australis* (Pa) and *Fucus vesiculosus* (Fv) was calculated as the fraction of the initial mass, as dry weight, remaining at time (t) and expressed as a percentage. Mass loss data were processed using non-linear regression analysis of the exponential model (Olson, 1963; Petersen and Cummins, 1974): $Mt = M_0 . e^{-kt}$ where the litter mass remaining after a given period of time (Mt) is calculated as a function of the initial mass (M_0) and the decay rate coefficient (k). In this study, the decay rates of *Phragmites australis* and *Fucus vesiculosus* were calculated per sampling site, per study area and for the whole Mira Channel, with the data obtained from day 0 to day 15 (k_{15}), from day 0 to day 30 (k_{30}) and from day 0 to day 60 (k_{60}). The decay rates obtained for both species were submitted to hypotheses testing under the null hypothesis of no significant differences in the decay rates of *P. australis* and *F. vesiculosus* along the full salinity gradient and between the two species, in the same salinity area at different time intervals. The test species, the salinity areas and time were considered fixed factors and orthogonal to each other. Hypothesis tests used Permutational Multivariate Analysis of Variance (Anderson, 2001), from the software PRIMER v6 (Clarke and Gorley, 2006), with the add-on PERMANOVA+ (Anderson et al., 2008). PERMANOVA+ partitions the variability from a dissimilarity matrix, in this case obtained with the Euclidean distance between samples, and tests individual terms, including interactions, using permutations (Anderson and ter Braak, 2003). The *F*-values in the main tests and the t-statistic in the pairwise comparisons were evaluated in terms of the significance among different groups, or levels, of the tested factor. Values of $p \leq 0.05$ reveal that the groups differ significantly.

2.4 Results

Table 2.1 shows the diminishing dry weight biomass of *Phragmites australis* and *Fucus vesiculosus* through time in the five study areas. The biomass loss through the leaching phase (day 3), considering the data for all five areas, was about 16% for *P. autralis* and 33% for *F. vesiculosus*. The leaching data were analyzed in a three-way analysis of variance, with areas and decaying substrates as fixed and crossed factors and sites as random factor, nested in areas. The interaction term [areas x substrate] and the factor area were found not significant (pseudo - $F = 1.0373$ with $p = 0.43$ and pseudo - $F = 0.5870$ with $p = 0.68$, respectively), indicating that the loss of biomass through leaching was independent of salinity for both species. A very strong significant difference was found between the amount of biomass loss of the decaying substrates (pseudo - $F = 779.72$; $p < 0.0001$). With increasing time, the remaining biomass difference between the two species increased in all study areas, as shown in Table 2.1 and Figure 2.2.

Figure 2.3 summarizes the temporal evolution of the remaining dry weight biomass for the whole Mira Channel and the pattern of biomass decay through time in each area for *P. australis* and *F. vesiculosus*. The differences in the remaining biomass between areas through time are not as clear for the macrophyte as they are for the alga. For *F. vesiculosus*, no differences were noticed between areas at day 3 (leaching) but from day 7 onwards, the remaining biomass was much larger in area 5 (limnetic area) than in all other areas. From day 15 onwards, a higher remaining biomass in area 4, when compared to areas 1 to 3, also became apparent. In areas 1 to 3, no algal biomass could be recovered at day 60 (cf. Figure 2.3). The biomass decay for this species was thus faster in the areas with higher mean salinity. The loss of biomass through time for *P. australis* was more subtle. Despite this, Table 2.1 shows that the highest loss of biomass occurred in the mid areas of the estuary (areas 3 and 4).

Table 2.1 *Phragmites australis* (Pa) and *Fucus vesiculosus* (Fv) dry weight remaining biomass for each site, area and the whole Mira Channel, at days 3, 7, 15, 30 and 60. The initial biomass was 3.000 g for all cases. The sign (-) denotes loss of sample, n.a. stands for non available due to decomposition of the whole experimental substrate and s.e. for standard error.

	3(Pa)	7(Pa)	15(Pa)	30(Pa)	60(Pa)	3(Fv)	7(Fv)	15(Fv)	30(Fv)	60(Fv)
Site 1	2.5505	2.7118	2.5499	2.2263	1.8683	2.0705	1.3706	0.8372	0.3318	0.0000
Site 2	2.5296	2.6166	2.3858	2.0642	1.8877	2.0540	1.4274	-	0.2988	0.0000
Site 3	2.5676	2.5266	2.4813	2.0734	1.8064	2.0540	1.3463	0.8005	0.1774	0.0000
Mean Area 1	**2.5492**	**2.6183**	**2.4724**	**2.1213**	**1.8541**	**2.0595**	**1.3814**	**0.8189**	**0.2693**	**n.a.**
s.e.	**0.0110**	**0.0535**	**0.0476**	**0.0526**	**0.0245**	**0.0055**	**0.0240**	**0.0183**	**0.0469**	**n.a.**
Site 4	1.9996	2.6166	2.4231	2.3432	1.9804	1.9996	1.4977	0.7635	0.3540	0.0000
Site 5	2.5861	2.5480	2.4508	1.9386	1.7979	2.0179	1.4662	0.8181	0.4706	0.0000
Site 6	2.4701	2.6296	2.3199	2.0687	1.9167	1.8552	1.2325	0.8102	0.1983	0.0000
Mean Area 2	**2.3519**	**2.5981**	**2.3979**	**2.1168**	**1.8983**	**1.9576**	**1.3988**	**0.7973**	**0.3410**	**n.a.**
s.e.	**0.1793**	**0.0253**	**0.0398**	**0.1193**	**0.0535**	**0.0515**	**0.0837**	**0.0170**	**0.0789**	**n.a.**
Site 7	2.5267	2.5266	2.3322	2.0972	2.0015	2.1313	1.7030	0.6881	0.2293	0.0000
Site 8	2.5284	2.5233	2.2720	1.9999	1.8267	2.0520	1.5953	1.0060	0.6393	0.0000
Site 9	2.4640	2.4640	-	-	-	1.9232	1.3914	-	-	-
Mean Area 3	**2.5064**	**2.5046**	**2.3021**	**2.0485**	**1.9141**	**2.0355**	**1.5632**	**0.8470**	**0.4343**	**n.a.**
s.e.	**1.4593**	**1.4578**	**1.6278**	**1.4485**	**1.3534**	**1.2076**	**0.9521**	**0.5989**	**0.3071**	**n.a.**
Site 10	2.4838	2.5235	2.3600	2.2061	1.7233	1.8310	1.5186	1.2025	1.0547	0.2122
Site 11	2.5353	2.4945	2.3258	2.0470	1.7387	2.0114	1.3675	1.0696	0.8270	0.0535
Site 12	2.5157	2.4658	2.3625	2.1685	2.0274	2.0535	1.4844	1.1669	0.5329	0.2927
Mean Area 4	**2.5116**	**2.4946**	**2.3495**	**2.1405**	**1.8298**	**1.9653**	**1.4568**	**1.1463**	**0.8049**	**0.1862**
s.e.	**0.0150**	**0.0166**	**0.0118**	**0.0480**	**0.0989**	**0.0682**	**0.0457**	**0.0397**	**0.1510**	**0.0703**
Site 13	2.5092	2.4607	2.3894	2.2449	1.9979	2.1119	2.0916	2.0337	1.2916	1.0264
Site 14	2.4708	2.4654	2.4196	2.3488	2.0482	1.9800	1.9642	2.0851	1.5942	1.1217
Site 15	2.5607	2.4496	-	2.2445	2.0023	2.0233	2.0460	-	1.6970	1.8770
Mean Area 5	**2.5135**	**2.4586**	**2.4045**	**2.2794**	**2.0161**	**2.0384**	**2.0339**	**2.0594**	**1.5276**	**1.3417**
s.e.	**0.0260**	**0.0047**	**0.0151**	**0.0347**	**0.0161**	**0.0388**	**0.0373**	**0.0257**	**0.1217**	**0.2690**
Mira channel										
Mean	**2.4865**	**2.5348**	**2.3902**	**2.1479**	**1.9016**	**2.0112**	**1.5668**	**1.1068**	**0.6926**	**0.3274**
s.e.	**0.0361**	**0.0199**	**0.0208**	**0.0335**	**0.0295**	**0.0219**	**0.0688**	**0.1368**	**0.1393**	**0.4145**

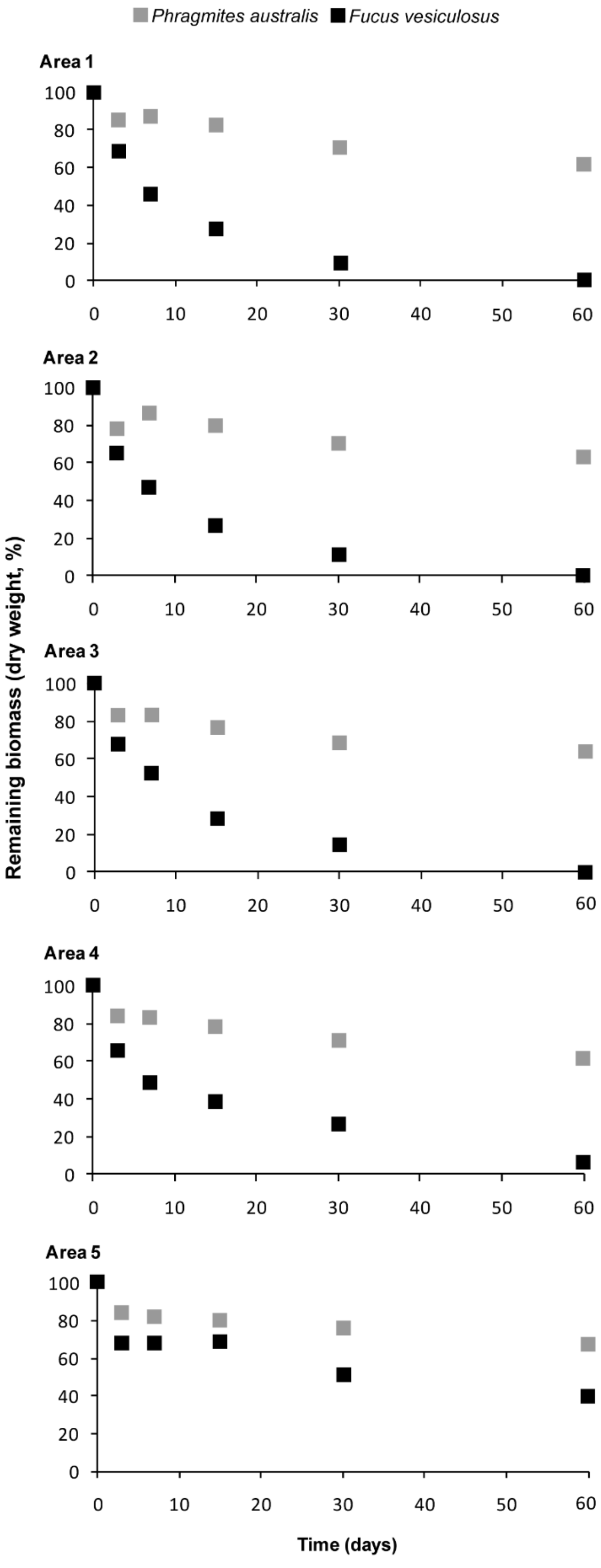

Figure 2.2 Evolution of the remaining biomass of *Phragmites australis* and *Fucus vesiculosus* during the 60-day decay period in areas 1 to 5, Mira Channel, Ria de Aveiro. The data from each area are plotted in separate graphs, in order to better compare the results obtained with each species. Each value corresponds to the mean for all replicates and all sampling sites per area.

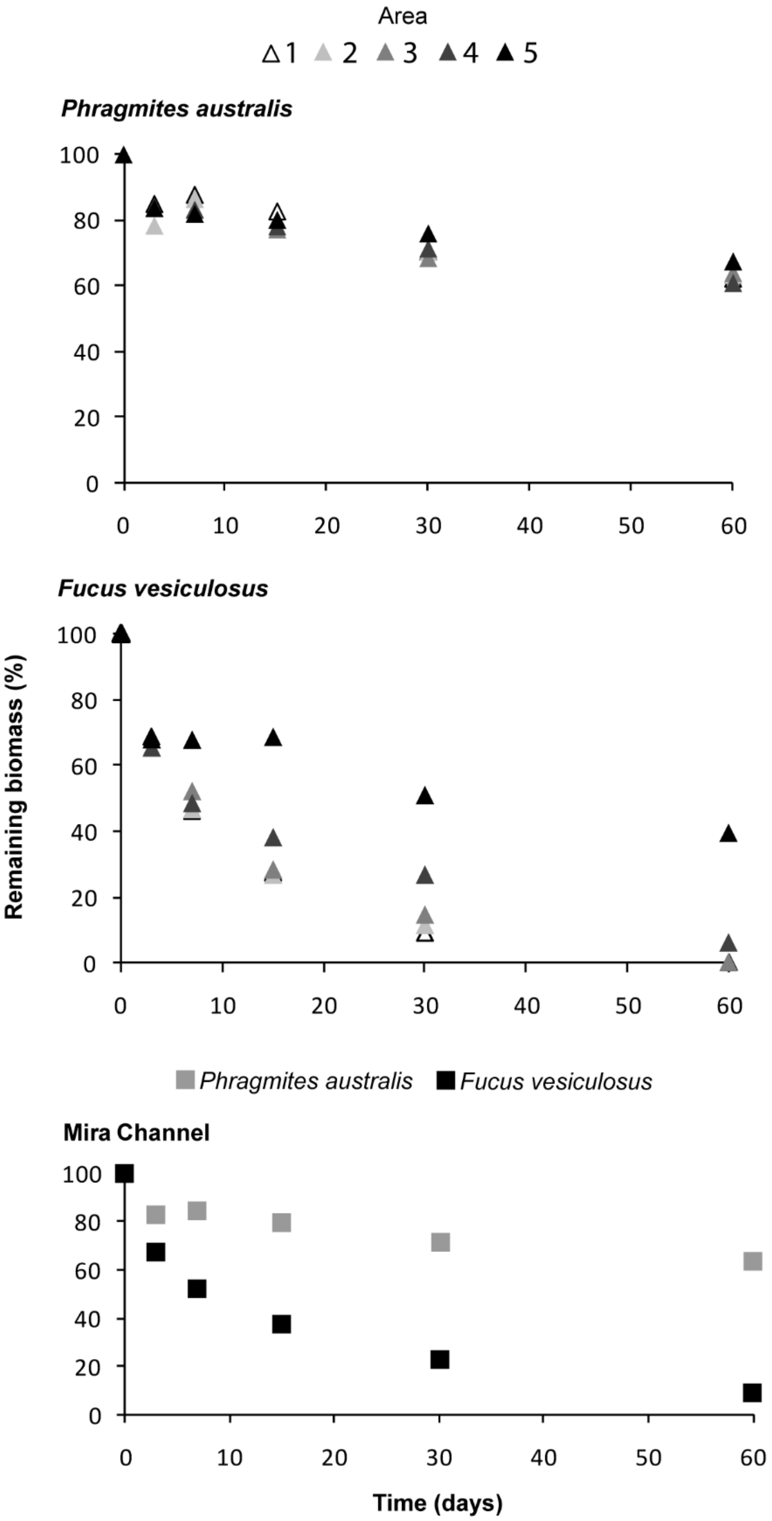

Figure 2.3 Evolution of the remaining biomass (dry weight) of *Phragmites australis* and *Fucus vesiculosus* during the 60-day decay period in areas 1 to 5, Mira Channel, Ria de Aveiro. The data obtained for each species are plotted in separate graphs, in order to better compare the results obtained in the 5 areas. The bottom summary graph shows the evolution of the mean values for the whole Mira Channel, for each species.

The remaining biomass data were modeled as a negative exponential decay function and Table 2.2 presents the detailed decay rates (k), for both substrates, calculated per site and area and considering various time intervals.The alga and the macrophyte presented an opposite trend in decomposition rate along the salinity gradient. The decomposition rates of the algae were always higher than those of the macrophyte and for both species, the k-values decreased with increasing time interval (Figure 2.4). Accounting for all sampling sites in the various areas, a k_{15} decay rate of 0.018 days^{-1}, a k_{30} decay rate of 0.013 days^{-1} and a k_{60} decay rate of 0.009 days^{-1} were obtained for *Phragmites australis*. For *Fucus vesiculosus*, a k_{15} decay rate of 0.076 days^{-1}, a k_{30} of 0.062 days^{-1} and a k_{60} decay rate of 0.034 days^{-1} were obtained. For the alga, k_{15}, k_{30} and k_{60} values also increased with increasing salinity and the highest values were obtained in areas near the mouth of the estuary, where the species occurs naturally. For *Phragmites australis*, the values of k_{15}, k_{30} and k_{60} increase with decreasing salinity and the highest values were obtained in the upper reaches of the estuary (areas 3 and 4), corresponding to the preferred natural distribution of *P. australis*.

Table 2.2 *Phragmites australis* (Pa) and *Fucus vesiculosus* (Fv) decay rates calculated for each site, area and the whole Mira Channel, from day 0 to day 15 (k_{15}) from day 0 to day 30 (k_{30}) and from day 0 to day 60 (k_{60}). The sign (-) denotes loss of sample, n.a. stands for non available due to decomposition of the whole experimental substrate.

	k_{15} (Fv)	k_{30} (Fv)	k_{60} (Fv)	k_{15} (Pa)	k_{30} (Pa)	k_{60} (Pa)
Site 1	0.091	0.078	n.a	0.013	0.011	0.009
Site 2	-	0.079	n.a	0.017	0.014	0.009
Site 3	0.094	0.094	n.a	0.016	0.013	0.010
Area 1	**0.092**	**0.084**	n.a	**0.015**	**0.012**	**0.009**
Site 4	0.094	0.077	n.a	0.019	0.011	0.008
Site 5	0.091	0.069	n.a	0.016	0.015	0.010
Site 6	0.097	0.092	n.a	0.019	0.014	0.009
Area 2	**0.094**	**0.079**	n.a	**0.018**	**0.013**	**0.009**
Site 7	0.096	0.088	n.a	0.019	0.014	0.009
Site 8	0.078	0.058	n.a	0.021	0.015	0.010
Site 9	-	-	n.a			
Area 3	**0.087**	**0.073**	**n.a.**	**0.020**	**0.015**	**0.009**
Site10	0.071	0.043	0.044	0.019	0.012	0.010
Site11	0.078	0.051	0.063	0.020	0.014	0.010
Site12	0.072	0.061	0.044	0.019	0.013	0.008
Area 4	**0.073**	**0.052**	**0.051**	**0.019**	**0.013**	**0.010**
Site 13	0.033	0.029	0.021	0.019	0.012	0.008
Site 14	0.034	0.024	0.018	0.018	0.011	0.007
Site 15	-	0.022	0.015	-	0.011	0.008
Area 5	**0.034**	**0.025**	**0.018**	**0.019**	**0.011**	**0.008**
Mira channel	**0.077**	**0.062**	**0.034**	**0.018**	**0.013**	**0.009**

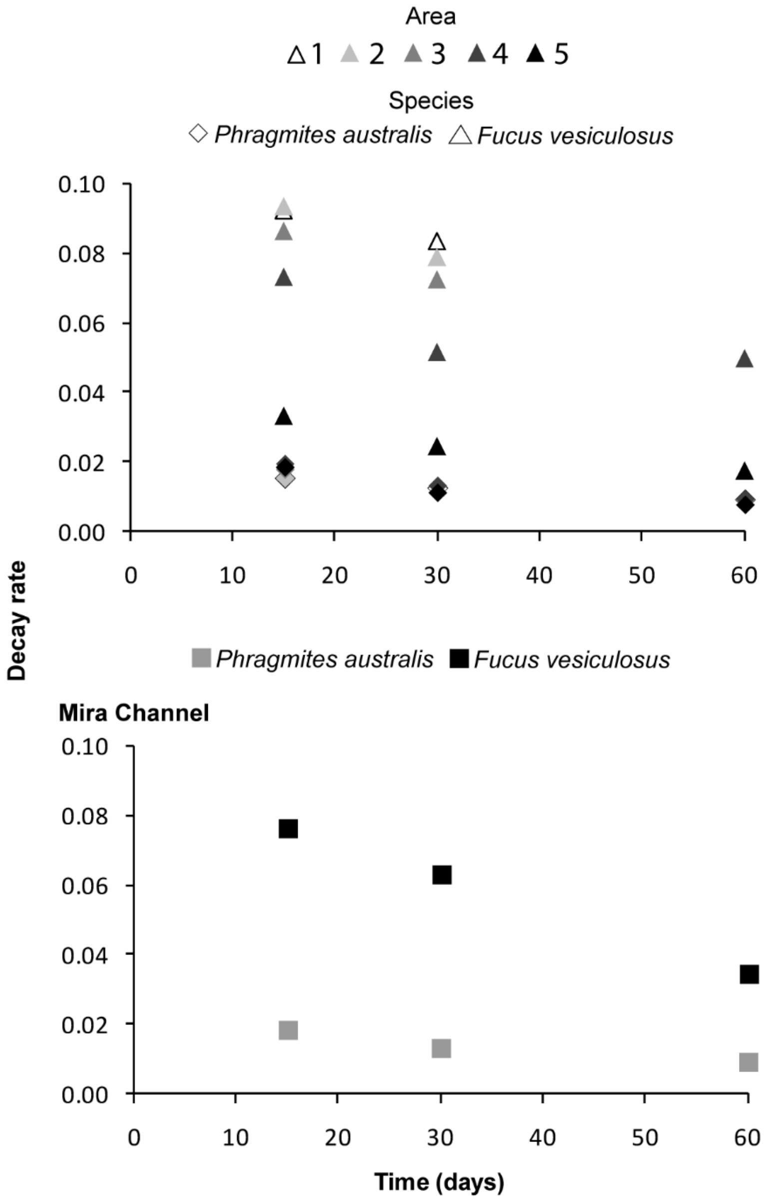

Figure 2.4 Evolution of the decay rate of *Phragmites australis* and *Fucus vesiculosus* during the 60-day decay period in areas 1 to 5, Mira Channel, Ria de Aveiro. The bottom summary graph shows the evolution of the mean values for the whole Mira Channel, for each species.

Table 2.3 summarizes the results obtained in the statistical comparison of the decay rates observed in the five study areas at days 15, 30 and 60 for both species. The main test always indicated significant differences in the decomposition rates among areas for the alga and significant differences only at day 15 for the macrophyte. For *F. vesiculosus* the decay rates observed at days 15 and 30 were significantly different between areas located at the extremities of the channel. At day 60 it was only possible to determine the decomposition rate in areas 4 and 5 as no biomass could be recovered in the other areas. For *Phragmites australis*, in general the decomposition rates among areas and with time were not significantly different. The exceptions were the decomposition rates between areas 1 and 4 at day 15, 3 and 5 and 4 and 5 at day 30 and between areas 1 and 5 at day 60.

Table 2.3 PERMANOVA main test *F* values (with associated significance in brackets) and t-values pair-wise comparisons (with associated significance in brackets) between areas for the decay rates calculated for day 0 to day 15 (k_{15}), day 0 to day 30 (k_{30}) and day 0 to day 60 (k_{60}) for *Fucus vesiculosus* (Fv) and *Phragmites australis* (Pa). The main test for Fv at day 60 is only calculated for areas 4 and 5. ns = stands for non-significant ($p > 0.05$).

	k_{15}(Fv)	k_{30}(Fv)	k_{60}(Fv)	k_{15}(Pa)	k_{30}(Pa)	k_{60}(Pa)
Main test	41.948 (0.0001)	14.193 (0.0007)	24.494 (0.0072)	4.257 (0.0419)	1.730 (ns)	1.891 (ns)
Pair-wise comparisons						
1 vs 2	0.551 (ns)	0.5071 (ns)	-	1.685 (ns)	0.507 (ns)	0.143 (ns)
1 vs 3	0.633 (ns)	0.800 (ns)	-	2.625 (ns)	1.503 (ns)	0.173 (ns)
1 vs 4	5.689 (0.0104)	4.299 (0.0123)	-	2.972 (0.0412)	0.650 (ns)	0.478 (ns)
1 vs 5	38.304 (0.0080)	10.113 (0.0005)	-	1.858 (ns)	1.249 (ns)	4.315 (0.0099)
2 vs 3	1.006 (ns)	0.432 (ns)	-	1.621 (ns)	0.708 (ns)	0.218 (ns)
2 vs 4	6.884 (0.0025)	3.203 (ns)	-	1.460 (ns)	0.047 (ns)	0.490 (ns)
2 vs 5	27.783 (0.0001)	7.525 (0.0021)	-	0.459 (ns)	1.563 (ns)	2.071 (ns)
3 vs 4	1.774 (ns)	1.614 (ns)	-	1.165 (ns)	1.296 (ns)	0.233 (ns)
3 vs 5	5.842 (0.0267)	4.108 (0.0258)	-	1.970 (ns)	4.280 (0.0237)	2.623 (ns)
4 vs 5	12.474 (0.0012)	4.858 (0.0078)	-	2.147 (ns)	2.739 (0.0486)	2.508 (ns)

2.5 Discussion

The evaluation of decay rates of macrophytes and macroalgae in estuarine systems is of great interest, since detritus formation and processing represent one of the main nutrient pathways in transitional ecosystems (McLusky and Elliott, 2004). The decomposition rate of reed litter in the Mediterranean Ecoregion is, in most cases, faster in freshwater ecosystems ([k = 0.034] van Dokkum et al., 2002; [k = 0.019] Pinna et al., 2004; [k = 0.036] Sangiorgio et al., 2004; [k = 0.014-0.029] Sangiorgio et al., 2008) than in brackish wetlands, lagoons and coastal lakes ([k = 0.004] Gessner, 2000; [k = 0.002] Menéndez et al., 2004; [k = 0.012] Costantini et al., 2009). The *Phragmites australis* decay rate obtained in the Mira Channel for this study [k_{min} = 0.007, k_{max} = 0.021] is within the range of literature values, considering the extension of the interecosystem comparisons on the indirect influence of water salinity to an intra-ecosystem comparison along the salinity gradient in

Mira Channel. Our data are also within the range determined by Quintino et al. (2009) in the same study area [k_{min} = 0.005, k_{max} = 0.036]. The inverse relationship between salinity and reed litter decay rates reported in this study has also been found in other ecosystems, such as desert saline rivers (Reice and Herbst, 1982), tidal marsh flats (Hemminga et al., 1991) and terrestrial ecosystems (Rietz and Haynes, 2003; Sardinha et al., 2003), as well as in laboratory and mesocosm experiments (Roache et al., 2006). Compared to the freshwater environment, there are fewer decomposition studies in marine and transitional waters (see namely a recent review by Banta et al., 2004) and very few studies have encompassed the full salinity gradient. Hunter (1976) studied the decomposing of *Fucus vesiculosus* in a rocky shore and a salt marsh (salinity not specified) and showed that the biomass loss occurred faster in the first four days of exposure in both environments. Over the 63 days of the experiment, the remaining biomass was also much lower in the rocky shore, 1%, than in the salt marsh, 34% of the initial dry weight. This trend is similar to that obtained in the present study, considering that the weight loss was also faster at the beginning of the experiment (leaching) and that after 60 days there was no biomass left in the areas with higher salinity (areas 1, 2 and 3), and only ca. 6% in the upper estuary (area 4) and 45% in freshwater (area 5). The decay rates of *Fucus vesiculosus* obtained in the present study in the euhaline (area 1) [k_{15} = 0.092, k_{30} = 0.084] and in the mesohaline areas (area 3) [k_{15} = 0.087, k30 = 0.073] are higher than the results obtained by Hunter (1976) in the rocky shore [k_{15} = 0.042, k_{30} = 0.038, k_{60} = 0.050] and in the salt marsh [k_{15} = 0.042, k_{30} = 0.033, k_{60} = 0.022] (values calculated from the graphs given in Hunter, 1976). However, the decay rates in both studies are higher in the areas with higher salinity and both diminish as the time interval considered for the calculation increases. Kristensen (1994), in an experimental study under a very different set up, also showed that the decomposition of the *F. vesiculosus* was faster than the decomposition of seagrass and tree leaves, which agrees with our own findings.

The use of leaf-bags with 5 mm mesh size enables the access of shredders to the decomposition substrates which means that the decomposition of *Phragmites australis* and *Fucus vesiculosus* in this study was due to the washing out of the soluble constituents (leaching), to the microbial conditioning and to the fragmentation by detritus feeders invertebrates.

During the leaching phase, salinity did not affect the biomass loss for both species, but the weight loss during this phase was much higher in *F. vesiculosus* than in *P. australis*. This can be explained considering that the initial decay of plant detritus depends on the size and lability of carbohydrates, phenolic (e.g. lignin) and organic nitrogen pools (Valiela et al., 1984; Twilley et al., 1986; Enríquez et al., 1993). According to Kristensen (1994), the aerobic decay in seawater is generally faster for macroalgae than for tree leaves as macroalgae have a higher protein content than leaves and most of its carbohydrates are aliphatic, non-lignified polysaccharides of high degradability, whereas leaf detritus are usually rich in decay-resistant lignocelluloses. Nevertheless, as showed by Bärlocher (1997), the weight loss during the leaching phase can be associated with the rupture of cell walls due to the oven heating of the plant material. Although pre-drying the plant material may induce enhanced and thus artificial weight loss during leaching, such trend was not always observed by Bärlocher (1997). The oven pre-drying of the plant material is commonly performed in many decomposition studies, as it allows the preparation of test substrates under controlled circumstances, which, in the case of the present study was very important, as our main goal was to compare the performance of the two substrates along the full salinity gradient.

After the leaching phase, the decomposition of both test substrates is mainly due to the microorganisms and invertebrates that colonize the detritus. The decomposition rate of *P. australis* was higher in the mid estuarine area, corresponding to the natural environment of the species. In *Phragmites australis*, abscission and collapse of plant material typically do not occur immediately following shoot senescence and death (Komínková et al., 2000). This implies that *P. australis* litter is exposed to an initial microbial colonization and starts to decay before its entry into the aquatic environment (Gessner, 2001). According toTanaka (1991), the inability of terrestrial fungi to survive in saline aquatic environments can explain why fungal populations decrease during the short time following submergence. This can further explain the decrease of the decomposition rate of *Phragmites australis* with increasing salinity. This trend was observed in this study and in the experiment carried out by Quintino et al. (2009), in which the differences in the decomposition rate of *Phragmites australis* between areas located along the full salinity gradient were more pronounced than in the present study. In the case of the alga, the microorganisms acting during the conditioning phase also appear adapted to saline conditions, thus explaining the higher decomposition rate of *Fucus vesiculosus* in the areas of higher salinity, where the alga occurs naturally, and much lower towards the freshwater environment.

The decomposition rates of *Fucus vesiculosus* and *Phragmites australis* decreased with time ($k_{15} > k_{30} > k_{60}$), with the decomposition rates of the alga always higher than those of the macrophyte. Algae have a higher nitrogen content than vascular marine plants and are attacked more rapidly by microorganisms and invertebrates, which results in a higher transfer efficiency of primary production to detritivores (Mann, 2000). This study also confirmed the findings of Quintino et al. (2009) who showed the difference in the decomposition rates vary according to the time interval considered for its calculation.

The influence of invertebrates in the decomposition of *Phragmites australis* and *Fucus vesiculosus* should be linked to the microorganisms, mainly fungi, which are associated with each kind of detritus. Conditioning of organic matter by microorganisms makes the detritus more palatable for detritus-feeders (Varga, 2001). These benefit from the fungal action, which promote the transformation of inedible material into edible compounds, but also from the fungi themselves, which provide micronutrients not present in litter tissues (Suberkropp et al., 1983; Graça et al., 1993). Similarly, the fragmentation of the organic matter by shredders also facilitates the microbial activity, enhancing the available detrital surface (Hargrave, 1970) and spreading microfungal spores (Rossi, 1985). Considering this, the action of invertebrates in the decomposition of both decaying substrates will be higher in the marine environment for *Fucus vesiculosus* and in the freshwater environment for *Phragmites australis*.

Estuarine ecosystems are ideal models to study *in situ* the influence of water salinity and associated descriptors on plant detritus decomposition, especially when a full salinity gradient can be appreciated within the same system without the influence of strong anthropogenic factors. This study was conducted under such conditions and showed that the evaluation of ecosystem functioning using litter decay rates in transitional waters needs to carefully consider a combination of factors such as the type of decaying substrate, the location of the study area in the salinity gradient and the time interval at which the decay rate is determined. Specifically, this study showed that the alga *Fucus vesiculosus*, distributed in the euhaline and polyhaline areas of the estuary, is unsuitable to evaluate decomposition in the oligohaline and limnetic reaches of the estuary, and conversely the macrophyte *Phragmites australis*, mainly distributed in the oligohaline and mesohaline part of the estuary, is unsuitable for assessing decomposition in the outer, euhaline areas.

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Chapter 3. Macroinvertebrates communities associated with the decomposition of *Phragmites australis* and *Fucus vesiculosus* in transitional systems

3.1 Abstract

The decomposition rates of a macrophyte (*Phragmites australis*) and an alga (*Fucus vesiculosus*) and the associated macrofauna communities were studied along a full salinity gradient, using the leaf-bag technique and four sampling times (days 3, 7, 15 and 30). A control was set up using an artificial substrate. A subsequent study conducted in the mesohaline part of the salinity gradient also included empty bags as procedure control. The decay rates of the alga and the macrophyte were significantly different, the alga decaying faster, and presented an opposite trend along the salinity gradient, with the faster decay rate for reed in the less saline areas and for the alga in the euhaline part of the gradient. The fauna associated with the decaying and the artificial substrate showed equally well the benthic succession from the marine to the freshwater areas, in all sampling times. Arthropods were dominant in all substrates along the estuarine gradient and replaced by annelids in freshwater. No significant differences were found between the benthic communities associated with *P. australis* and *F. vesiculosus*, despite the strong differences in the decay rates, suggesting that these do not seem to be primarily related to the benthic colonizers. Although the organic substrates sustained a more abundant fauna, the benthic communities did not show significant differences between the organic and the artificial substrates, especially at the level of the species composition, suggesting that the macroinvertebrates may colonize both substrates to feed on the biofilm and/or to seek shelter. The strongly impoverished benthic community sampled by the empty bags reinforced this idea.

Keywords: *Phragmites australis*; *Fucus vesiculosus*; Functional indicators, Macroinvertebrate Communities; Salinity gradient; Ria de Aveiro.

3.2 Introduction

Benthic macroinvertebrates play an important role in many ecological processes in aquatic ecosystems (McCall and Soster, 1990; Griffiths, 1991). Transitional waters are extremely productive systems, with large quantities of organic matter available to decomposition (among others, McLusky and Elliott, 2004). In experimental studies conducted in the freshwater environment, litter decomposition starts with a weight loss due to the washing out of the organic matter's soluble constituents (leaching), followed by the modification of the organic matrix by microorganisms as a result of enzymatic activities (conditioning) and finally the physical break down of the coarser detritus mediated by shredder feeding (fragmentation) (Petersen and Cummins, 1974; Rossi, 1985; Webster and Benfield, 1986; Hargrave, 1970; Mancinelli, 2012).

Macroinvertebrates have long received attention on impact assessment and water management studies, being one of the diagnostic biological elements included in the European Water Framework Directive (WDF, 2000/60/EC). Indices were developed namely based in the species tolerance/sensitivity to organic enrichment. However, transitional waters are naturally stressed and characterized by highly dynamic physical, chemical and hydro-morphologic conditions and by species with a higher level of tolerance to change, being more difficult to develop suitable quality indicators for these systems than for the marine environment (Elliott and Quintino, 2007). In recent years several biotic indices have been proposed to be used as ecological quality indicators for the marine and estuarine systems (AMBI, Borja et al., 2000, BENTIX, Simboura and Zenetos, 2002, BQI, Rosenberg et al., 2004, BOPA, Dauvin and Ruellet, 2007). The estuarine quality paradox however leads authors to suggest the importance to use functional as well as structural indicators of change in transitional waters (Elliott and Quintino, 2007).

In the freshwater environment, several bio-monitoring studies have included both functional and structural approaches, namely leaf-litter decomposition rates and structural characteristics of the associated benthic invertebrate community (Pascoal et al., 2003; Bergfur et al., 2007; Castela et al., 2008). In estuarine ecosystems, decomposition studies were far less common (Twilley et al., 1986; Rossi and Costantini, 2000; Menéndez and Sanmartì, 2007; Sangiorgio et al., 2008;), and a recent study conducted in Mira Channel, Ria de Aveiro, Portugal, comprising the full salinity gradient and using *Phragmites australis* and *Fucus vesiculosus* as test species, indicated that the decaying substrate, the part of the salinity gradient where decomposition is taking place and the time interval for the calculation of the decomposition rate, all interfere with the final result and should be taken in consideration if using decomposition rates as a measure of estuarine ecosystem functioning (Quintino et al., 2009; Lopes et al., 2011). Here, we take that analysis further by studying the macrofauna associated with the decomposition of *P. australis* and *F. vesiculosus* in experiments conducted in Mira Channel, covering all levels of the Venice System (1959) classification of brackish waters, and in two other channels representing the mesohaline region, Canelas and Salreu. The study used a control, consisting of an artificial substrate made up of plastic pieces, and a procedure control, consisting of empty mesh bags. With these studies we aim to test the null hypotheses that from the euhaline to the limnetic area no significantly different macrofauna communities are associated with the experimental substrates at a given sampling time as well as along the study period and that no significant differences exist between the macrofauna community captured in the leaf-bags with the decaying organic substrates, with the artificial control substrate, and in the empty mesh bags.

3.3 Material and methods

3.3.1 Study area

Ria de Aveiro is located on the Northwestern coast of Portugal, between 40°38′N and 40°57′N and presents a maximum of 10 km width and 45 km length. This system is characterized by extensive intertidal mud and sand flats, salt marshes and islands. It includes four main channels, Mira, Ílhavo, Espinheiro and S. Jacinto, all of which receive freshwater inputs. This study was conducted in Mira, Canelas and Salreu Channels. Mira Channel is a narrow 20 km long channel, running south from the entrance (Figure 3.1). It is one of the most pristine channels in Ria de Aveiro (Castro et al., 2006) and the salinity gradient ranges from fully marine at the mouth to freshwater at the head, receiving continuous freshwater input from a small system of ponds and drainage channels. Canelas and Salreu channels are smaller, connected with the Laranjo Basin, and located in the central area of Ria de Aveiro (cf. Figure 3.1).

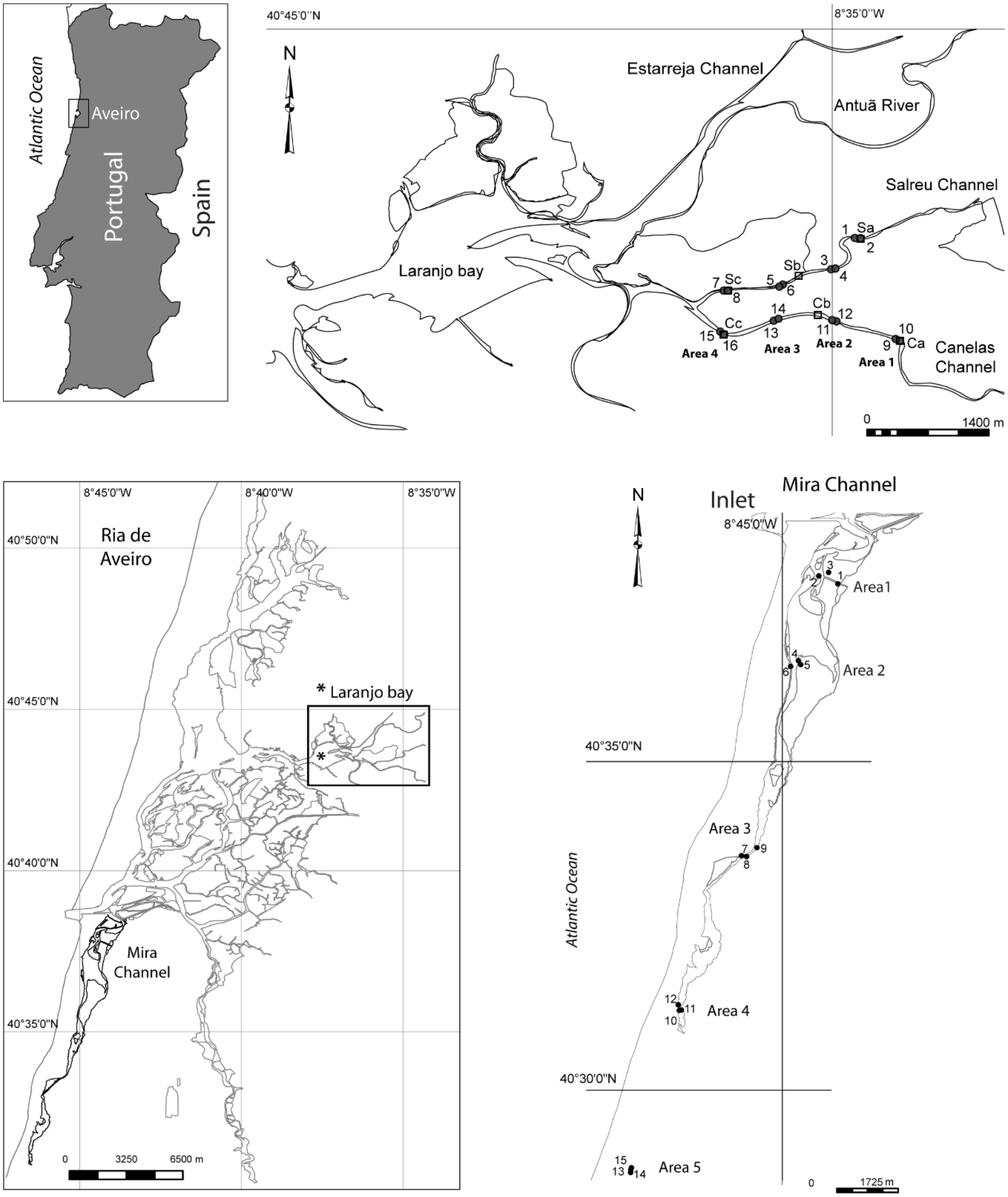

Figure 3.1 Positioning of the sampling sites in the Mira, Canelas and Salreu Channels, Ria de Aveiro, Western Portugal, for the study of benthic macroinvertebrate communities. Also shown are the sites for the study of bottom water salinity in Canelas and Salreu Channels along a total tidal cycle (Ca to Cc and Sa to Sc, respectively).

3.3.2 Field and laboratory procedures

The study in Mira Channel was performed during winter 2009 (January - March), in a total of fifteen sites arranged in five areas with three sites per area. The five areas spread across all levels of the salinity gradient according to the Venice System (1959) for the Classification of Estuarine Waters: euhaline (area 1); polyhaline (area 2); mesohaline (area 3); oligohaline (area 4) and limnetic (area 5) (Figure 3.1). The mean salinity values (with standard deviation) obtained over a complete tidal cycle in the five areas were respectively 34.6 ± 1.52, 30.2 ± 3.48, 16.2 ± 6.59, 2.4 ± 2.12 and 0.0 (Quintino et al., 2009). An experimental field study of the decomposition of dry *P. australis* and *F. vesiculosus* was undertaken simultaneously in all fifteen sites (Lopes et al., 2011), using the leaf-bag technique (Petersen and Cummins, 1974). At the beginning of the experiment (day 0), all the 5 mm mesh bags with 3.0 g of *P. australis* leaves, of *F. vesiculosus* and of an artificial substrate used as control (plastic pieces) were placed in the field sites, at the bottom, in the subtidal. In each site, four replicates of each substrate were collected over time in days 3, 7, 15 and 30. Each replicate was placed in separate plastic containers, brought to the laboratory, washed through a 0.5 mm mesh sieve and the residue preserved in 70% ethanol. Macroinvertebrates were sorted and identified to species level whenever possible. At the end of the experiment, the biological data matrix included the macroinvertebrate species/taxa and their abundance per replicate, per site and per sampling time for each substrate. During winter 2011 (February - April) a confirmation study was conducted in the mid part of the salinity gradient, the mesohaline region, in two other channels of Ria de Aveiro, Canelas and Salreu. The mean salinity over a complete tide cycle was 13.8 ± 4.55 in Salreu and 12.8 ± 4.43 in Canelas. This confirmation study used the same experimental organic decaying substrates and the control substrate but included more samples and added a procedure control, consisting of empty mesh bags. Four areas per channel and two sampling sites per area were established (Figure 3.1). In each sampling time, three replicates of each substrate and the empty bags were collected and treated as mentioned before for the study of the associated macroinvertebrate communities. In total, in the Salreu and Canelas Channels, 48 replicates per substrate were obtained per sampling occasion, whereas the mesohaline region of the Mira Channel was studied using a total of 12 replicates per experimental substrate and per sampling moment. The decay of *F. vesiculosus* and *P. australis* followed the same methodology used by Lopes et al. (2011) in the decomposition study of these two species in the Mira Channel. The decay rate (k) for each species was obtained by modeling the remaining biomass as a negative exponential decay function, from day 0 to 7 (k_7), 0 to 15 (k_{15}) and 0 to 30 (k_{30}). Bottom water samples were also collected simultaneously every 30 min during a period of 12 h, in the three sites per channel, in order to measure salinity over a tidal cycle (sites Ca to Cc in Canelas and Sa to Sc in Salreu, Figure 3.1).

3.3.3 Data analysis

The benthic macrofauna data were represented by the abundance of species per replicate, per site and per sampling time for each substrate and empty mesh bags. The resemblance matrix between samples was obtained with the Bray-Curtis similarity coefficient, following a square root transformation of the original data. Synthesis descriptors, namely the mean number of species/taxa and the mean abundance of specimens per study area, sampling time and substrate were calculated.

In Mira Channel, a few samples didn't have macrofauna associated and, in order to keep those samples in the analysis, prior to the calculation of the Bray-Curtis resemblance matrix, a dummy variable was added to all, with the abundance of 1 individual (Clarke et al., 2006a). The macrofauna community data was analyzed in an experimental design with four factors, using the salinity classes, time and substrate as fixed and orthogonal factors and the sampling sites as a random factor nested in the salinity classes, under the null hypothesis of no significant differences in the benthic community among the levels of the main factors and their interaction terms. In this analysis, salinity had five levels (euhaline, polyhaline, mesohaline, oligohaline and limnetic), time had four levels (days 3, 7, 15 and 30) and substrates had three levels (macrophyte, alga and control).

The study conducted in Salreu and Canelas showed that many replicates had low species richness or no macrofauna, specially the empty mesh bags. Due to such low taxa richness, the use of a dummy variable would artificially increase the similarity between samples which could mask the potential differences. Instead, we opted to combine the three replicates from each site at each sampling time into a single sample. This minored the problem of having many replicates without fauna and increased the sample redundancy. Each site was then represented by a single sample from averaging the species abundance of the original three replicate mesh-bags. A second analysis averaged the species at the level of the area, using all six individual mesh-bags from the two sites sampled per area. This second approach produced a matrix where no single sample was devoided of species, but did not allow to analyze the data using areas nested in the channels, given that each area was represented by a single sample. In the first case, the data was analyzed in an experimental design with three factors, with channels (two) and substrates (four) fixed and orthogonal and areas (four), random, nested in channels. In the second case, a two-way analysis was used to test the null hypothesis of no significant differences between the macrofauna associated with the substrates and between channels, with channels and substrates as fixed and orthogonal factors. This same model was used following a presence-absence transformation, to analyze the selectivity of the macrofauna species for the organic substrates, the control substrate and the empty bags.

In the decomposition experiment conducted in Canelas and Salreu channels, the remaining biomass of *P. australis* and *F. vesiculosus* was analyzed in an experimental model with three factors, with channels and substrates fixed and orthogonal and areas random and nested in the channels, to test the null hypothesis of no significant differences in the remaining biomass of the two substrates and between channels. The remaining biomass of the two substrates was plotted against time and this null hypothesis was tested separately for each sampling occasion (days 3, 7, 15 and 30). Time was never used as a factor here given the clear reduction of the biomass of both substrates as the experiments developed.

Hypothesis testing was performed by Permutation Multivariate Analysis of Variance (Anderson, 2001), using the software PRIMER v6 (Clarke and Gorley, 2006), with the add-on PERMANOVA+ (Anderson et al., 2008). This method allows the partitioning of the variability from a resemblance matrix and test individual terms, including interactions, using permutations (Anderson and ter Braak, 2003). To run the PERMANOVA tests we considered 9999 Monte Carlo permutations (Anderson et al., 2008). The F-values in the main tests and the t-statistic in the pairwise comparisons were evaluated in terms of the significance among levels of the tested factor. Values of $p \leq 0.05$ reveal that the groups differ significantly. The benthic data was also represented in ordination analyses, using Non-metric Multidimensional Scaling (NMDS).

3.4 Results

3.4.1 Loss of biomass and decay rates

The biomass loss and the decay rates of the macrophyte *P. australis* and the alga *F. vesiculosus* in Mira Channel were studied in detail by Lopes et al. (2011). According to these authors, the difference in the remaining biomass between *P. australis* and *F. vesiculosus* increased with time and the decomposition rates differed along the salinity gradient. For the alga, the decomposition rate was highest near the mouth of the estuary, corresponding to the preferential distribution area of this species, and decreased towards freshwater. For *P. australis*, the fastest decay was observed in the mid estuary, where it occurs naturally. In Canelas and Salreu Channels, with increasing time, the difference in the remaining biomass between the two decaying species (*F. vesiculosus* and *P. australis*) also increased in all study areas, as shown in Figure 3.2. The remaining biomass of each substrate along time showed a similar trend in both channels, with *F. vesiculosus* loosing biomass faster than *P. australis*. This reflects in the decomposition rate, as shown in Table 3.1. The decay rate (k) was always higher for *F. vesiculosus*, considering the various time intervals (k_7, k_{15} and k_{30}). The interaction term [channel × substrate] and the main factor channel were never found significant in any sampling time whereas the main factor substrate was significant from day 3 onwards (Table 3.A given as supporting information). The differences in the remaining biomass between the alga and the macrophyte were then of the same magnitude in the two channels and never found significant at sampling moments, including day 3, in the beginning of the experiment. From day 15 onwards, areas nested in the channels were also found significantly different, indicating that the loss of biomass in the decaying substrates was different throughout the areas in both channels. This may be due to salinity differences from area 1 to area 4 in each channel (Figure 3.3). Despite the location of all study areas in the mesohaline class, the mean salinities in the areas 1 and 4 considering a complete tidal cycle were 10 and 17 in Canelas Channel (sites Ca and Cc, Figure 3.3) and 11 and 18 in Salreu Channel (sites Sa and Sc, Figure 3.3). The maximum value of salinity measured along the tidal cycle in Canelas and Salreu Channels was 24 and 23, respectively.

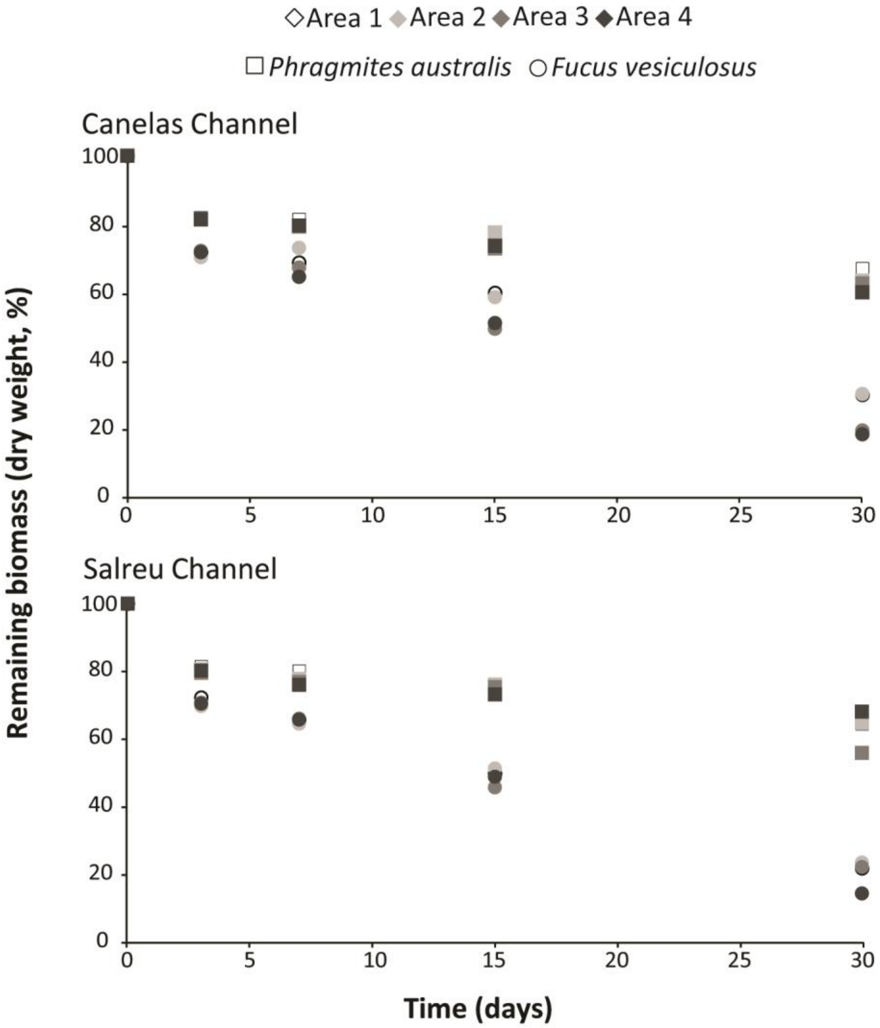

Figure 3.2 Evolution of the remaining biomass of *Phragmites australis* and *Fucus vesiculosus* during the 30-day decay period in areas 1 to 4 in Canelas and Salreu Channels, Ria de Aveiro. Each value corresponds to the mean of all replicates and all sampling sites per area.

Table 3.1 *Phragmites australis* (Pa) and *Fucus vesiculosus* (Fv) decay rates calculated per site, per area and for the whole Canelas and Salreu Channels, from day 0 to day 7 (k_7) from day 0 to day 15 (k_{15}) and from day 0 to day 30 (k_{30}).

	Canelas Channel						**Salreu Channel**					
	k7 (Pa)	**k15 (Pa)**	**k30 (Pa)**	**k7 (Fv)**	**k15 (Fv)**	**k30 (Fv)**	**k7 (Pa)**	**k15 (Pa)**	**k30 (Pa)**	**k7 (Fv)**	**k15 (Fv)**	**k30 (Fv)**
Site 1	0.0366	0.0220	0.0155	0.0663	0.0425	0.0395	0.0370	0.0218	0.0155	0.0678	0.0513	0.0467
Site 2	0.0363	0.0237	0.0162	0.0610	0.0388	0.0414	0.0377	0.0263	0.0182	0.0667	0.0499	0.0552
Area 1	**0.0365**	**0.0229**	**0.0159**	**0.0637**	**0.0407**	**0.0405**	**0.0374**	**0.0241**	**0.0169**	**0.0673**	**0.0506**	**0.0510**
Site 3	0.0414	0.0232	0.0175	0.0505	0.0425	0.0421	0.0458	0.0235	0.0168	0.0704	0.0507	0.0510
Site 4	0.0386	0.0210	0.0165	0.0649	0.0388	0.0384	0.0372	0.0224	0.0162	0.0724	0.0493	0.0463
Area 2	**0.0400**	**0.0221**	**0.0170**	**0.0577**	**0.0407**	**0.0403**	**0.0415**	**0.0230**	**0.0165**	**0.0714**	**0.0500**	**0.0487**
Site 5	0.0382	0.0258	0.0172	0.0651	0.0499	0.0468	0.0423	0.0240	0.0229	0.0673	0.0566	0.0534
Site 6	0.0407	0.0247	0.0191	0.0670	0.0528	0.0631	0.0447	0.0237	0.0182	0.0685	0.0541	0.0493
Area 3	**0.0395**	**0.0253**	**0.0182**	**0.0661**	**0.0514**	**0.0550**	**0.0435**	**0.0239**	**0.0206**	**0.0679**	**0.0554**	**0.0514**
Site 7	0.0395	0.0254	0.0183	0.0764	0.0521	0.0592	0.0472	0.0273	0.0168	0.0652	0.0502	0.0623
Site 8	0.0395	0.0240	0.0198	0.0662	0.0493	0.0515	0.0417	0.0240	0.0149	0.0721	0.0538	0.0606
Area 4	**0.0395**	**0.0247**	**0.0191**	**0.0713**	**0.0507**	**0.0554**	**0.0445**	**0.0257**	**0.0159**	**0.0687**	**0.0520**	**0.0615**
Channel	**0.0389**	**0.0237**	**0.0175**	**0.0647**	**0.0458**	**0.0478**	**0.0417**	**0.0241**	**0.0174**	**0.0688**	**0.0520**	**0.0531**

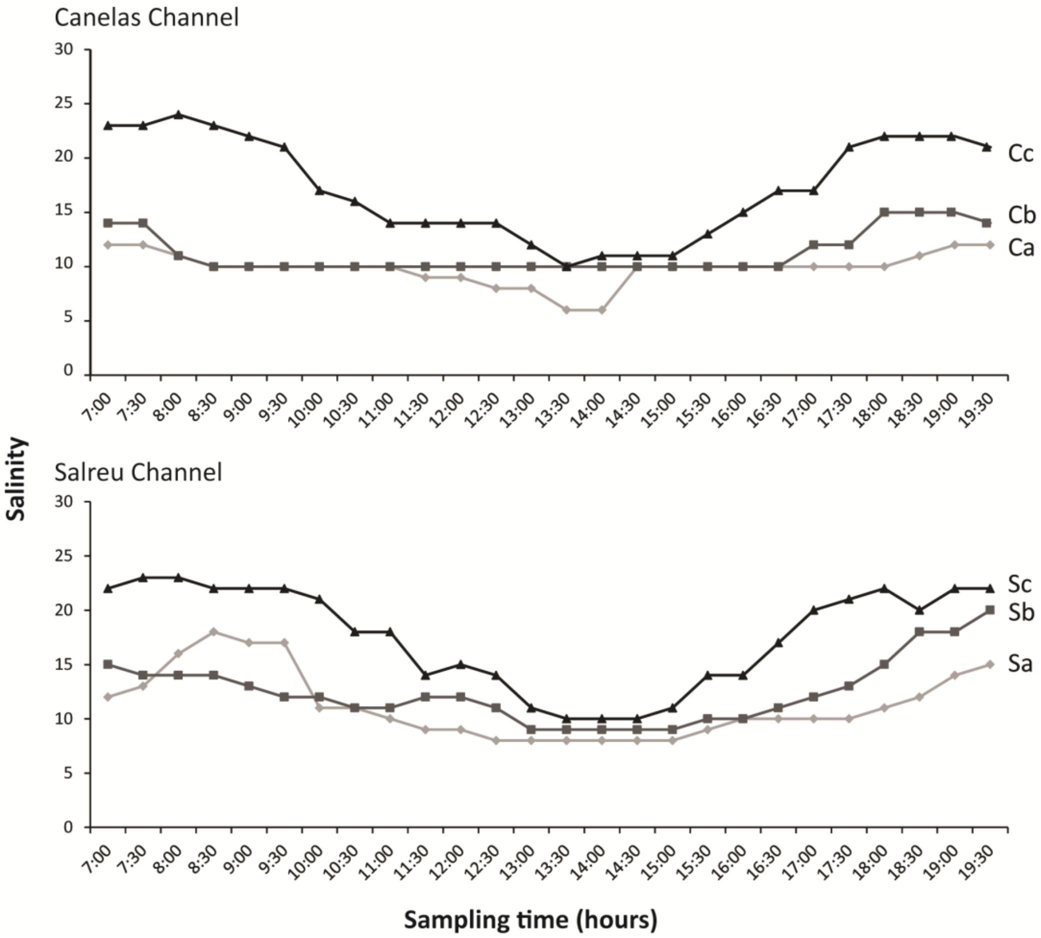

Figure 3.3 Temporal variation of bottom water salinity during a tidal cycle in Canelas and Salreu Channels, Ria de Aveiro. See Figure 3.1 for the positioning of sites Sa to Sc (Salreu) and Ca to Cc (Canelas).

3.4.2 Benthic macrofauna associated with the decay process

In all areas of the Mira Channel, the evolution of the mean taxa richness (*S*) and abundance (*N*) through time showed a similar trend in the decaying and the control substrate, indicating that much of the overall dynamic aspects of the community were equally seen in the artificial and in the decaying substrates (Figure 3.4). The benthic community generally presented higher taxa richness and abundance in the alga bags. For the three substrates, the maximum mean taxa richness was obtained at day 30 in the estuarine areas (areas 1 to 4) and at day 7 in the freshwater (area 5). The single exception was for *F. vesiculosus* in the euhaline region (area 1) in which the maximum taxa richness was obtained earlier, at day 7. The maximum mean abundance for *P. australis* was obtained at day 30 in the euhaline and oligohaline regions (areas 1 and 4) and at day 15 in the polyhaline, mesohaline and limnetic regions (areas 2, 3 and 5). This was not identical to the control substrate, in which the maximum mean abundance was reached at day 30 in the euhaline, polyhaline and oligohaline regions (areas 1, 2 and 4), at day 15 in the mesohaline regions (area 3) and at day 7 in the limnetic region (area 5). For *F. vesiculosus* the maximum mean abundance was obtained at day 7 in the euhaline and polyhaline regions (areas 1 and 2) and successively later, from the mesohaline to the oligohaline region (areas 3 and 4), in which the maximum abundance was obtained at days 15 and 30, respectively. In the limnetic region (area 5) the maximum mean abundance was reached at day 15. The oligohaline area presented the highest taxa richness and abundance for all substrates through time. In Canelas and Salreu Channels, the mean taxa richness and mean abundance were higher in the *P. australis* samples, followed by *F. vesiculosus* and the control substrate, and finally lower in the empty bags at all sampling times. The two descriptors increased along time in all substrates and empty bags. This trend was also seen in the mesohaline area of Mira Channel, at least for species richness (Table 3.2).

The fauna associated with the decaying and the control substrates in Mira Channel showed equally well the benthic succession from the marine to the freshwater areas. This is shown on the ordination diagrams of Figure 3.5, representing separately the *P. australis*, the *F. vesiculosus* and the control samples through time. The three diagrams show the separation of the euhaline and polyhaline samples from the oligohaline in the horizontal dimension, with most of the mesohaline samples in between, while the vertical dimension opposes the limnetic to the estuarine samples. The five dominant taxa in each salinity area are almost the same in the three substrates although with different relative proportions (Tables 3.B to 3.F given as supporting information).

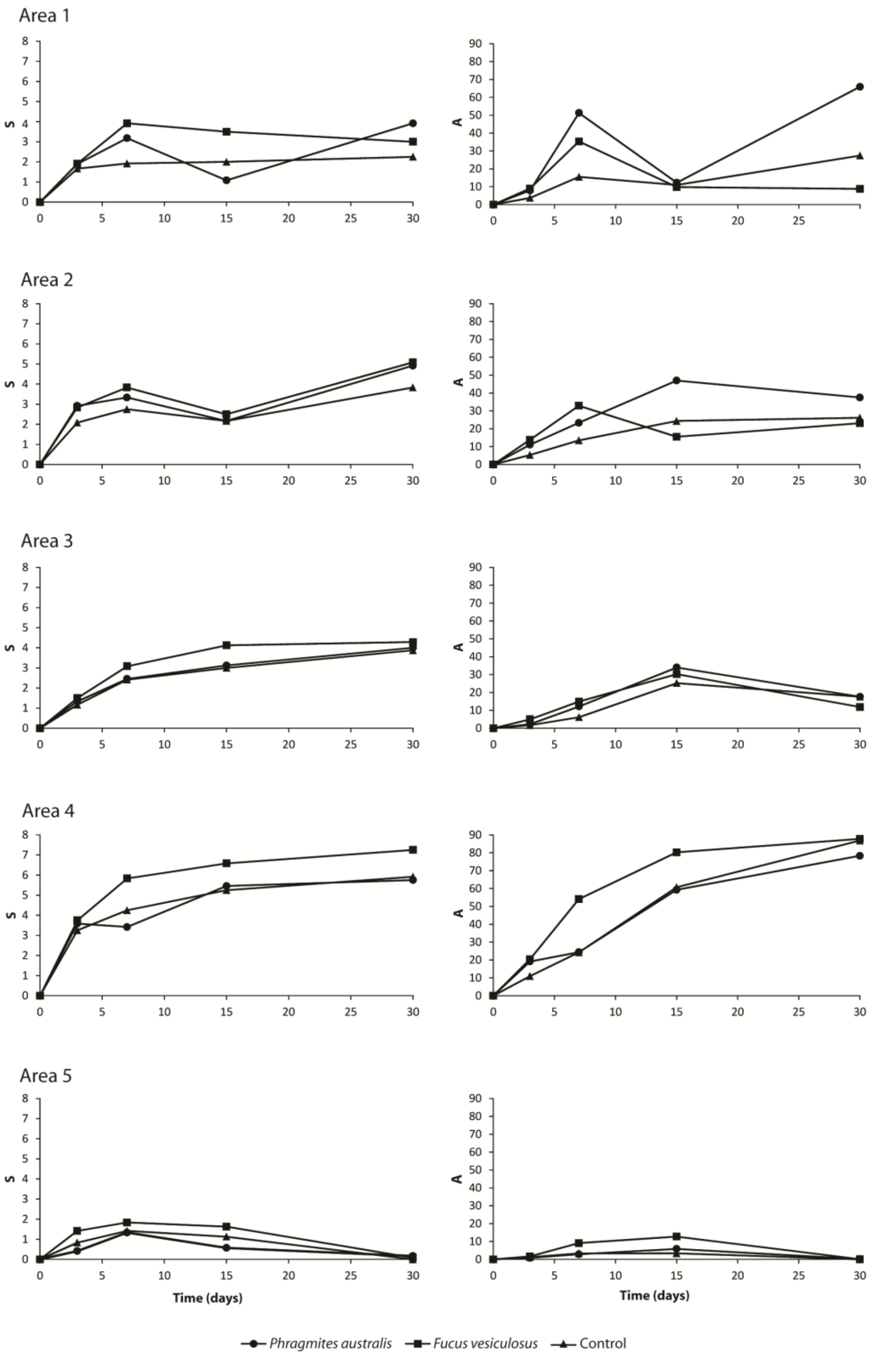

Figure 3.4 Evolution of the mean taxa richness (*S*) and mean abundance (*N*) of the benthic fauna samples in *Phragmites australis*, *Fucus vesiculosus* and the control samples at days 3, 7, 15 and 30, in areas 1 to 5 in Mira Channel. Area 1 - euhaline; area 2 - polyhaline; area 3 - mesohaline; area 4 - oligohaline; and area 5 - limnetic. Each data point in the graphs corresponds to the mean of all the individual samples obtained per site and per area.

Table 3.2 The mean taxa richness (S, with total taxa richness in brackets) and mean abundance (N) calculated with the benthic data obtained in the *Phragmites australis* (Pa), *Fucus vesiculosus* (Fv), control substrate (C) and empty bags (EB) sampled at days 3, 7, 15 and 30 in Canelas and Salreu Channels (4 areas × 2 sites × 3 replicates) and the mesohaline area of the Mira Channel (3 sites × 4 replicates). n.a. non available.

	Canelas Channel					**Salreu Channel**						**Mira Channel (mesohaline area)**				
	3	**7**	**15**	**30**	**Mean**	**3**	**7**	**15**	**30**	**Mean**	**Overall mean**	**3**	**7**	**15**	**30**	**Mean**
S_{Pa}	3.2	3.6	3.8	4.8	3.8 (14)	2.8	4.3	4.8	5.2	4.2 (14)	4.0	1.3	2.5	3.1	4.0	2.6 (17)
S_{Fv}	3.4	4.0	3.7	4.0	3.8 (17)	3.3	4.4	4.9	5.0	4.4 (16)	4.1	1.5	3.1	4.1	4.3	3.0 (23)
S_{C}	2.3	3.0	2.9	2.8	2.7 (14)	2.2	3.2	4.3	4.2	3.5 (16)	3.1	1.2	2.4	3.0	3.9	2.5 (21)
S_{EB}	0.9	1.4	2.0	2.1	1.5 (13)	0.9	2.4	1.1	4.0	2.1 (14)	1.8	n.a.	n.a.	n.a.	n.a.	n.a.
N_{Pa}	15.9	20.0	33.7	78.0	36.0	7.3	25.1	46.5	84.3	40.8	38.4	2.3	12.2	34.0	17.6	14.7
N_{Fv}	10.4	17.9	27.8	40.7	24.0	5.8	22.9	42.7	45.2	29.1	26.6	5.0	14.9	30.3	11.9	14.5
N_{C}	7.8	12.0	16.6	22.4	14.4	5.8	17.3	27.4	45.5	23.8	19.1	1.7	6.2	25.3	17.6	10.9
N_{EB}	1.2	3.2	4.5	8.9	4.4	1.0	4.2	2.1	16.5	5.9	5.2	n.a.	n.a.	n.a.	n.a.	n.a

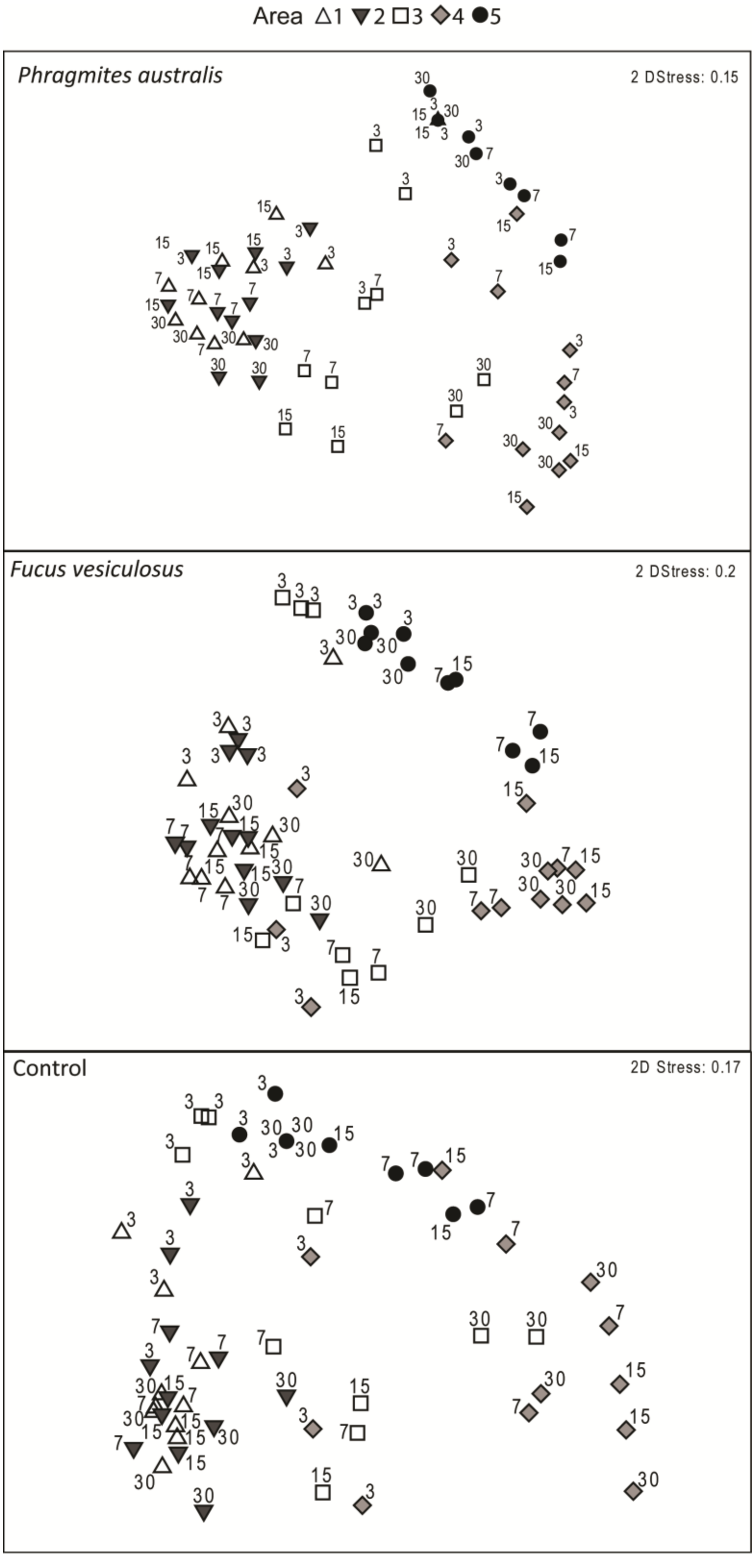

Figure 3.5 Ordination diagram (NMDS) of the benthic data obtained in the *Phragmites australis*, *Fucus vesiculosus* and control substrate samples at days 3, 7, 15 and 30 in Mira Channel.

Arthropods were dominant in all substrates along the estuarine gradient being replaced by annelids in the freshwater (Figure 3.6). In the Canelas and Salreu Channels, arthropods were also the dominant group (Figure 3.7).

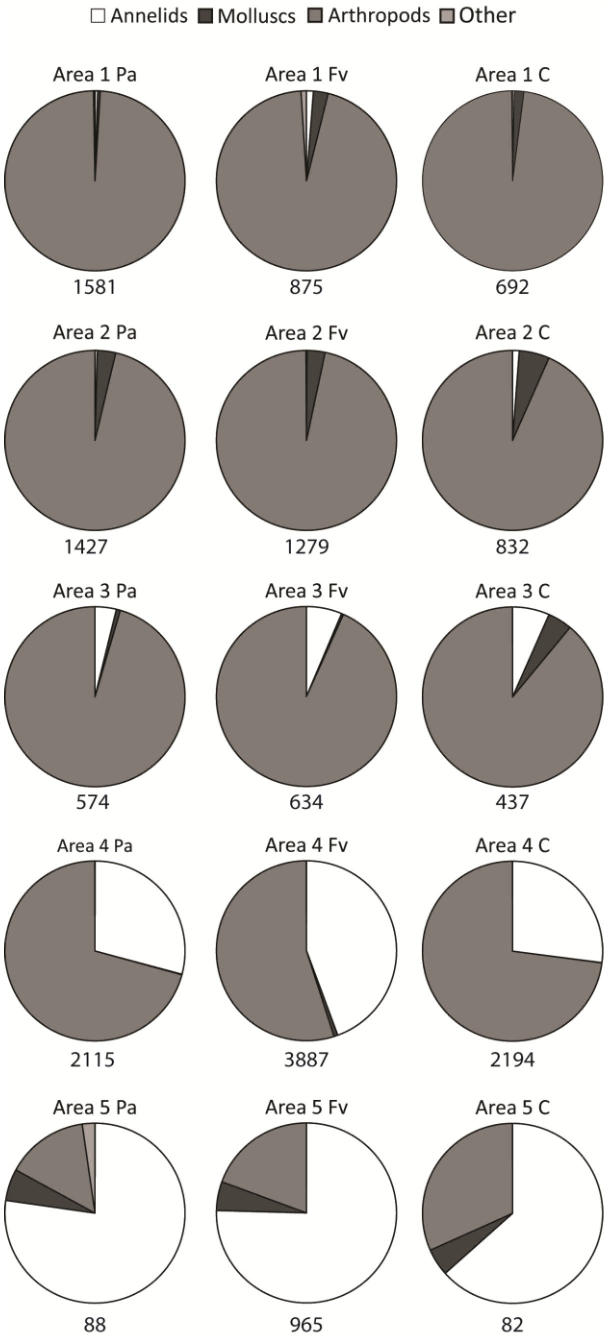

Figure 3.6 Pie charts representing the proportion of Annelids, Molluscs, Arthropods and other groups, in the mesh-bags with *Phragmites australis* (Pa), *Fucus vesiculosus* (Fv) and control substrate (C) across the study areas in Mira Channel (area 1 - euhaline; area 2 - polyhaline; area 3 - mesohaline; area 4 - oligohaline; area 5 - limnetic). The values close to each pie chart indicate total abundance. Tables 3.4 to 3.8 show the detailed distribution of abundance per species.

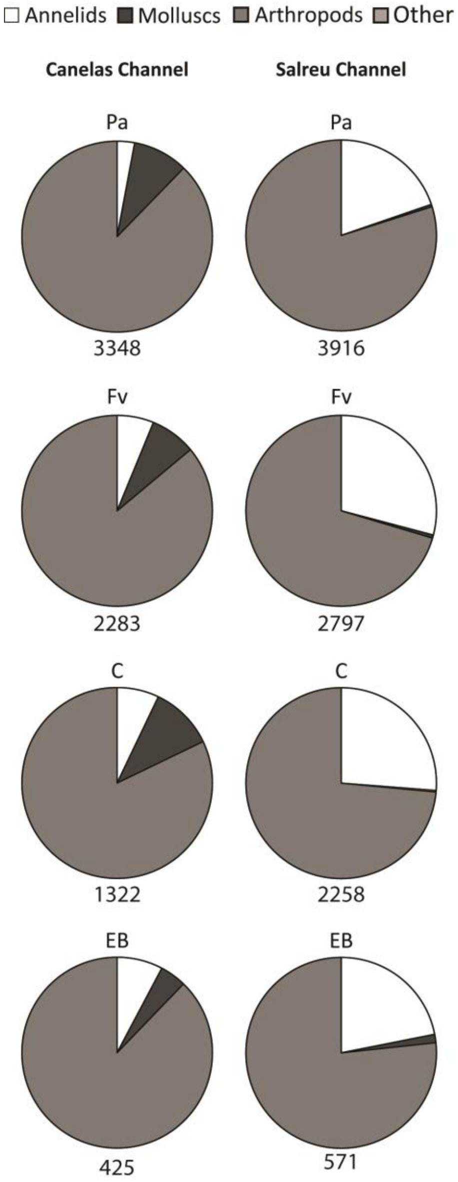

Figure 3.7 Pie charts representing the proportion of Annelids, Molluscs, Arthropods and Other groups, in the mesh-bags with *Phragmites australis* (Pa), *Fucus vesiculosus* (Fv), control substrate (C) and the empty bags (EB), across the study areas in Canelas and Salreu Channels (mesohaline). The values close to each pie chart indicate total abundance. Tables 3.9 to 3.10 show the detailed distribution of abundance per species.

The analysis of the data obtained in Mira Channel showed a significant interaction term [salinity × time × substrate] (Table 3.G, supporting information). The pairwise comparisons between salinity areas were then made separately for each substrate and each sampling time (Table 3.3) and the pairwise comparisons between the benthic macrofauna associated with the macrophyte, the alga and the control substrate were made separately for each sampling time and each salinity area (Table 3.4).

No significant differences were found between the euhaline and the polyhaline areas for any of the substrates and all the sampling times, which is in good agreement with the position of the sampling points representing these two salinity areas in the ordination diagrams shown in Figure 3.5. From the mesohaline to the limnetic area, the macrofauna associated with the alga was significantly different between all salinity areas and at all sampling times, with the exception of areas 4 and 5 at day 15. The same pattern was not found for *P. australis* and for the control substrate (Table 3.3).

Concerning the pairwise comparisons between substrates (Table 3.4), in most cases no significant differences were found between the macrofauna sampled in the three experimental substrates. Near the estuarine entrance significant differences in the macroinvertebrates associated with the alga and the control were noticed from day 7 onwards and between all substrates at day 30. In the poly-, meso- and oligohaline areas, significant differences were only found between the fauna associated with the macrophyte and the alga and the macrophyte and the control at day 3 and between the alga and the control in the oligohaline area at day 7. In the limnetic area no significant differences were ever found in the benthic communities associated with all substrates (cf. Table 3.4). In the study conducted in Salreu and Canelas Channels, when we opted to combine the three replicates from each site at each time in a single sample, the interaction term [channel × substrate] was only found significant in day 15 (pseudo-F = 2.4554; p = 0.0092). In days 3 and 7, in the beginning of the experiment, the alga and the macrophyte were the only substrates with no significant differences in the associated macrofauna. At day 15, considering the significant interaction between the channels and the substrates, the comparison between substrates was made separately for the two channels. In Canelas, no significant differences were found between all the substrates. In Salreu, only the macrophyte and the alga were significantly different. At day 30, significant differences were found between all substrates. In the second analysis, combining all the six replicates per area, the interaction term [channel × substrate] was never significant. In days 3 and 7, the only significant differences found were alga-empty bags and macrophyte-empty bags. In day 15, significant differences were found between the two organic substrates andempty bags and between the control and empty bags. All substrates were found significantly different in day 30. When combining the replicates at the level of the area, the resulting samples had more species than the individual replicates (see Table 3.2), which increased the redundancy between samples. This operation made the samples representing the control substrate more similar to those from the organic substrates, which did not happen when the analysis combined the replicates only at the level of the sites.

Table 3.3 PERMANOVA t-values frompairwise comparisons with associated significance considering the benthicmacrofauna associated with *Phragmites australis* (Pa), *Fucus vesiculosus* (Fv) and the control substrate (C) in Mira Channel at days 3, 7, 15 and 30. ns - stands for non-significant ($p > 0.05$); *$p \leq 0.05$; **$p \leq 0.001$. Area 1 - euhaline; area 2 - polyhaline; area 3 - mesohaline; area 4 - oligohaline; and area 5 - limnetic.

Salinity areas	**Pa (3)**	**Pa (7)**	**Pa (15)**	**Pa (30)**	**Fv (3)**	**Fv (7)**	**Fv (15)**	**Fv (30)**	**C (3)**	**C (7)**	**C (15)**	**C (30)**
1, 2	0.800 (ns)	1.432 (ns)	1.157 (ns)	1.588 (ns)	1.221 (ns)	1.273 (ns)	1.129 (ns)	1.227 (ns)	0.269 (ns)	0.890 (ns)	1.342 (ns)	1.546 (ns)
1, 3	1.518 (ns)	3.889*	2.128*	4.391**	2.063*	4.002*	4.691*	2.140*	1.724 (ns)	2.523*	4.824*	4.561**
1, 4	1.765 (ns)	2.581*	1.567 (ns)	3.121*	2.437*	3.956*	2.426*	2.670*	1.908*	2.641*	1.964*	2.682*
1, 5	1.741 (ns)	3.788*	1.138 (ns)	6.268**	1.791*	3.614*	2.331*	3.108*	1.526 (ns)	3.520*	2.555*	10.290**
2, 3	2.253*	3.183*	3.292*	3.629*	3.207*	2.874*	3.083*	2.017*	1.976*	2.134*	4.903*	1.956*
2, 4	2.272*	2.316*	2.103*	3.029*	3.415*	3.156*	2.700*	2.983*	1.934*	2.229*	2.503*	1.866*
2, 5	3.060*	3.291*	2.170*	6.310*	3.418*	2.668*	2.407*	4.358**	1.796*	2.841*	3.142*	2.956*
3, 4	2.102*	1.987*	1.856 (ns)	1.823 (ns)	3.864**	3.246*	2.487*	2.111*	2.831*	2.561*	1.947*	1.524 (ns)
3, 5	1.543 (ns)	2.645*	2.174 (ns)	7.150**	3.054*	2.499*	2.497*	4.769**	2.361*	3.206*	2.328*	4.838*
4, 5	2.315*	1.491 (ns)	0.949 (ns)	3.538*	3.409*	2.479*	1.387 (ns)	4.569**	2.100*	2.186*	1.239*	2.835 *

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When the macrofauna data was analyzed following a presence/absence transformation, some of the significant differences in the previous analysis were eliminated. When keeping the sites per area, only the empty bags were found significantly different from the other substrates, while the same analysis with the quantitative data differentiated the control substrate from the organic substrates. When combining the replicates per area, in days 3 and 30, none of the substrates were found significantly different and, in days 7 and 15, significant differences were found only between the empty bags and the other substrates. This implies that only the analysis of the quantitative data and keeping the combination of replicates at the level of the sites was capable of distinguishing the two organic substrates from the artificial substrate, implying that the difference between these substrates was due to the relative abundance of the species. This is shown in Tables 3.H and 3.I (supporting information), detailing the abundance distribution per species in the various experimental substrates in Canelas and Salreu Channels.

Table 3.4 PERMANOVA t-values frompairwise comparisons with associated significance between the benthicmacrofauna associatedwith *Phragmites australis* (Pa), *Fucus vesiculosus* (Fv) and the control substrate (C) leaf-bags in Mira Channel (salinity areas 1 to 5) at days 3, 7, 15 and 30. ns - stands for non-significant ($p > 0.05$). Area 1 - euhaline; area 2 - polyhaline; area 3 - mesohaline; area 4 - oligohaline; and area 5 - limnetic.

Substrates	Time	Area 1	Area 2	Area 3	Area 4	Area 5
Pa vs Fv	**3**	1.644 (ns)	2.390*	2.400*	3.004*	2.112 (ns)
Pa vs C	**3**	1.535 (ns)	2.485*	2.136*	2.773*	1.472 (ns)
Fv vs C	**3**	0.927 (ns)	1.678 (ns)	2.505 (ns)	1.560 (ns)	1.339 (ns)
Pa vs Fv	**7**	1.599 (ns)	1.131 (ns)	0.682 (ns)	1.434 (ns)	1.122 (ns)
Pa vs C	**7**	2.036 (ns)	1.306 (ns)	1.114 (ns)	1.116 (ns)	0.913 (ns)
Fv vs C	**7**	2.183*	1.751 (ns)	1.135 (ns)	2.537*	1.147 (ns)
Pa vs Fv	**15**	2.055 (ns)	1.671 (ns)	0.232 (ns)	0.986 (ns)	1.073 (ns)
Pa vs C	**15**	1.082 (ns)	1.412 (ns)	0.800 (ns)	1.350 (ns)	0.952 (ns)
Fv vs C	**15**	3.668*	1.739 (ns)	0.462 (ns)	0.951 (ns)	1.197 (ns)
Pa vs Fv	**30**	2.409*	1.259 (ns)	1.039 (ns)	1.074 (ns)	1.000 (ns)
Pa vs C	**30**	2.467*	1.558 (ns)	1.417 (ns)	1.036 (ns)	0.866 (ns)
Fv vs C	**30**	3.462*	1.343 (ns)	0.929 (ns)	1.128 (ns)	1.000 (ns)

3.5 Discussion

In a comparative decomposition study of a macrophyte (*P. australis*) and a marine alga (*F. vesiculosus*), using the leaf-bag technique (Petersen and Cummins, 1974), along a full salinity gradient, Lopes et al. (2011) concluded that the alga and the macrophyte presented an opposite trend in the decomposition rate. The biomass decay for the algawas faster in the areas with higher mean salinity and for *P. australis* the highest loss of biomass occurred in the mid-upper areas of the estuary, so in those areas where both species occurs naturally. Whether such opposing trends would reflect upon the benthic community associated with the decomposition process was one of the objectives of this study. In order to test this, a comparison of the macrofauna sampled in the leaf-bags along the estuarine gradient was undertaken and, at a later stage, a more detailed comparison of the macrofauna sampled by different substrates was also performed, within the same salinity class, the mesohaline area, in the Canelas and Salreu Channel.

In the study conducted along the full salinity gradient, in Mira Channel, no significant differences were found between the euhaline and the polyhaline areas considering the macrofauna sampled in all substrates, organic and the control. From the mesohaline to the limnetic areas, the macrofauna associated with the alga was significantly different between all salinity areas. This pattern was similar although not as clear for *P. australis* and for the control substrate. Thus, macroinvertebrate communities associated with the leaf-bags containing the organic substrates and the control substrate, showed equally well the benthic succession from the marine to the freshwater areas. These results are consistent with a previous study carried out by Quintino et al. (2011) in the same areas, comparing the macrofauna sampled by leaf-bags of *P. australis* and by regular corer sediment samples. According to these authors, the superficial sediment grain-size was relatively similar among the salinity areas which suggested that the salinity gradient and associated hydrological descriptors were the environmental variables most related to the benthic community spatial distribution. Rodrigues et al. (2011) conducted a wider study of the benthic communities in Ria de Aveiro and also concluded that the spatial distribution of macrofauna was more related to hydrodynamic descriptors and to salinity than to the sediment grain size descriptors. Although the fauna sampled in association with the various substrates varied along the salinity areas, within the same salinity area, and during the decomposition process, the differences were much less pronounced, except in the euhaline area, where the fauna associated with the alga was soon differentiated from the other substrates. This suggests that the trends of the decay process of the alga and the macrophyte along Mira Channel would be more related to the salinity gradient and associated environmental conditions than to the macrofauna community.

In general, this study showed no significant differences between the benthic communities associated with *P. australis* and *F. vesiculosus*, despite the strong differences in the decay rates of both substrates. This reinforces the idea that there was not an intrinsic relationship between the decomposition process and the associated macroinvertebrate communities. This is in agreement with studies conducted in freshwater by Ribas et al. (2006) and Moulton et al. (2010), who evaluated the macrofaunal effects on leaf litter breakdown rates and concluded that the rates did not differ between the coarse mesh cages and the fine coarse mesh cages, which should preclude the access of the macroinvertebrates, suggesting that macroinvertebrates do not influence leaf litter decomposition. Other study carried out by Leroy and Marks (2006) compared the decomposition rates of five native riparian tree species across three streams and concluded that leaf quality was more important than stream differences in determining the decomposition rate, and that although invertebrate assemblages differed among streams, leaf decomposition was most affected by substrate quality, not stream-to-stream differences in the shredder assemblage.

This study showed detectable differences in the macrofauna colonizing mesh-bags with substrates, organic or artificial, and empty mesh-bags. The mesh-bags acted as a fauna attractor, which was significantly enhanced when an artificial substrate was supplied, and the most abundant fauna was sampled in the mesh-bags containing the organic decaying substrates. In other studies in freshwater ecosystems, leaf-bags have also been described as attractors for bottom macroinvertebrates, constituting an additional supply of food mainly for shredder guilds (Cortes et al., 1997; Sangiorgio et al., 2010). Our results are also in accordance with Gerrish and Bristow (1979). Those authors showed that the density of macroinvertebrates associated with three species of aquatic macrophytes (*Myriophyllum exalbescens*, *Potamogeton richardsonii* and *Vallisneria americana*) was different, whereas no differences were detected between the population associated with *P. richardsonii* and an artificial substrate, morphologically similar. The authors admit that the macroinvertebrates colonized the organic and the artificial substrates to feed on the biofilm of microorganisms or to take shelter. The pattern of the decomposition process in aquatic systems is determined by the synergy between the trophic activity of invertebrates and the enzymatic activity of the microorganisms (Rossi, 1985). Conditioning of organic matter by microorganisms (mainly bacteria and fungi) promotes the transformation of inedible material into edible compounds and makes the detritus more palatable for detritus-feeders (Varga, 2001) which benefit from the fungal action and from the fungi themselves (Graça et al., 1993; Suberkropp et al., 1983).

The fragmentation of the organic matter by shredders also facilitates the microbial activity, enhancing the available detrital surface (Hargrave, 1970) and spreading microfungal spores (Rossi, 1985). A study of the bacterial communities associated with the decomposition of *F. vesiculosus* in transitional waters was carried out by Martins et al. (2012), simultaneously with the study of the decomposition of *P. australis* and *F. vesiculosus* in Mira Channel by Lopes et al. (2011). Study indicated that in the limnetic area the alga was behaving as an artificial substrate since no significant differences were found in the bacterial community over time for the alga and the artificial substrate, contrary to the areas nearest to the mouth of the estuary. This supports the decomposition results reported by Lopes et al. (2011), as well as the results obtained in our present study. According to Martins et al. (2012), in the euhaline area, significant differences were found between the bacterial communities extracted from the alga and the control, except in day 3. In the mesohaline and the limnetic areas, significant differences between the two substrates took longer to develop. These results could indicate that the bacterial succession was faster on the alga in the euhaline area, where the alga naturally occurs and where the decomposition was also faster. Also, in the present study, the macrofauna community associated with the alga differentiated from the other substrates relatively soon only in the euhaline area. However, in the mesohaline and limnetic areas, despite the differences in the bacterial communities between the alga and the control (Martins et al., 2012), this study reports no significant differences in the macrofauna associated with both substrates. The importance of microflora on decomposition was also demonstrated by Sabetta et al. (2000), who conducted a study in a volcanic lake focusing on the feeding interactions between macroinvertebrates detritivores and decomposing fungi, as drivers of *P. australis* decomposition. These authors concluded that when the access of the macroinvertebrates to the leaf bags was precluded, the decomposition rate was positively related to the number of fungi on the decaying litter. The feeding activity of detritivores changed both the species richness and composition of the fungal community on the litter. The substrate reduction due to intense animal feeding appeared to limit the ability of fungi to regrow after grazing, resulting in an inverse relationship between the number of fungi and the decomposition rate.

This study also showed that differences between the macrofauna colonizing the organic and the inorganic substrates become less apparent if only the species composition information was considered, indicating that the species were not selective between the organic substrates and the artificial (control) substrate. Overall, these results suggest that the macroinvertebrates colonize the organic and the artificial substrates to feed on the biofilm and/or to seek shelter but the decomposition rates of the two substrates, namely along the salinity gradient, do not seem to be primarily related to the macroinvertebrates colonizers.

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3.6 Supporting information

Table 3.A PERMANOVA main test Pseudo-*F* values and associated significance (p) for the remaining biomass of the *Phragmites australis* and *Fucus vesiculosus* in Canelas and Salreu Channels (areas 1 to 4) at days 3, 7, 15 and 30.

Day 3					
Source	**df**	**SS**	**MS**	**Pseudo-*F***	***p***
Channel (Ch)	1	2.41E-03	2.41E-03	0.50589	0.5099
Substrate (Su)	1	2.0816	2.0816	1157.5	0.0001
Ar (Ch)	6	2.86E-02	4.77E-03	0.70893	0.6426
ChxSu	1	6.56E-04	6.56E-04	0.36488	0.5648
SuxAr (Ch)	6	1.08E-02	1.80E-03	0.26719	0.9498
Residual	80	0.53845	6.73E-03		
Total	95	2.6626			
Day 7					
Source	**df**	**SS**	**MS**	**Pseudo-F**	**p**
Channel (Ch)	1	8.32E-02	8.32E-02	3.8396	0.0924
Substrate (Su)	1	3.005	3.005	126.96	0.0002
Ar (Ch)	6	0.13001	2.17E-02	1.4412	0.2185
ChxSu	1	2.91E-03	2.91E-03	0.12285	0.7428
SuxAr (Ch)	6	0.14202	2.37E-02	1.5743	0.1712
Residual	80	1.2028	1.50E-02		
Total	95	4.566			
Day 15					
Source	**df**	**SS**	**MS**	**Pseudo-F**	**p**
Channel (Ch)	1	0.13807	0.13807	1.7652	0.2292
Substrate (Su)	1	11.198	11.198	396.11	0.0001
Ar (Ch)	6	0.46929	7.82E-02	6.3897	0.0001
ChxSu	1	0.16927	0.16927	5.988	0.0531
SuxAr (Ch)	6	0.16961	2.83E-02	2.3094	0.0423
Residual	80	0.97928	1.22E-02		
Total	95	13.123			
Day 30					
Source	**df**	**SS**	**MS**	**Pseudo-F**	**p**
Channel (Ch)	1	5.93E-02	5.93E-02	0.45007	0.5208
Substrate (Su)	1	35.563	35.563	299.21	0.0001
Ar (Ch)	6	0.79097	0.13183	4.0726	0.0017
ChxSu	1	0.10431	0.10431	0.87759	0.3763
SuxAr (Ch)	6	0.71313	0.11886	3.6718	0.0021
Residual	80	2.5896	3.24E-02		
Total	95	39.82			

Table 3.B Relative abundance (A) per taxon in the *Phragmites australis* (Pa), *Fucus vesiculosus* (Fv) and control substrate (C) samples in the area 1 of the Mira Channel, ranked from highest to lowest. The letters before the species/taxon name refer to the Phylum: A - Annelids; Ar - Arthropods; M - Molluscs; C - Cnidarians; E – Echinoderms.

			Area 1	
			A (%)	
	Taxa	**Pa**	**Fv**	**C**
Ar	*Gammarus insensibilis*	81.5	40.2	83.5
Ar	*Gammarus chevreuxi*	13.9	39.1	8.1
Ar	*Gammarus* sp.		5.7	1.4
Ar	Microdeutopus sp.	0.1	4.1	1.2
Ar	*Melita palmata*	1.8	1.4	
M	*Peringia ulvae*	0.4	2.4	1.4
Ar	*Idotea chelipes*	0.7	0.7	0.4
Ar	Tanidaidacea sp1	0.1	1.6	0.7
E	*Amphiura filiformis*	0.2	1.0	0.1
Ar	Chironomidae	0.3	0.7	
Ar	Coenagrionidae		0.1	1.3
A	*Tharyx marioni*	0.3	0.3	0.3
Ar	*Microdeutopus gryllotalpa*		0.4	0.4
Ar	*Carcinus maenas*	0.1	0.3	0.1
A	Oligochaeta		0.6	
Ar	*Janiropsis breviremis*		0.1	0.4
Ar	*Schistomysis ornata*		0.3	0.1
M	*Mytilus galloprovincialis*	0.1	0.1	
A	*Eteone picta*	0.1		
A	*Sigalion* sp.	0.1	0.1	
M	*Hydrobia neglecta*	0.1		0.1
Ar	*Monocorophium acherusicum*		0.3	
Ar	*Harmathoe impar*		0.1	
A	*Platynereis dumerilii*	0.1		
A	*Streblospio shrubsolii*		0.1	
M	*Physa acuta*			0.1
M	*Tellina tenuis*	0.1		
Ar	*Heterotanais oerstedtii*		0.1	
C	Anthozoa	0.1		

Table 3.C Relative abundance (A) per taxon in the *Phragmites australis* (Pa), *Fucus vesiculosus* (Fv) and control substrate (C) samples in the area 2 of the Mira Channel, ranked from highest to lowest. The letters before the species/taxon name refer to the Phylum: A - Annelids; Ar - Arthropods; M - Molluscs.

			Area 2 **A (%)**	
	Taxa	**Pa**	**Fv**	**C**
Ar	*Gammarus insensibilis*	62.4	33.2	63.6
Ar	*Gammarus chevreuxi*	18.4	30.5	20.7
Ar	*Melita palmata*	9.2	6.9	2.2
Ar	*Gammarus* sp.	1.7	9.8	1.4
M	*Peringia ulvae*	3.2	3.0	5.4
Ar	*Microdeutopus* sp.	1.3	3.8	1.8
Ar	*Leptocheirus pilosus*		6.8	0.1
Ar	*Idotea chelipes*	2.7	1.4	1.4
Ar	*Microdeutopus gryllotalpa*	0.1	2.1	0.4
A	*Streblospio shrubsolii*	0.4		0.4
A	*Tharyx marioni*		0.1	0.8
Ar	*Janiropsis breviremis*		0.5	0.4
Ar	Chironomidae	0.1	0.4	0.1
Ar	*Monoorophium acherusicum*	0.2	0.3	0.1
Ar	*Carcinus maenas*		0.3	0.2
M	*Cerithiopsis tubercularis*	0.1		
M	*Hydrobia neglecta*		0.2	
Ar	*Urothoe brevicornis*			0.2
Ar	*Lekanesphaera* sp.		0.2	
Ar	*Paramysis bacescoi*	0.1		
M	*Mytilus galloprovincialis*			0.2
M	*Venerupsis decussata*	0.1	0.1	
A	*Hediste diversicolor*	0.1		
Ar	*Aora* sp.			0.1
Ar	*Lekanesphaera hookeri*		0.1	
Ar	*Lekanesphaera monodi*		0.1	
Ar	*Palaemon serratus*		0.1	
M	*Cerastoderma edule*			0.1
M	*Corbicula fluminea*			0.1
Ar	*Musculus costulatus*			0.1
Ar	Acarina		0.1	

Table 3.D Relative abundance (A) per taxon in the *Phragmites australis* (Pa), *Fucus vesiculosus* (Fv) and control substrate (C) samples in the area 3 of the Mira Channel, ranked from highest to lowest. The letters before the species/taxon name refer to the Phylum: A - Annelids; Ar - Arthropods; M - Molluscs.

			Area 3	
			A (%)	
	Taxa	**Pa**	**Fv**	**C**
Ar	*Melita palmata*	74.0	60.8	60.9
Ar	*Idotea chelipes*	8.4	10.1	9.8
Ar	*Leptocheirus pilosus*	7.7	4.4	7.6
A	*Hediste diversicolor*	1.9	4.3	2.5
Ar	*Microdeutopus gryllotalpa*		6.0	2.1
Ar	*Gammarus insensibilis*	1.6	2.5	3.2
Ar	*Janiropsis breviremis*		4.1	1.8
A	Oligochaeta	1.6	0.5	1.6
A	*Streblospio shrubsolii*	0.3	1.1	2.3
Ar	Chironomidae	1.4	1.1	0.9
M	*Hydrobia neglecta*			3.2
M	*Peringia ulvae*	0.7	0.4	1.1
Ar	*Gammarus chevreuxi*	0.3	1.1	0.2
Ar	*Microdeutopus* sp.	0.3	0.7	0.2
Ar	*Cyathura carinata*		0.5	0.9
Ar	*Lekanesphaera hookeri*	0.5	0.2	0.2
A	Nereidae (juveniles)		0.5	0.2
Ar	*Lekanesphaera* sp.		0.4	0.5
Ar	*Paragnathia formica*	0.2	0.4	0.2
Ar	*Heterotanais oerstedtii*	0.3	0.4	
Ar	*Amphithoe* sp.	0.2	0.2	0.2
Ar	*Monocorophium acherusicum*	0.2	0.2	0.2
Ar	*Corophium multisetosum*	0.3		
Ar	*Bathyporeia sarsi*		0.2	
Ar	*Lekanesphaera monodi*		0.2	

Table 3.E Relative abundance (A) per taxon in the *Phragmites australis* (Pa), *Fucus vesiculosus* (Fv) and control substrate (C) samples in the area 4 of the Mira Channel, ranked from highest to lowest. The letters4before the species/taxon name refer to the Phylum: A - Annelids; Ar - Arthropods; M - Molluscs.

			Area 4	
			A (%)	
	Taxa	**Pa**	**Fv**	**C**
Ar	*Leptocheirus pilosus*	39.5	22.8	42.8
A	Oligochaeta	22.7	38.2	20.4
Ar	Chironomidae	10.4	8.2	9.9
Ar	*Lekanesphaera hookeri*	9.8	8.2	4.5
A	*Streblospio shrubsolii*	5.7	3.8	3.9
Ar	*Corophium volutator*	2.9	1.7	4.6
Ar	*Gammarus chevreuxi*	2.3	3.2	2.4
Ar	*Lekanesphaera monodi*		4.8	1.3
Ar	*Heterotanais oerstedtii*	1.8	2.0	2.1
Ar	*Corophium multisetosum*	2.4	1.1	1.9
A	*Platynereis dumerili*		2.0	2.1
Ar	*Cyathura carinata*	0.9	1.0	1.2
Ar	*Melita palmata*	0.1	1.0	1.7
A	*Alkmaria romijni*	0.4	0.3	0.2
M	*Peringia ulvae*		0.6	
A	*Hediste diversicolor*	0.3	0.2	0.2
Ar	*Gammarus insensibilis*		0.3	0.3
Ar	Caenidae	0.2	0.2	
Ar	Coenagrionidae		0.2	0.2
A	*Tharyx marioni*			0.1
Ar	*Gammarus* sp.	0.1		
Ar	*Microdeutopus* sp.	0.1		
M	*Physa acuta*		0.1	
Ar	Diptera			
Ar	Elmidae		0.1	

Table 3.F Relative abundance (A) per taxon in the *Phragmites australis* (Pa), *Fucus vesiculosus* (Fv) and control substrate (C) samples in the area 5 of the Mira Channel, ranked from highest to lowest. The letters before the species/taxon name refer to the Phylum: A - Annelids; Ar - Arthropods; M - Molluscs.

			Area 5 **A (%)**	
	Taxa	**Pa**	**Fv**	**C**
A	Oligochaeta	77.3	59.1	53.7
Ar	Chironomidae	12.5	9.1	20.7
A	Nereidae (juveniles)		14.2	
A	*Platynereis dumerilii*		2.2	9.8
Ar	Coenagrionidae		3.4	6.1
M	*Physa acuta*	3.4	2.2	2.4
M	*Potamopyrgus jenkinsi*		2.2	1.2
Ar	Caenidae		2.2	
Ar	*Idotea chelipes*		1.3	1.2
Ar	Stratiomyidae		1.3	
M	*Peringia ulvae*		0.4	1.2
Ar	Hydrophilidae		0.4	1.2
Ar	*Melita palmata*			2.4
Ar	*Janiropsis breviremis*	1.1	0.4	
Ar	*Lekanesphaera hookeri*		0.9	
Ar	Copepoda	2.3		
M	*Lymnaea peregra*	1.1		
M	Nudibranchia		0.4	
M	*Planorbis corneus*	1.1		
Ar	Ephemeroptera	1.1		
Ar	Simuliidae		0.4	

Table 3.G PERMANOVA main test Pseudo-*F* values and associated significance for the benthic macrofauna sampled in the *Phragmites australis*. *Fucus vesiculosus* and control substrate leaf-bags in Mira Channel (areas 1 to 5) at days 3, 7, 15 and 30.

			Mira Channel		
Source	**df**	**SS**	**MS**	**Pseudo-F**	**P(MC)**
Salinity (Sa)	4	5.37E+05	1.34E+05	11.105	0.0001
Time (Ti)	3	87816	29272	6.8957	0.0001
Substrate (Su)	2	19100	9550.1	6.1672	0.0001
Si (Sa)	10	1.26E+05	12582	17.211	0.0001
SaxTi	12	2.18E+05	18189	4.233	0.0001
SaxSu	8	30004	3750.4	2.3983	0.0001
TixSu	6	33136	5522.6	4.1857	0.0001
TixSi (Sa)	27	1.17E+05	4351.4	5.9523	0.0001
SuxSi (Sa)	20	31843	1592.2	2.1779	0.0001
SaxTixSu	24	81163	3381.8	2.5611	0.0001
TixSuxSi (Sa)	52	68741	1321.9	1.8083	0.0001
Residual	499	3.65E+05	731.04		
Total	667	1.79E+06			

Table 3.H Total abundance (N) and relative abundance (A) per taxon in the *Phragmites australis* (Pa), *Fucus vesiculosus* (Fv), control substrate (C) and empty bags (EB) samples in the Canelas Channel, ranked from highest to lowest. The letters before the species/taxon name refer to the Phylum: A - Annelids; Ar - Arthropods; M – Molluscs.

		Canelas Channel							
		Pa		**Fv**		**C**		**EB**	
	Taxa	**N**	**A (%)**	**N**	**A (%)**	**N**	**A (%)**	**N**	**A (%)**
Ar	*Gammarus chevreuxi*	2564	76.6	1451	63.3	830	62.8	244	57.4
M	*Peringia ulvae*	312	9.3	175	7.7	142	10.7	19	4.5
Ar	Chironomidae	123	3.7	254	11.1	136	10.3	79	18.6
Ar	*Heterotanais oerstedtii*	88	2.6	84	3.7	68	5.1	38	8.9
Ar	*Leptocheirus pilosus*	74	2.2	59	2.6	22	1.7	3	0.7
Ar	*Lekanesphaera hookeri*	74	2.2	121	5.3	25	1.9	6	1.4
A	Oligochaeta	28	0.8	54	2.4	47	3.6	8	1.9
A	*Alkmaria romijni*	47	1.4	48	2.1	27	2.0	12	2.8
A	*Hediste diversicolor*	22	0.7	34	1.5	11	0.8	10	2.4
A	*Streblospio shrubsolii*	2	0.1	6	0.3	9	0.7	3	0.7
Ar	*Paragnathia formica*	6	0.2	4	0.2			1	0.2
Ar	*Monocorophium insidiosum*	2	0.1			2	0.2		
Ar	Diptera			1		1	0.1		
Ar	Copepoda	2	0.1						
Ar	*Idotea chelipes*					1	0.1		
Ar	Acarina					1	0.1		
M	*Lymnea peregra*								
Ar	Hemiptera								
Ar	Dolichopodidae	1							
Ar	Dysticidae	1							
Ar	Elmidae	1							
Ar	*Melita palmata*	1							
Ar	Caenidae							1	0.2
Ar	Onychiuridae							1	0.2

Table 3.I Total abundance (N) and relative abundance (A) per taxon in the *Phragmites australis* (Pa), *Fucus vesiculosus* (Fv), control substrate (C) and empty bags (EB) samples in the Salreu Channel, ranked from highest to lowest. The letters before the species/taxon name refer to the Phylum: A - Annelids; Ar - Arthropods; M – Molluscs.

		Salreu Channel							
		Pa		**Fv**		**C**		**EB**	
	Taxa	**N**	**A (%)**	**N**	**A (%)**	**N**	**A (%)**	**N**	**A (%)**
Ar	*Gammarus chevreuxi*	2794	71.3	1193	42.7	1293	57.3	190	33.3
A	*Alkmaria romijni*	423	10.8	432	15.4	319	14.1	55	9.6
Ar	Chironomidae	190	4.9	615	22.0	241	10.7	159	27.8
A	*Hediste diversicolor*	218	5.6	121	4.3	156	6.9	41	7.2
A	Oligochaeta	86	2.2	221	7.9	79	3.5	24	4.2
Ar	*Heterotanais oerstedtii*	79	2.0	84	3.0	83	3.7	71	12.4
A	*Streblospio shrubsoli*	46	1.2	40	1.4	38	1.7	5	0.9
Ar	*Leptocheirus pilosus*	30	0.8	29	1.0	27	1.2	2	0.4
Ar	*Lekanesphaera hookeri*	27	0.7	33	1.2	9	0.4	9	1.6
M	*Hydrobia ulvae*	15	0.4	14	0.5	6	0.3	8	1.4
Ar	*Paragnathia formica*	4	0.1	9	0.3	2	0.1	3	0.5
Ar	Diptera			1		1		2	0.4
Ar	*Corophium multisetosum*	2	0.1			1			
Ar	Corixidae			1		1			
Ar	Copepoda			2	0.1				
Ar	Elmidae					1			
Ar	Collembola					1			
Ar	*Corophium volutator*			1					
Ar	*Procambarus clarkii*			1					
Ar	*Corophium acherusicum*	1							
Ar	*Corophium* sp.	1							
Ar	*Melita palmata*	1							
Ar	Dolichopodidae							1	0.2
Ar	*Idotea chelipes*							1	0.2

Chapter 4. Variability of body size descriptors in benthic invertebrates along a full salinity gradient sampled by leaf-bags and corer

4.1 Abstract

The abundance distribution of the macrobenthic community by the ISS index body size classes and the variability of length, weight and length-weight relationships of Arthropods, Molluscs and Annelids were studied in a system without anthropogenic stresses, the Mira Channel, using different sampling techniques, the leaf-bag technique with *Phragmites australis* and two mesh-sizes (1 mm and 5 mm) and a hand-held corer. The same study was carried out considering individual species with at least three distribution areas along the full salinity gradient. The dominant species of the sub-set of measured specimens were not the same of the original macrobenthic fauna sampled in the 1 mm leaf-bag and corer samples due to the exclusion of some taxa, mainly Annelids. Despite the changes in the species dominance, the sub-set of measured specimens was able to show the benthic succession from the marine to the freshwater areas showed by the original macrobenthinc fauna sampled. The body size abundance distribution of the benthic macroinvertebrates according to the ISS size classes did not show any particular trend in all samplers, indicating that a salinity range from 0 to 35 will not act like a masking factor when this index is applied in areas affected by anthropogenic stress. Along the salinity gradient significant differences were found in the length, weight and length-weight relationships of Annelids, Arthropods and Molluscs between the upstream and the downstream areas of the channel, which could be related with the species succession along the estuarine gradient. At the species level, significant differences were found in the length and weight of the Arthropods *Microdeutopus gryllotalpa* in the 1 mm leaf-bags and *Gammarus insensibilis* and *Melita palmata* 5 mm leaf-bags, in both cases, explained by their preferential distribution areas. The length-weight relationships of the Arthropods *G. insensibilis* and *M. palmata* between their distribution areas were significant in the 5 mm leaf-bags but not in the 1 mm leaf-bags suggesting that this mesh-size could confine the circulation of the spcimens, acting like a trap, which interfears with the corporeal condition of these species. In the 5 mm mesh-bags individuals circulate freely, representing in a better way the body size structure of these species in the different salinity areas. In the corer samples no significant differences were found in the length-weight relationships of the Annelid *Streblospio shrubsolii* along its distribution areas.

Keywords: Body size descriptors; Benthic macroinvertebrates; Leaf-bag technique; Corer samples; Salinity gradient; Ria de Aveiro.

4.2 Introduction

The hight dinamism of the physicochemical and hydro-morphologic conditions make from estuaries complex and naturally stressed ecosystems characterized by a mosaic of habitats with species particularly well adapted to variability and with a high level of tolerance to change (Elliott and Quintino, 2007). Salinity is one of the most important factors conditioning macrobenthic species richness and distribution (Quintino and Rodrigues, 1989; Rodrigues and Quintino, 1993; Uwadiae, 2009; Rodrigues et al., 2006; 2011; Whitfield et al., 2012), resulting in a succession of species from marine to freshwater environments (Quintino et al., 2011; Lopes et al., 2013).

Individual size distributions have been widely recognized as important components of guild or community structure (Kerr and Dickie, 2001; White et al., 2007) being studied in marine ecosystems (Warwick, 1984), lakes (Hanson, 1990; Rodriguez and Magnan, 1993), streams and rivers (Bourassa and Morin, 1995; Solimini et al., 2001; Basset et al., 2004) and transitional waters (Reizopoulou et al., 1996; Basset et al., 2004, 2008). They are simple, non-taxonomic based and energy-related descriptors, independent of species specific resource requirements and species composition and based on individual biomass data.

With this study we aim to evaluate the effect of a full salinity gradient in a system without antropogenic stresses and in which salinity constitutes the main factor affecting the macrobenthic distribution. Thus, this study was carried out in Mira Channel, one of the most pristine channels in Ria de Aveiro (Moreira et al., 1993; Castro et al., 2006). The macrobenthic fauna used in this study was collected with different sample techniques, the leaf-bag technique with *Phragmites australis* and two mesh-sizes (1 mm and 5 mm) and a hand-held corer. The abundance distribution of the macrobenthic communities by the ISS index (Bassett et al., 2012) body size classes was evaluated along the salnity gradient for each sampler and the variability of the length, the weight and the length-weight relationships of Arthropods, Molluscs and Annelids was tested along the salinity gradient under the null hypothesis of no significant differences in these body size descriptors along a full salinity gradient. The same null hypothesis was tested for species with at least three disribution areas along the salinity gradient.

The representativeness of the data used was also evaluated. Considering that body length and weight were determined only for complete specimens, not damage or broken, the loss of information should be considered and studied in order to insure the representativeness of the measured specimens. To achieve this objective, the benthic macrofauna sampled and the sub-set of specimens measured were analysed in terms of dominant species and their ability to show the natural replacement of species along the salinity gradient in the leaf-bag and corer samples.

4.3 Material and methods

4.3.1 Study area

Ria de Aveiro is located on the Northwestern coast of Portugal, between 40º38`N and 40º57`N. With a maximum width and length of 10 and 45 km, covers an area of 83 km^2 and 66 km^2 at high and low spring tide, respectively. This transitional system is separated from the ocean by a sand bar and is formed by four main channels, Mira, Ílhavo, Espinheiro and São Jacinto. All the channels receive freshwater inputs and connect with the sea an artificial inlet (Figure 4.1). This study was conducted in the Mira Channel, a narrow 20 Km long, running south from the entrance. This channel presents a full salinity gradient, ranging from fully marine at the mouth to freshwater at the head and is considered as one of the most pristine channels in Ria de Aveiro (Moreira et al., 1993, Castro et al., 2006).

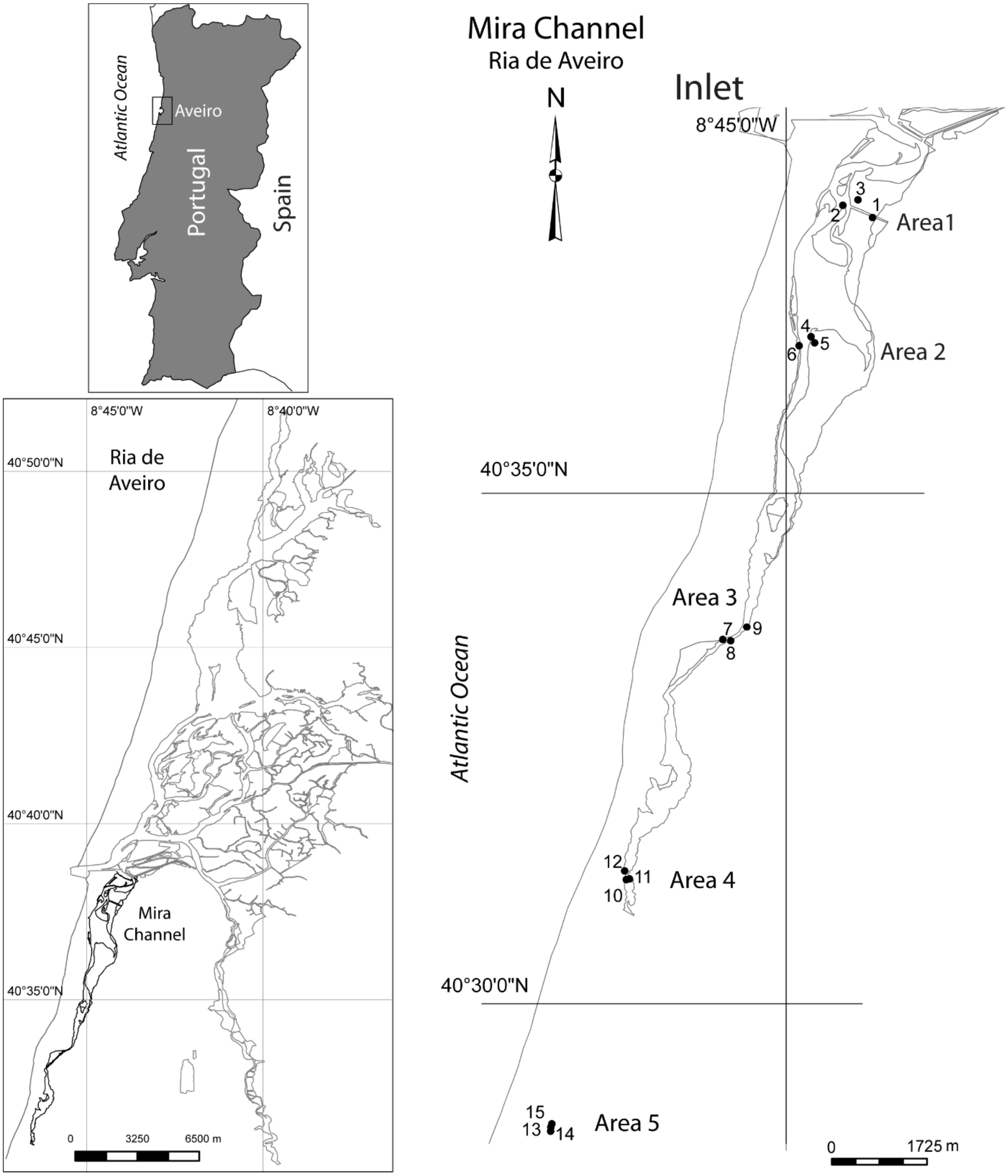

Figure 4.1 Positioning of the study sites in the Mira Channel, Ria de Aveiro, Western Portugal (sites 1 to 15, nested in areas 1 to 5). Area 1 - euhaline; area 2 - polyhaline; area 3; area 4 - Oligohaline and area 5 - limnetic.

4.3.2 Field and laboratory procedures

This study is based on an experimental field study of the decay of *Phragmites australis* leaves undertaken in the same sites, using the leaf-bag technique (Petersen and Cummins, 1974, Quintino et al., 2009). This study was carried out during winter 2008 (January-March), in a total of fifteen sampling sites distributed in five areas (area 1 to area 5), three sites per area, along the Mira Channel (Figure 4.1). The five areas cover all levels of the salinity gradient according to the Venice System (1959) for the Classification of Estuarine Waters: euhaline (area 1); polyhaline (area 2); mesohaline (area 3); oligohaline (area 4) and limnetic (area 5) (cf. Figure 4.1). The mean salinity (with standard deviation) obtained over a complete tidal cycle in the five areas (areas 1 to 5) was, respectively, 34.6±1.52, 30.2±3.48, 16.2±6.59, 2.4±2.12 and 0.0 (Quintino et al., 2009). The benthic macrofauna was sampled using the leaf-bag technique with *Phragmites australis* leaves and two mesh sizes (1 mm and 5 mm). At the beginning of the experiment (day 0), all the leaf-bags were placed in the field sites, at the bottom, in the subtidal, and replicates were collected over time, at days 3, 7, 15, 30 and day 60. At each sampling time, four replicates of the leaf-bags were collected per site and placed in separate plastic containers. Four sediment samples were also collected per site with a 0.01 m^2 hand-held corer for the study of the benthic community. The leaf-bags macrofauna samples were brought to the laboratory, washed through a 0.5 mm mesh-sieve and the residue preserved in 70% ethanol. The sediment samples were sieved through 1 mm mesh-sieve and the material retained in the sieve was fixed in 4% buffered formalin and stained with Rose Bengal. In the laboratory, all samples were individually hand-sorted, identified using binocular stereoscopic and optical microscopes to species level whenever possible, measured and then weighted after 72 hours in the dry oven at 60ºC. Body length measurements were carried out on complete individuals and obtained to the nearest 0.01 mm using a LEICA MZ12 stereoscope and an image analysis software (Leica Qwin, Leica, Germany). The largest individuals were measured with a Vernier caliper to the nearest 0.5 mm. For Anthozoa, Hirudinea and Turbellaria was measured the total body length. For the Insecta, Crustacea and Polychaeta the body length was measured as the distance from the anterior part of the head to the end of the last abdominal segment (excluding cerci, antennae and other appendages). The body length was measured as total shell length for Gastropoda and as valve length at the longest point for Bivalvia (Rosati et al., 2012). The specimens were individually weighted to the nearest ±1 µg on a Sartorius (MC 5) microanalytic balance.

At the end of the experiment, was elaborated the biological data matrix including the macroinvertebrate species/taxa and their abundance per replicate and per site for the sediment samples and also per sampling time for the 1 mm and 5 mm leaf-bags. The length and weight data matrix was also organized per replicate and per site for the sediment samples and also per sampling time for the leaf-bags.

4.3.3 Data analysis

The original benthic macrofauna data sampled in the 1 mm leaf-bags and the sub-set of benthic macroinvertebrates measured in the 1 mm and 5 mm leaf-bags and corer samples were represented by the abundance of species per replicate and per site. The resemblance matrix between samples was obtained with the Bray-Curtis similarity coefficient, following a square root transformation of the original data. The macrofauna community data was analyzed in an experimental design with two factors, using salinity classes as fixed and the sampling sites as a random factor nested in the salinity classes, under the null hypothesis of no significant differences in the benthic community along the salinity gradient. The benthic data was also represented in ordination analyses, using Non-metric Multidimensional Scaling (NMDS). The original macrobenthic fauna associated with the 5 mm leaf-bags and the corer samples were already studied by Quintino et al. (2011).

Data on individual masses were logarithmically transformed ($\log_2$ µg) and grouped into size classes (octaves) to describe the size structure of benthic macroinvertebrate guilds. Then, in order to simplify the data, the macroinvertebrate size spectra were divided into only 6 classes (CL1–CL6) by clustering the original abundance octaves into groups of three suggested for the calculation of the ISS index (Basset et al., 2012). This procedure was carried out separately for the leaf-bags and corer samples.

The length and weight of Annelids, Arthropods and Molluscs were tested along the salinity gradient in an experimental design with two factors, using salinity areas as fixed and sites as a random factor nested in areas, under the null hypothesis of no significant differences in the length and weight of each group along the salinity gradient. The same statistical analyses was made for species with at least three distribution areas along the salinity gradient and five individuals per area. Hypothesis testing was performed by Permutation Multivariate Analysis of

Variance (Anderson, 2001), using the software PRIMER v6 (Clarke and Gorley, 2006), with the add-on PERMANOVA+ (Anderson et al., 2008). This method allows partitioning the variability from a resemblance matrix and test individual terms, including interactions, using permutations (Anderson and ter Braak, 2003). To run the PERMANOVA tests we considered 9999 Monte Carlo permutations (Anderson et al., 2008). The pseudo-*F* values in the main tests and the t-statistic in the pairwise comparisons were evaluated in terms of the significance among levels of the tested factor. Values of $p \leq 0.05$ reveal that the groups differ significantly.

Length-weight relationships were described as $M = aL^b$, where M = body weight, L = body length; *a* = specific body weight, and *b* = regression slope and calculated for Annelids, Arthropods, Molluscs and species with at least three distribution areas along the salinity gradient, sampled in the 1 mm and 5 mm leaf-bags and the corer samples. To obtain such equations, data regarding the body weight and length of all individuals belonging to each group and species were logarithmically transformed ($\log_2$) and the linear regression was then computed. An ANOVA test was performed using the statistical sofware SPSS in order to test length-weight relationships in each salinity area. The significant regression slopes were statistically tested under the null hypothesis of no significant differences in the length-weight relationships of each group and species along the full salinity gradient using ANCOVA model and the aov() function in R with the F test.

4.4 Results

4.4.1 Macrobenthic community

The 1 mm leaf-bag samples, considering the original data sampled, showed the benthic succession from the marine to the freshwater areas. The horizontal axis of the ordination diagram of Figure 4.2 opposes the estuarine and the freshwater samples and the vertical axis separates the euhaline, polyhaline, mesohaline and oligohaline areas. Significant differences were found in the macrofauna along the salinity gradient (pseudo-$F = 10.804$, $p = 0.0001$) between all the areas excepting between the euhaline and the polyhaline areas (1,2: $t = 1.0225$, $p = 0.402$; 1,3: $t = 2.262$, $p = 0.007$; 1,4: $t = 3.279$, $p = 0.002$; 1,5: $t = 3.549$, $p = 0.001$; 2,3: $t =$

1.964, *p* = 0.019; 2,4: *t* = 3.528, *p* = 0.001; 2,5: *t* = 3.751, *p* = 0.001; 3,4: *t* = 4.554, *p* = 0.001; 3,5: *t* = 5.888, *p* = 0.0001; 4,5: *t* = 6.712, *p* = 0.0001). Along the salinity gradient the Arthropod *Melita palmata* was dominant in the euhaline, polyhaline and mesohaline area. The second dominant species were the Annelids *Mediomastus fragilis, Capitella* spp. and *Hediste diversicolor* in the euhaline, polyhaline and mesohaline areas, respectively. In the oligohaline area, the Arthropods *Leptocheirus pilosus* and *Gammarus chevreuxi* were dominant, being replaced by the Chironomidae and Oligochaeta in the limnetic area (Table 4.A, given as supporting information). The dominant species represent 43.0%, 44.8%, 72.4%, 68.0% and 68.5% of the sampled community in the areas 1, 2, 3, 4 and 5, respectively. The ability of the macrobenthic fauna to reflect the salinity gradient and the species dominance for the 5 mm leaf-bags and the corer samples were already published by Quintino et al. (2011). In this case, the pairwise comparisons between areas also showed the same result for both datasets. The benthic community was significantly different between all salinity areas, except between areas 1 and 2 (euhaline and polihaline areas), located closer to the mouth of the estuary.

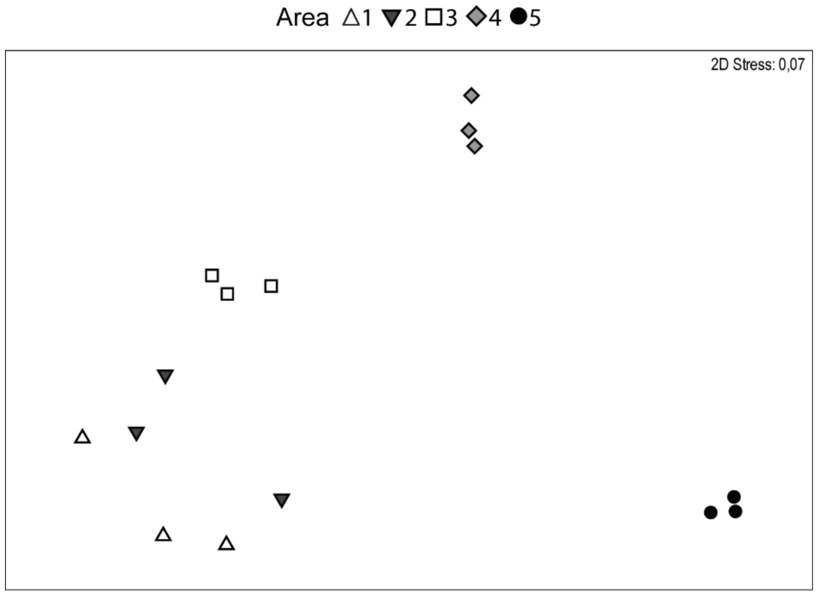

Figure 4.2 Ordination diagram (NMDS) of the original benthic macroinvertebrates data obtained in the 1 mm *Phragmites australis* leaf-bags along the full salinity gradient (areas 1 to 5). The five study areas are represented by symbols, without discriminating the sampling sites within each area.

In the study of the decay of *Phragmites australis* in which this work is based (Quintino et al., 2009), a total of 89 taxa were sampled, 85 in the 1 mm leaf-bags, 70 in the 5 mm leaf-bags and 50 in the corer samples. However, due to the bad condition of some taxa, in the 1 mm and 5 mm leaf-bags and in the corer samples were only measured 62, 52 and 33 taxa, respectively, corresponding to 72.9%, 74.3% and 66.0% of the total taxa sampled. From the taxa not measured in the 1 mm and 5 mm leaf-bags and the corer samples, 54.6%, 50.0% and 63.2% were Annelids (mainly *Capitella* spp., *Mediomastus fragilis*, Oligochaeta and *Tharyx marioni*), 22.7%, 14.3% and 5.3% were Arthropods, 4.6%, 7.1% and 15.8% were Molluscs and 18.2%, 28.6% and 15.8% other groups (

Table 4.B, given as supporting information).

The dominant taxa changed along the salinity gradient in all samplers (Tables 4.C to 4.D, given as supporting information). In the 1 mm leaf-bags, *Gammarus insensibilis* and *Melita palmata* were the dominant species in the euhaline and polyhaline areas, in the mesohaline area *Melita palmata* and *Hediste diversicolor* were dominant, being replaced by *Leptocheirus pilosus* and *Gammarus chevreuxi* in the oligohaline area and by the insects Chironomidae and Empididae in the limnetic area. The dominant species represent 66.8%, 74.4%, 75.4%, 75.7% and 66.2% of the community in the areas 1, 2, 3, 4 and 5, respectively (Table 4.C, given as supporting information). In the 5 mm leaf-bags *Gammarus insensibilis* and *Melita palmata* were the dominant species in the euhaline, polyhaline and mesohaline areas, being replaced by *Gammarus chevreuxi* and *Leptocheirus pilosu*s in the oligohaline area and by Chironomidae and Caenidae in the limnetic area (Table 4.D, given as supporting information). The dominant species represent 63.0%, 90.6%, 86.2%, 94.1% and 67.4% of the total individuals of the community in area 1, 2, 3, 4 and 5, respectively. In the corer samples, the taxa *Mediomastus fragilis* and *Angulus tenuis* were dominant in the euhaline area, being replaced by the annelids *Hediste diversicolor* and *Mediomastus fragilis* in the polyhaline area, in the mesohaline area the species *Mediomastus fragilis* was replaced by the species *Streblospio shrubsolii*, in the oligohaline the *Streblospio shrubsolii* remains dominant but the annelid *Hediste diversicolor* was replaced by the amphipod *Corophium multisetosum* and both species were replaced by the insect Chironomidae and the bivalve *Corbicula fluminea* in the freshwater (Table 4.E, given as supporting information). The dominant species represent 78.2%, 48.2%, 79.6%, 80.5% and 96.5% of the total individuals of the community in area 1, 2, 3, 4 and 5, respectively.

The sub-set of benthic macroinvertebrates measured in the 1 mm and 5 mm leaf-bags and the corer samples showed equally well the benthic succession from the marine to the freshwater areas as is shown in the ordination diagrams of Figure 4.3. In the 1 mm and 5 mm leaf-bags, the horizontal axis opposes the estuarine and the limnetic areas and the vertical axis separates the euhaline, polyhaline, mesohaline and oligohaline areas. In the corer samples, the horizontal axis separates the areas along the salinity gradient, with the euhaline and the limnetic areas in opposite sites and the polyhaline, mesohaline and oligohaline areas in the middle. Significant differences were found in the benthic macrofauna between all the areas for the 1 mm and 5 mm leaf-bags and for the corer samples, excepting between the euhaline and the polyhaline areas (Table 4.1). For the corer samples significant differences were also found between the polyhaline and the mesohaline areas (cf. Table 4.1). These results show the same patterns observed in the original data sampled in the 1 mm leaf-bags and the 5 mm leaf-bags and sediment samples as was shown by Quintino et al. (2011).

The average values of Bray-Curtis similarity between the salinity areas considering the taxa abundance were 26.44, 29.10 and 19.58 for the 1 mm and the 5 mm leaf-bags and for the corer samples, respectively. The great percentage of taxa in the 1 mm and 5 mm leaf-bags and in the corer samples had a very restricted spatial distribution resulting in a high heterogeneity of the community along the salinity gradient and low average values of Bray-Curtis similarity between areas, 23.63, 22.62 and 19.76 for the 1 mm and the 5 mm leaf-bags and for the corer samples, respectively.

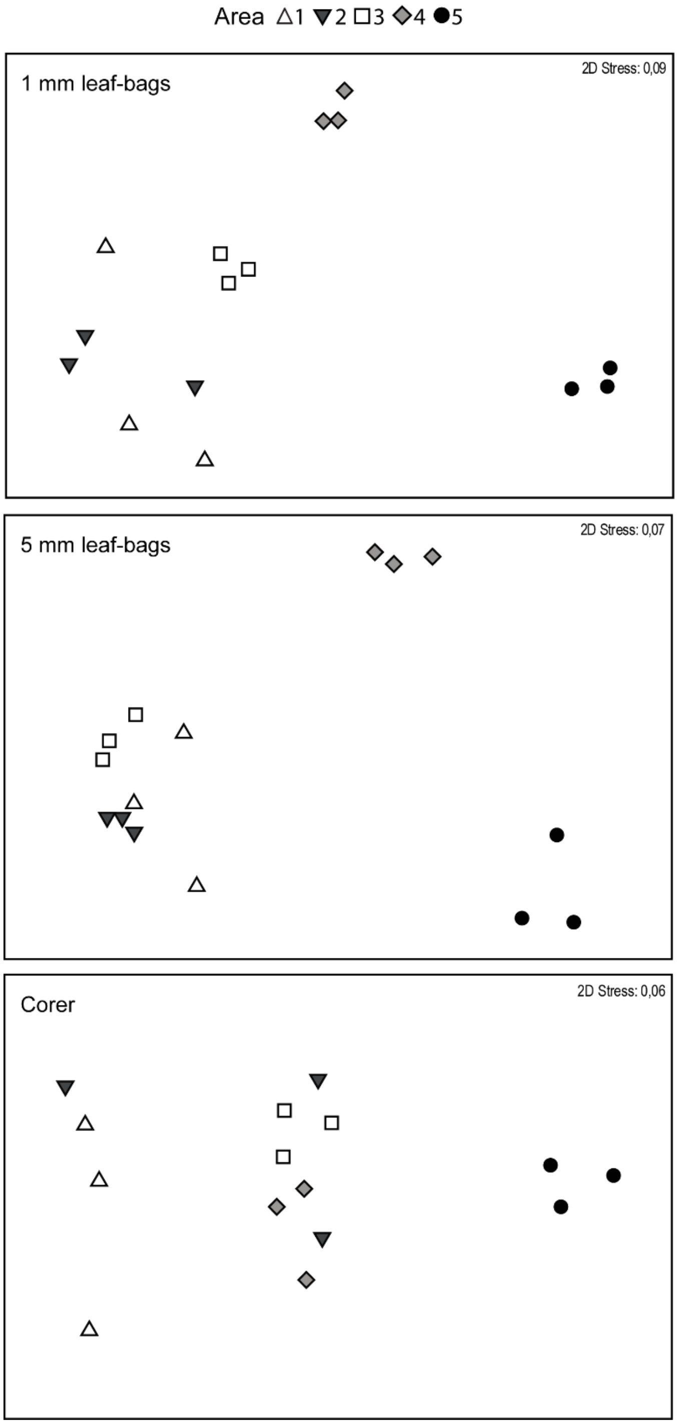

Figure 4.3 Ordination diagram (NMDS) of the measured benthic macroinvertebrates data obtained in the 1 mm and 5 mm leaf-bags with *Phragmites australis* and the corer samples along the full salinity gradient (areas 1 to 5). The five study areas are represented by symbols, without discriminating the sampling sites within each area.

Table 4.1 PERMANOVA main test *F* values (with associated significance in brackets) and t-values pairwise comparisons (with associated significance in brackets) between areas for the abundance and taxa richness for 1 mm and 5 mm leaf-bags and the corer samples along the full salinity gradient. ns - stands for non-significant ($p > 0.05$), * $p \leq 0.05$, ** $p \leq 0.01$; *** $p \leq 0.001$. Area 1 - euhaline; area 2 - polyhaline; area 3 - mesohaline; area 4 - oligohaline; and area 5 - limnetic.

	1mm	**5mm**	**corer**
Main test	12.6490***	9.1357***	7.4209***
Pairwise comparisons			
1, 2	1.2932 (ns)	1.5243 (ns)	1.5768 (ns)
1, 3	2.2187**	1.8481*	2.0052*
1, 4	3.2413**	2.6329*	2.242**
1, 5	3.3868**	2.2591**	2.8597**
2, 3	2.3465**	3.5979**	1.1398 (ns)
2, 4	4.7842**	5.4125***	2.6421**
2, 5	4.7239***	3.5366***	3.7034***
3, 4	4.5177***	4.9668***	2.7268*
3, 5	5.3735***	3.4677***	4.5048***
4, 5	6.5532***	3.0637**	4.6019**

4.4.2 Body size descriptors

An overall analysis of the sub-set of specimens and taxa measured was done for the 1 mm and 5 mm leaf-bags and corer samples. The macroinvertebrate size spectra were divided into the 6 body size classes (C1–C6) used to calculate the ISS index (Basset el al., 2012) by clustering the original abundance octaves into groups of three (Table 4.2). Along the salinity gradient any particular trend was observed in the macrobenthic abundance distribution by the 6 body size classes for all the samplers (cf. Table 4.2). In the 1 mm leaf-bags the euhaline, oligohaline and limnetic areas were characterized by spcimens belonging to size class C3 and the poly- and mesohaline areas by specimens from size class C4. In the 5 mm leaf-bags all the areas, excepting the limnetic, were characterized by highest abundances of specimens belonging to size class C4. In the limnetic area size class C3 presented the highest abundance. In the corer samples, the euhaline, polyhaline and mesohaline areas were characterized by individuals from size classes C3, C4 and C5, respectively, while the oligohaline and limnetic areas were dominated by specimens belonging to the size class C3 (cf. Table 4.2).

Table 4.2 Size distribution of the macroinvertebrates abundance in each body size class along the full salinity gradient for the 1 mm and 5 mm leaf-bags and corer samples. Area 1 - euhaline; area 2 - polyhaline; area 3 - mesohaline; area 4 - oligohaline; area 5 – limnetic. The boxes correspond to the body size class with the highest abundance per salinity area.

		Body size classes					
	Area	1	2	3	4	5	6
1mm	1	0	2	143	74	7	0
	2	0	5	216	237	19	0
	3	0	10	347	474	36	7
	4	0	81	1148	363	19	1
	5	2	406	2290	803	66	1
5mm	1	0	5	69	88	22	16
	2	0	6	91	178	125	6
	3	0	2	171	643	64	19
	4	0	26	780	913	264	2
	5	0	141	537	378	101	6
Corer samples	1	0	1	78	27	33	12
	2	0	1	7	14	2	3
	3	0	0	13	9	14	13
	4	0	9	96	57	5	7
	5	0	40	542	49	10	12

Considering the different morphology of the main groups that constitute the macroinvertebrate benthic community, Annelids, Arthropods and Molluscs, the length, weight and length-weight relationships were studied separately for each group. The statistical analysis of length and weight of all groups is shown in Table 4.3. Significant differences were found in the Annelids length between the euhaline and oligohaline areas for the 1 mm leaf-bag samples and between the euhaline and the mesohaline, oligohaline and limnetic areas and between the mesohaline and the limnetic area for the 5 mm leaf-bag samples. In terms of weight, significant differences were found in the 1 mm leaf-bags between the polyhaline and the oligohaline and the limnetic areas and between the oligohaline and the limnetic areas (cf. Table 4.3). For Arthropods, significant differences were found in the macroinvertebrates length associated with the corer samples between the oligohaline and limnetic areas (cf. Table 4.3). No significant differences were found in the length and weight of Molluscs along the salinity gradient for all samplers (cf. Table 4.3).

When the length and weight variability along the salinity gradient was tested using species with at least three distribution areas along the salinity gradient and five individuals per area, in the 1 mm leaf-bags, no significant differences were found in the length and weight of Chironomidae (areas 2, 4 and 5), *Gammarus insensibilis* (areas 1 to 3), *Melita palmata* (areas 1 to 3) and *Physa acuta* (areas 3 to 5) (length - Chironomidae: pseudo-F = 0.526, p = 0.618; *Gammarus insensibilis*: pseudo-F = 0.437, p = 0.661; *Melita palmata*: pseudo-F = 0.219, p = 0.811; and *Physa acuta*: pseudo-F = 1.406, p = 0.297; weight – Chironomidae: pseudo-F = 0.161, p = 0.855; *Gammarus insensibilis:* pseudo-F = 1.041, p = 0.396; *Melita palmate*: pseudo-F = 1.368, p = 0.323; *Physa acuta:* pseudo-F = 1.143, p = 0.359). Significant differences were found in the length and weight of the Arthropod *Microdeutopus gryllotalpa* (areas 1 to 3) (length: pseudo-F = 3.771, p = 0.045; weight: pseudo-F = 6.594, p = 0.008) between the euhaline and the polyhaline areas (1,2 - length: t = 1.461, p = 0.001; weight: t = 4.482, p = 0.001), with smaller individuals in the euhaline area (Table 4.6). In the 5 mm leaf-bags no significant differences were found in the length of the Arthropods *Gammarus insensibilis* (areas 1 to 3) and *Melita palmata* (areas 1 to 4) along the salinity gradient (*G. insensibilis*: pseudo-F = 0.494, p = 0.635; *M. palmata:* pseudo-F = 2.605, p = 0.122). In terms of weight, significant differences were found for the Arthropod *M. palmata* (pseudo-F = 12.461, p = 0.0004) between the oligohaline and euhaline, polyhaline and mesohaline areas (1,4: t = 4.315, p = 0.003 ; 2,4: t = 3.961, p = 0.001; 3,4: t = 5.660, p = 0.001) with smaller individuals in the oligohaline area (Table 4.6). In the corer samples, no significant difference were found in the length and weight of the Annelid *Streblospio shrubsolii* along its distribution areas (areas 1, 3 and 4) (length: pseudo-F = 0.196, p = 0.831; weight: pseudo-F = 0.395, p = 0.693).

Table 4.3 PERMANOVA main test *F* values (with associated significance in brackets) and t-values pairwise comparisons with associated significance between areas for the length and weight of Annelids, Arthropods and Molluscs associated with the 1 mm an 5 mm leaf-bags and corer samples along the salinity gradient (areas 1 to 5). ns - non-significant ($p > 0.05$); * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

		Length (mm)			**Weight (mg)**		
		1mm	**5mm**	**corer**	**1mm**	**5mm**	**corer**
Annelids	Main test	4.213*	5.640*	1.927 (ns)	3.704*	0.667 (ns)	1.245 (ns)
	Pairwise comparisons						
	1, 2	2.121 (ns)	1.606 (ns)		1.696 (ns)		
	1, 3	2.593 (ns)	6.112*		0.292 (ns)		
	1, 4	2.722*	2.980*		2.019 (ns)		
	1, 5	1.794 (ns)	3.284*		1.578 (ns)		
	2, 3	1.120 (ns)	1.400 (ns)		0.971 (ns)		
	2, 4	1.191 (ns)	0.184 (ns)		8.056***		
	2, 5	1.980 (ns)	0.366 (ns)		8.816***		
	3, 4	0.376 (ns)	2.133 (ns)		1.295 (ns)		
	3, 5	0.722 (ns)	11.311**		1.701 (ns)		
	4, 5	1.152 (ns)	1.054 (ns)		9.530***		
Arthropods	Main test	1.059 (ns)	3.006 (ns)	5.520*	0.951 (ns)	0.894 (ns)	0.777 (ns)
	Pairwise comparisons						
	1, 2			0.119 (ns)			
	1, 3			1.219 (ns)			
	1, 4			0.731 (ns)			
	1, 5			1.211 (ns)			
	2, 3			0.590 (ns)			
	2, 4			1.455 (ns)			
	2, 5			1.437 (ns)			
	3, 4			1.400 (ns)			
	3, 5			0.842 (ns)			
	4, 5			4.763*			
Molluscs	Main test	2.070 (ns)	0.984 (ns)	1.900 (ns)	0.273 (ns)	0.561 (ns)	1.614 (ns)

The length-weight relationships for Annelids, Arthropods and Molluscs in each salinity area are shown in Figure 4.4 for the 1 mm and 5 mm leaf-bags and corer samples. The length-mass relationships of all groups were always significant, excepting for Annelids from the corer samples in the polyhaline area (Table 4.4).

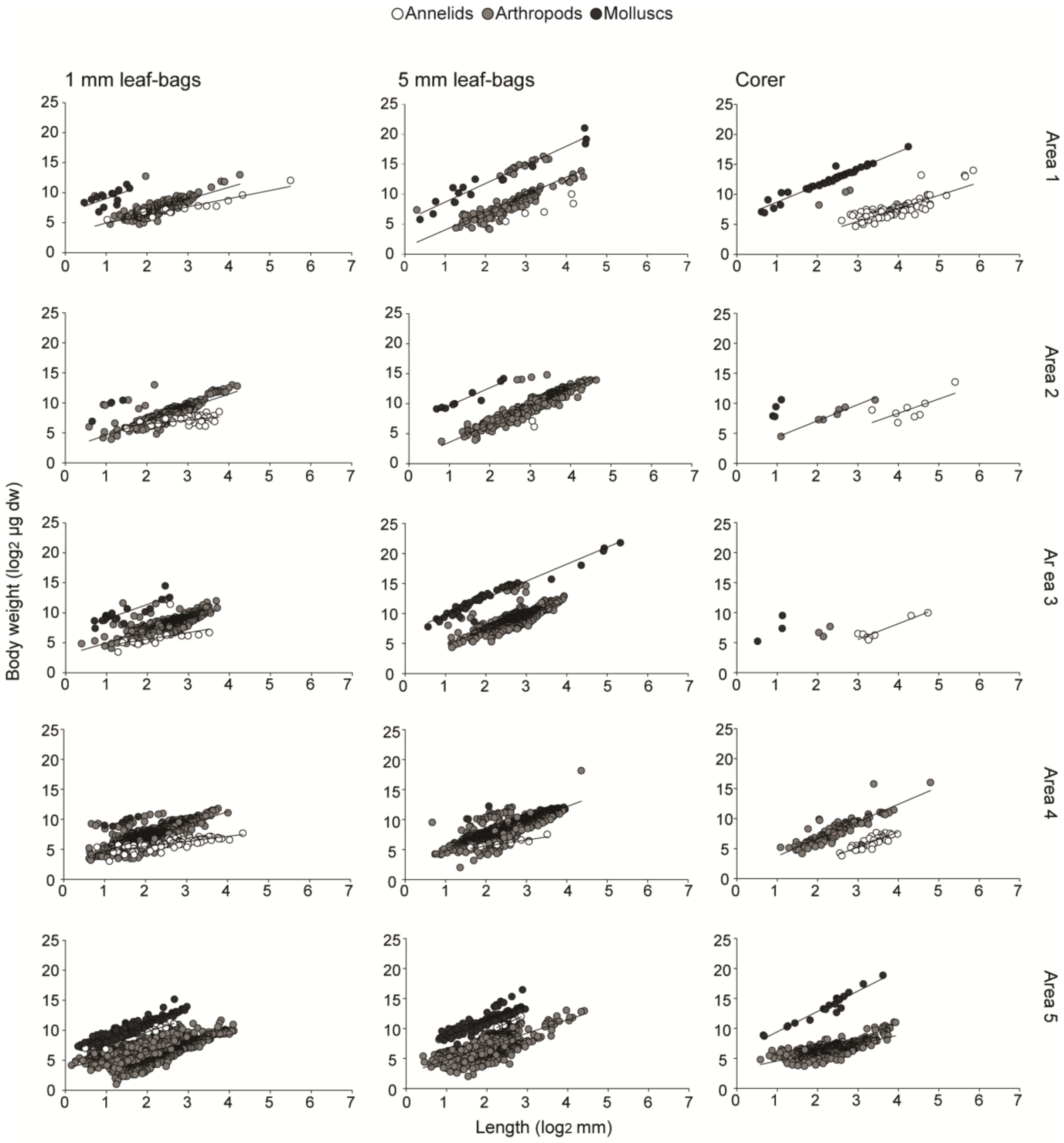

Figure 4.4 Length-weight relationships of Annelids, Arthropods and Molluscs along the salinity gradient (areas 1 to 5) for the 1 mm and 5 mm leaf-bags and corer samples. Each symbol represents an individual. Lines represent the length-weight linear regression.

Table 4.4 Parameters of the length-weight regressions (Dry weight = aLength^{b}) and PERMANOVA main test F-values and associated significance (ns - stands for non-significant ($p > 0.05$), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$) for Annelids, Arthropods and Molluscs with at least five individuals in the 1 mm and 5 mm leaf-bags and the corer samples along the salinity gradient (areas 1 to 5). b - regression slope; r^2 - coefficient of determination; n - number of individuals. Area 1 - euhaline; area 2 - polyhaline; area 3 - mesohaline; area 4 - oligohaline; and area 5 – limnetic. n.a. – not available.

		Annelids				**Arthropods**				**Molluscs**			
	Area	**b**	**r²**	**n**	**F-value**	**b**	**r²**	**n**	**F-value**	**b**	**r²**	**n**	**F-value**
1 mm	1	1.363	0.899	13	98.311***	1.980	0.586	200	183.928***	2.414	0.376	13	6.710*
	2	0.669	0.314	32	13.798*	2.292	0.681	439	933.161***	n.a	n.a	4	n.a
	3	1.108	0.247	18	5.232*	1.994	0.647	745	1598.775***	2.439	0.759	22	63.474***
	4	0.837	0.595	76	108.522***	2.196	0.641	1500	9403.158***	2.077	0.882	6	30.826**
	5	2.169	0.942	12	162.172***	1.373	0.299	3290	717.924***	2.459	0.868	230	1489.119***
5 mm	1	2.203	0.850	5	16.306*	2.935	0.511	179	183.928***	3.100	0.943	16	230.529***
	2	n.a.	n.a	2	n.a	2.951	0.874	394	933.161***	2.956	0.899	9	60.839***
	3	-	-	-	-	2.421	0.660	826	1598.775***	2.828	0.951	55	1005.918***
	4	0.999	0.898	9	60.360***	2.494	0.827	1971	403.158***	n.a	n.a	2	n.a
	5	2.978	0.968	5	127.727**	2.244	0.420	993	717.824***	2.555	0.768	139	450.733***
Corer	1	2.161	0.693	95	197.009***	n.a	n.a	3	n.a	2.786	0.952	47	1113.664***
	2	2.382	0.492	8	5.729 (ns)	2.704	0.972	7	182.045***	n.a	n.a	4	n.a
	3	2.613	0.882	8	41.973**	n.a	n.a	3	n.a	n.a	n.a	3	n.a
	4	2.532	0.737	37	97.962***	2.884	0.804	126	508.388***	-	-	-	-
	5	-	-	-	-	3.133	0.583	624	868.646***	3.404	0.940	21	293.548***

The linear regression slopes of Annelids, Arthropods and Molluscs were tested under the null hypothesis of no significant differences in the linear regression of each group along the salinity gradient for all the samplers and significant differences were found mainly between the upstream areas (oligohaline and limnetic) and the other salinity areas for all groups and also between the oligohaline and the limnetic areas mainly for Arthropods in the leaf-bags and corer samples and for Annelids in the 1 mm leaf-bags (Table 4.5).

Table 4.5 PERMANOVA main test F values and associated significance (ns - stands for non-significant (*p* > 0.05), * *p* ≤ 0.05, ** *p* ≤ 0.01, *** *p* ≤ 0.001) between the linear regressions slopes (b) along the salinity gradient (areas 1 to 5) for Annelids, Arthropods and Molluscs with 5 or more individuals sampled in the 1 mm and 5 mm leaf-bags and corer samples. Area 1 - euhaline; area 2 - polyhaline; area 3 - mesohaline; area 4 - oligohaline; and area 5 – limnetic. n.a. – not available.

		1,2	**1,3**	**1,4**	**1,5**	**2,3**	**2,4**	**2,5**	**3,4**	**3,5**	**4,5**
1mm	Annelids	5.448*	0.006 (ns)	13.25**	2.404 (ns)	0.738 (ns)	0.81 (ns)	12.186**	0.557*	7.371*	9.072*
	Arthropods	5.955*	0.021 (ns)	2.568 (ns)	5.517*	3.407 (ns)	0 (ns)	24.4 ***	3.385 (ns)	21.99***	72.62***
	Molluscs	n.a.	1.116 (ns)	0.004 (ns)	2.238 (ns)	n.a.	n.a.	n.a.	0.014 (ns)	3.502 (ns)	0.159 (ns)
5mm	Annelids	n.a.	n.a.	48.064**	209.1*	n.a.	n.a.	n.a.	n.a.	n.a.	3.106 (ns)
	Arthropods	0.156 (ns)	2.992 (ns)	4.116*	4.375*	18.18***	26.74***	17.833***	2.2 (ns)	0.584 (ns)	4.655*
	Molluscs	0.032 (ns)	2.721 (ns)	n.a.	8.946**	0.33 (ns)	n.a.	0.001 (ns)	n.a.	0.995 (ns)	n.a.
corer	Annelids	2.751 (ns)	0.701 (ns)	0.277 (ns)	n.a.	0.775 (ns)	5.363*	n.a.	0.154 (ns)	n.a.	n.a.
	Arthropods	n.a.	n.a.	n.a.	n.a.	n.a.	0.003 (ns)	9.076**	n.a.	n.a.	91.19***
	Molluscs	n.a.	n.a.	n.a.	2.378 (ns)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

The same analysis was made considering the species with at least three distribution areas and five individuals per area. In the 1 mm leaf-bags, the length-weight relationships of the Arthropods Chironomidae, *Gammarus chevreuxi*, *Melita palmata*, *Microdeutopus gryllotalpa* and the Mollusc *Physa acuta* were significant in all their distribution areas (Table 4.6). When the length-weight relationship of each taxon was tested between areas, no significant differences were found (Table 4.7). In the 5 mm leaf-bags, the length-weight relationships of the Arthropods *Gammarus insensibilis* and *Melita palmata* was significant in all the salinity areas (Table 4.6). Between areas, for *G. insensibilis* significant differences were found between the euhaline and the poly- and mesohaline areas and for *M. palmata*, significant differences were found between the euhaline and the meso- and oligohaline areas (cf. Table 4.7). The regression slope value for the *G. insensibilis* increased from the euhaline to the mesohaline areas (cf. Table 4.6). For the *M. palmata*, an opposite trend was observed. In this case, the regression slope value decreased from the euhaline to the oligohaline areas (cf. Table 4.6). In the corer samples, the Annelid *Streblospio shrubsolii* showed a significant length-weight relationship in the euhaline and oligohaline but not in the mesohaline area. In this case, no significant differences were found in the length-weight relationship between the euhaline and oligohaline areas (cf. Table 4.7).

Table 4.6 Parameters of the length-weight regressions (Dry weight = $aLength^b$) and PERMANOVA main test F-values and associated significance (ns - stands for non-significant ($p > 0.05$), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$) for species with at least thee distribution areas along the salinity gradient sampled in the 1 mm and 5 mm leaf-bags and corer samples along the salinity gradient. b - regression slope; r^2 - coefficient of determination; n - number of individuals; L – average length; W – average weight. Area 1 - euhaline; area 2 - polyhaline; area 3 - mesohaline; area 4 - oligohaline; and area 5 – limnetic.

Taxon	Area	b	r²	n	L (mm)	W (mg)	*F*-value
1 mm leaf-bags							
Chironomidae	2	6.057	0.9777	5	5.72	0.109	45.563**
	4	2.092	0.4461	43	5.12	0.095	43.789***
	5	2.057	0.5642	1940	5.12	0.109	3730.762***
Gammarus insensibilis	1	2.45	0.909	99	5.69	0.432	295.112***
	2	2.663	0.848	147	6.59	0.736	591.741***
	3	3.072	0.8359	35	7.93	0.973	112.258***
Melita palmata	1	2.244	0.5858	52	6.03	0.311	57.677***
	2	2.538	0.7237	208	6.47	0.403	395.872***
	3	2.248	0.7383	573	6.39	0.343	1204.026***
Microdeutopus gryllotalpa	1	2.978	0.5922	10	4.13	0.106	25.814**
	2	2.327	0.7676	64	4.68	0.154	120.619***
	3	1.573	0.5747	11	4.69	0.138	5.689*
Physa acuta	3	2.936	0.9996	5	3.46	2.387	255.323**
	4	2.077	0.8821	6	2.75	0.865	43.703**
	5	1.637	0.5281	161	3.45	1.960	223.45***
5 mm leaf-bags							
Gammarus insensibilis	1	2.821	0.9384	54	8.13	1.516	117.805***
	2	3.046	0.9315	311	9.90	2.144	816.325***
	3	3.185	0.8832	73	9.28	1.749	259.248***
Melita palmata	1	3.081	0.7605	72	7.23	0.592	154.563***
	2	2.867	0.8106	57	6.64	0.487	180.415***
	3	2.404	0.7897	702	6.56	0.529	1602.152***
	4	1.991	0.6814	9	5.90	0.260	9.218*
Corer							
Streblospio shrubsolii	1	1.255	0.3790	12	9.15	0.100	10.573**
	3	10.307	0.2188	6	9.343	0.067	1.252 (ns)
	4	2.532	0.7370	37	10.23	0.084	62.773***

Table 4.7 PERMANOVA main test *F* values and associated significance (ns - stands for non-significant ($p > 0.05$), * $p \leq 0.05$, ** $p \leq 0.01$, ** $p \leq 0.001$) between the linear regressions slopes (b) along the salinity gradient (areas 1 to 5) for species with at least three distribution areas sampled in the 1 mm and 5 mm leaf-bags and the sediment. Area 1 - euhaline; area 2 - polyhaline; area 3 - mesohaline; area 4 - oligohaline; and area 5 – limnetic. n.a. – not available.

	Taxon	**1,2**	**1,3**	**1,4**	**2,3**	**2,4**	**2,5**	**3,4**	**3,5**	**4,5**
1 mm	Chironomidae	n.a.	n.a.	n.a.	n.a.	0,649 (ns)	0,121 (ns)	n.a.	n.a.	0,875 (ns)
	Gammarus chevreuxi	1,808 (ns)	0,008 (ns)	n.a.	0,357 (ns)	n.a.	n.a.	n.a.	n.a.	n.a.
	Melita palmata	3,022 (ns)	0,047 (ns)	n.a.	1,309 (ns)	n.a.	n.a.	n.a.	n.a.	n.a.
	Microdeutopus gryllotalpa	0,049 (ns)	0,410 (ns)	n.a.	0,476 (ns)	n.a.	n.a.	n.a.	n.a.	n.a.
	Physa acuta	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	33,332 (ns)	0,066 (ns)	0,203 (ns)
5 mm	*Gammarus insensibilis*	7,965**	10,204**	n.a.	0,019 (ns)	n.a.	n.a.	n.a.	n.a.	n.a.
	Melita palmata	0,164 (ns)	4,301*	5,675*	0,121 (ns)	2,082 (ns)	n.a.	2,082 (ns)	n.a.	n.a.
corer	*Streblospio shrubsolii*	n.a.	n.a.	0,616 (ns)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

4.5 Discussion

Salinity is one of the most important factors conditioning benthic macroinvertebrates species richness and distribution. This was observed in several estuaries all over the world (Quintino and Rodrigues, 1989; Rodrigues and Quintino, 1993; Uwadiae, 2009; Rodrigues et al., 2006; Whitfield et al., 2012) and also in Mira Channel as was demosntrated by Quintino et al. (2011) and Lopes et al. (2013). These studies carried out in Mira Channel showed the benthic succession from the marine to the freshwater areas using the leaf-bag technique with 5 mm leaf-bags and different substrates (*Phragmites australis*, *Fucus vesiculosus* and a control substrate) and corer samples. In this study, in the 5 mm leaf-bags, despite the exclusion of some individuals and even some taxa from the original data, due to the impossibility of measure them, the sub-set of individuals measured were able to show equally well the benthic succession from the marine to the freshwater areas showed by Quintino et al. (2011) when considered all the macrobenthic fauna sampled. In this case, the dominant species remained the same of the original data in each salinity area. In the 1 mm leaf-bags, the measured specimens also represented the salinity gradient showed by the original data. However, in the original data, the Annelids *Mediomastus fragilis*, *Capitella* spp. and *Hediste diversicolor* were the second dominant species in the euhaline, polyhaline and mesohaline areas, respectively, and these species were not measured. Thus, in this case, the dominant species changed. In the corer samples, due to the dominance of Annelids, the loss of information was higher. Despite that, the measured specimens were able to show the benthic succession from the marine to the freshwater areas observed by Quintino et al. (2011). Thus, in the case of the 1 mm leaf-bag and, mainly, in the corer samples, despite both show the species sucession along the salinity gradient, the representativeness of the measured individuals was affected as some dominant species were not measured. Thus, the macrobenthic community used in the body size study was compromised and the results should be considered with some precautions.

The body size abundance distribution of the benthic macroinvertebrates by the ISS size classes along the salinity gradient did not show any particular trend from the marine to the freshwater environment for the leaf-bag and corer samples. The non-taxonomic index ISS is based on the macrobenthic abundance distribution by the body size classes, on the the sensitivity/tolerance of macroinvertebrate body size classes to stress and the taxa richness correction factor (Basset et al., 2012). Thus, the fact that the body size abundance distribution of the benthic macroinvertebrates by the ISS size classes in a salinity range from 0 to 35 did not show any particular trend, suggests that these salinities will not act like a masking factor when this index is applied in areas affected by an arthropogenic stress. The same did not happen in hypersaline ecosystems as was observed by Barbone et al. (2007). In this case, the size structure of the sediment benthic macroinvertebrates was influenced by high salinities, being smaller under higher salinity condition.

Along the salinity gradient significant differences were found in the length and weight of Annelids and Arthropods. These differences occur between the upstream (oligohaline and limnetic) and the downstream (euhaline and polyhaline) areas of the channel, suggesting that these difeferences should be related with the species sucession along the estuarine gradient. At the species level, significant differences were found in the length and weight of the Arthropod *Microdeutopus gryllotalpa* between the euhaline and polyhaline areas in the 1mm leaf-bags. In the 5 mm leaf-bags the weight of the Arthropod *Melita palmata* was found significantly different between the oligohaline and the other areas. In both cases, the average length and weight of the individuals was lower in the areas with the lowest abundances revealing that the euhaline and oligohaline areas are not the preferencial distribution areas of the *M. gryllotalpa* and *M. palmata*, respectively, and that salinity affects their growth. This explanation is reinforced by the fact that, no significant differences were found in the weight and length of both species between areas with higher abundances.

Transitional waters are very complex ecosystems and is known that water temperature (West et al., 1997), resource availability (Basset and Glazier, 1995) competitive interactions among species (Basset and Rossi, 1990) and chemical pollution (Basset, 1993) affect the body size and body condition of individuals, potentially leading to bias in length-weight models. Most of the available information on length-weight relationships in transitional waters comes from studies dealing with life cycles (Abrantes et al., 1999; Perez et al., 2007), somatic growth and/or population growth rates (Bachelet, 1980; Kater et al., 2008) and production of benthic macrofauna (Arias and Drake, 1994; Gillet and Torresani, 2003). However any information was found in what concens to the length-weigh relationships in macroinvertebrate communities along a full salinity gradient in the same system. In the 1 mm and 5 mm leaf-bags and the corer samples, significant differences were found in the length-weight relationship of Annelids, Arthropods and Molluscs along the salinity gradient. These differences occurred essentially between the upstream areas (oligohaline and limnetic) and the other salinity areas, which should be related with the species succession along the salinity gradient. A clear example that could explain the significant differences in the length-weight relationships of Arthropods between the upstream areas (oligohaline and limnetic) and the other part of the Mira Channels is the presence of Chironomidae and other insects larvae in the upstream areas. Larvae have an elongated shape, similar to Annelids, inducing an alteration in the length-weight relationship of Arthropods in this particular areas, comparatively to the other areas. When this analysis was carried out considering species individually, no significant differences were found in the length-weight relationships between areas for the Arthropods Chironomidae, *Gammarus chevreuxi*, *Melita palmata*, *Microdeutopus gryllotalpa* and the Mollusk *Physa acuta* sampled in the 1 mm leaf-bags, suggesting that salinity is not interfering in the corporeal condition of these species. However, in the 5 mm leaf-bags, the length-weight relationships of the Arthropods *Gammarus insensibilis* and *Melita palmata* showed to be influenced by the salinity gradient with the regression slope values of the *Gammarus insensibilis* increasing from the euhaline to the mesohaline areas and for the *Melita palmata* the regression slope values showed and opposite trend, decreasing from the euhaline to the oligohaline areas. These differeces in the regression slopes of both species should be related with the peferencial distribution areas of each species, indicating that salinity was interferaing in the corporeal condition of these species. The fact that this is observed in the 5 mm leaf-bags and not in the 1 mm leaf-bags should be related with the fact that the 1 mm mesh-size bags could could confine the circulation of the spcimens, acting like a trap, which interfears with the corporeal condition of these species. In the 5 mm mesh-bags the individuals circulate freely, representing in a better way the body size structure of these species in the different salinity areas. Thus, the use of leaf-bags with 1 mm mesh-size to study the body size structure of the community should be done with some precautions. In the corer sample, the length-weight relationship of the Annelid *Streblospio shrubsolii* did not show significant diffeneces between the euhaline and the oligohaline areas.

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4.6 Supporting information

Table 4.A Relative abundance (A) per taxon of the original samples collected by the 1 mm leaf-bags with *Phragmites australis* leaves along the full salinity gradient (area 1 to 5) in the Mira Channel ranked from highest to lowest. Area 1 - euhaline; area 2 - polyhaline; area 3 - mesohaline; area 4 - oligohaline; and area 5 - limnetic. The letters before the species/taxon name refer to the Phylum: An - Annelids; Ar - Arthropods; M – Molluscs ans O – Others.

Area 1			Area 2			Area 3			Area 4			Area 5		
	Taxon	A (%)		Taxon	A (%)		Taxon	A (%)		Taxon	A (%)		Taxon	A (%)
Ar	*Gammarus insensibilis*	26.1	Ar	*Melita palmata*	25.5	Ar	*Melita palmata*	63.3	Ar	*Leptocheirus pilosus*	50.7	Ar	Chironomidae	44.9
A	*Mediomastus fragilis*	16.9	A	*Capitella* spp.	19.3	A	*Hediste diversicolor*	9.1	Ar	*Gammarus chevreuxi*	17.3	A	Oligochaeta	23.7
Ar	*Melita palmata*	12.9	Ar	*Gammarus insensibilis*	18.8	Ar	*Leptocheirus pilosus*	8.1	Ar	*Corophium multisetosum*	8.6	Ar	Cladocera	7.7
A	*Capitella spp.*	9.5	A	Oligochaeta	10.1	A	*Polydora cornuta*	4.8	A	*Streblospio shrubsolii*	6.8	Ar	Empididae	7.3
A	Nemertea	6.0	Ar	*Microdeutopus gryllotalpa*	7.0	Ar	*Gammarus insensibilis*	3.3	A	Oligochaeta	5.2	Ar	Caenidae	6.3
Ar	*Heterotanais oerstedti*	5.0	A	*Tharyx marioni*	6.6	M	*Peringia ulvae*	1.9	Ar	Chironomidae	2.8	M	*Physa acuta*	5.1
Ar	*Gammarus chevreuxi*	3.5	A	*Mediomastus fragilis*	5.8	A	*Capitella* spp.	1.6	Ar	*Cyathura carinata*	1.8	Ar	Hydroptilidae	1.0
M	*Peringia ulvae*	3.5	A	Nemertea	2.0	A	*Streblospio shrubsolii*	1.1	A	*Hediste diversicolor*	1.7	M	*Limnea peregra*	0.6
Ar	*Microdeutopus gryllotalpa*	2.5	A	*Streblospio shrubsolii*	0.8	A	Nemertea	1.1	A	*Alkmaria romijni*	1.6	M	*Planorbis corneus*	0.5
A	*Spio filicornis*	1.5	Ar	Chironomidae	0.4	A	Oligochaeta	0.8	Ar	*Heterotanais oerstedii*	1.4	O	Anthozoa	0.5
A	Oligochaeta	1.2	M	*Peringia ulvae*	0.4	Ar	*Lekanesphaera levii*	0.8	Ar	*Lekanesphaera hookeri*	1.0	O	Turbellaria	0.3
A	*Tharyx marioni*	1.2	Ar	*Monocorophium acherusicum*	0.4	Ar	*Monocorophium acherusicum*	0.6	O	Phoronida	0.4	Ar	Psychodidae	0.3
A	Nematoda	1.2	Ar	*Pholoe inornata*	0.4	Ar	*Idotea sp.*	0.6	Ar	Caenidae	0.2	Ar	Simuliidae	0.3
A	*Prionospio* sp.	1.0	A	*Scoloplos armiger*	0.4	Ar	*Microdeutopus gryllotalpa*	0.5	Ar	*Melita palmata*	0.2	A	Hirudinea	0.2
A	*Microphthalmus* sp.	1.0	Ar	*Monocorophium insidiosum*	0.3	A	*Alkmaria romijni*	0.5	Ar	Empididae	0.1	M	*Corbicula fluminea*	0.2
Ar	Chironomidae	0.7	Ar	*Gammarus chevreuxi*	0.2	M	*Scrobicularia plana*	0.5	M	*Physa acuta*	0.1	M	*Pomatopyrgus jenkinsi*	0.2
A	*Malacoceros* sp.	0.7	A	*Hediste diversicolor*	0.2	Ar	Chironomidae	0.3	Ar	*Gammarus insensibilis*	0.1	Ar	Ostracoda	0.2
A	*Owenia fusiformis*	0.7	A	Nematoda	0.2	Ar	*Heterotanais oerstedii*	0.2	Ar	Simuliidae	0.1	Ar	Elmidae	0.2
M	*Abra alba*	0.7	Ar	*Idotea* sp.	0.2	Ar	*Gammarus chevreuxi*	0.1	M	*Ancylus lacustris*	0.1	Ar	Baetidae	0.1
O	*Acrocnida brachiata*	0.7	Ar	*Lekanesphaera levii*	0.1	Ar	*Corophium multisetosum*	0.1	A	*Ampharete grubei*	0.1	Ar	Tipulidae sp2	0.1
A	*Syllidia armata*	0.5	M	*Scrobicularia plana*	0.1	Ar	*Cyathura carinata*	0.1	A	*Melinna palmata*	0.1	Ar	Anthomyidae	0.1
M	*Cerastoderma edule*	0.5	A	*Prionospio* sp.	0.1	Ar	Simuliidae	0.1				A	Nematoda	0.1
O	Phoronida	0.2	A	*Glycera tridactyla*	0.1	A	Hirudinea	0.1				Ar	Copepoda	0.1
Ar	*Pholoe inornata*	0.2	M	*Mytilus galloprovincialis*	0.1	Ar	*Monocorophium insidiosum*	0.1				Ar	Hydrophilidae	0.1
A	*Glycera tridactyla*	0.2	Ar	*Carcinus maenas*	0.1	M	*Mytilus galloprovincialis*	0.1				Ar	*Janira maculosa*	0.05
A	*Eumida sanguinea*	0.2	Ar	*Jaera albifrons*	0.1	A	Sabellidae	0.1				M	*Scrobicularia plana*	0.03
A	*Harmothoe extenuata*	0.2	Ar	*Palaemon serratus*	0.1	Ar	Gnatiidae	0.1				Ar	Gyrinidae	0.03
A	*Spiophanes bombyx*	0.2										Ar	Hydropsychidae	0.03
M	*Angulus tenuis*	0.2										M	*Hydrobia ulvae*	0.02
M	*Venerupis pullastra*	0.2										Ar	*Idotea* sp.	0.02
Ar	*Caprella acanthifera*	0.2										M	*Ancylus lacustris*	0.02
												Ar	*Carcinus maenas*	0.02
												Ar	Brachycentridae	0.02
												Ar	Ceratopogonidae	0.02
												Ar	Dytiscidae	0.02
												Ar	Ecnomidae	0.02
												Ar	Ephydridae	0.02
												Ar	Sciomyzidae	0.02
												Ar	Trichoptera sp1	0.02

Table 4.B Total abundance (N) of the taxa not measured in the 1 mm and 5 mm leaf-bags and the sediment samples in the Mira Channel ranked from highest to lowest. Area 1 - euhaline; area 2 - polyhaline; area 3 - mesohaline; area 4 - oligohaline; and area 5 - limnetic. The letters before the species/taxon name refer to the Phylum: A - Annelids; Ar - Arthropods; M – Molluscs; O - Others.

1 mm leaf-bags			5 mm leaf-bags			Corer		
Taxa		N	Taxa		N	Taxa		N
A	Oligochaeta	1656	A	Oligochaeta	1288	A	Oligochaeta	150
A	*Capitella* spp.	115	O	Nemertea	24	A	*Hediste diversicolor*	58
A	*Hediste diversicolor*	98	A	*Hediste diversicolor*	23	O	Phoronida	48
A	*Mediomastus fragilis*	102	A	*Mediomastus fragilis*	17	A	*Tharyx marioni*	38
O	Nemertea	38	A	*Capitella* spp.	12	O	Nematoda	31
O	Nematoda	11	A	*Melinna palmata*	10	A	*Capitella* spp.	25
O	Phoronida	8	O	Nematoda	3	A	*Microphthalmus sp.*	11
A	*Microphthalmus* sp.	4	O	Phoronida	3	A	*Notomastus latericeus*	7
A	*Scoloplos armiger*	4	Ar	Hydrophilidae	2	O	Nemertea	4
O	Copepoda	4	A	*Alkmaria romijni*	1	A	*Scolelepis squamata*	3
Ar	Hydrophilidae	4	A	*Ampharete grubei*	1	M	*Nassarius reticulatus*	3
A	*Malacoceros* sp.	3	A	*Eumida sAguinea*	1	A	*Prionospio* sp.	2
O	*Acrocnida brachiata*	3	Ar	*RhithropAopeus harrisii*	1	Ar	*AmphibalAus improvisus*	2
A	*Syllidia armata*	2	O	*Ophiotrix fragilis*	1	M	*Kurtiella bidentata*	2
A	*Ampharete grubei*	1	M	*Nassarius reticulatus*	1	A	*Glycera fallax*	1
A	*EumidasAguinea*	1				A	*Malacoceros* sp.	1
A	*Harmothoe extenuata*	1				A	*Melinna palmata*	1
A	*Melinna palmata*	1				A	*Owenia fusiformis*	1
A	*SpiophAes bombyx*	1				A	*Parougia caeca*	1
Ar	*Caprella acAthifera*	1				M	*Ruditapes decussatus*	1
Ar	Dytiscidae	1						
Ar	Ephydridae	1						
M	*Tellina tenuis*	1						

Table 4.C Relative abundance (A) per taxon of the measured specimens collected by 1 mm leaf-bags with *Phragmites australis* leaves along the full salinity gradient (area 1 to 5) in the Mira Channel ranked from highest to lowest. Area 1 - euhaline; area 2 - polyhaline; area 3 - mesohaline; area 4 - oligohaline; and area 5 - limnetic. The letters before the species/taxon name refer to the Phylum: A - Annelids; Ar - Arthropods; M – Molluscs ans O – Others.

Area 1			Area 2			Area 3			Area 4			Area 5		
	Taxa	A%	Taxa		A%	Taxa		A%	Taxa		A%	Taxa		A%
Ar	*Gammarus insensibilis*	43.81	Ar	*Melita palmata*	43.79	Ar	*Melita palmata*	72.99	Ar	*Leptocheirus pilosus*	55.91	Ar	Chironomidae	54.37
Ar	*Melita palmata*	23.01	Ar	*Gammarus insensibilis*	30.95	Ar	*Leptocheirus pilosus*	11.21	Ar	*Gammarus chevreuxi*	21.16	Ar	Empididae	11.83
Ar	*Heterotanais oerstedtii*	8.41	Ar	*Microdeutopus gryllotalpa*	13.47	Ar	*Gammarus insensibilis*	4.46	Ar	*Corophium multisetosum*	9.35	Ar	Cladocera	11.32
Ar	*Gammarus chevreuxi*	6.19	A	*Tharyx marioni*	5.26	M	*Peringia ulvae*	1.78	A	*Streblospio shrubsolii*	3.54	Ar	Caenidae	9.78
Ar	*Microdeutopus gryllotalpa*	4.42	Ar	Chironomidae	1.05	Ar	*Microdeutopus gryllotalpa*	1.40	Ar	Chironomidae	2.72	Ar	Physa acuta	4.51
A	*Tharyx marioni*	1.77	Ar	*Monocorophium acherusicum*	0.84	Ar	*Streblospio shrubsolii*	1.40	Ar	*Cyathura carinata*	2.02	Ar	Hydroptilidae	1.93
M	*Venerupis pullastra*	1.77	A	*Streblospio shrubsolii*	0.84	Ar	*Idotea* sp.	1.27	Ar	*Heterotanais oerstedtii*	1.64	Ar	*Idotea* sp.	0.84
M	*Abra alba*	1.33	Ar	*Monocorophium insidiosum*	0.63	Ar	*Monocorophium acherusicum*	0.89	A	*Alkmaria romijni*	1.33	M	*Limnea peregra*	0.64
Ar	Chironomidae	1.33	Ar	Hydroptilidae	0.42	A	*Alkmaria romijni*	0.76	Ar	*Lekanesphaera hookeri*	1.07	O	Anthozoa	0.53
Ar	Hydroptilidae	1.33	M	*Peringia ulvae*	0.42	M	*Physa acuta*	0.64	M	*Physa acuta*	0.38	M	*Planorbis corneus*	0.50
M	*Peringia ulvae*	1.33	A	*Pholoe synophthalmica*	0.42	Ar	*Lekanesphaera hookeri*	0.51	Ar	Caenidae	0.25	Ar	Psychodidae	0.48
A	*Prionospio* sp.	1.33	A	*Alkmaria romijni*	0.21	Ar	*Lekanesphaera levii*	0.51	Ar	Hydroptilidae	0.19	O	Turbellaria	0.48
M	*Cerastoderma edule*	0.88	Ar	*Carcinus maenas*	0.21	Ar	Chironomidae	0.38	Ar	*Melita palmata*	0.19	Ar	Simuliidae	0.42
A	*Glycera tridactyla*	0.88	Ar	*Gammarus chevreuxi*	0.21	M	*Scrobicularia plana*	0.38	Ar	Empididae	0.06	M	*Corbicula fluminea*	0.34
A	*Spio filicornis*	0.88	Ar	*Idotea* sp.	0.21	Ar	*Heterotanais oerstedtii*	0.25	Ar	*Gammarus insensibilis*	0.06	A	Hirudinea	0.34
A	*Owenia fusiformis*	0.44	Ar	*Jaera albifrons*	0.21	Ar	*Corophium multisetosum*	0.13	Ar	Gyrinidae	0.06	M	*Pomatopyrgus jenski*	0.34
A	*Pholoe synophthalmica*	0.44	Ar	*Lekanesphaera hookeri*	0.21	Ar	*Cyathura carinata*	0.13	Ar	Simuliidae	0.06	Ar	Elmidae	0.28
M	*Tellina tenuis*	0.44	M	*Mytillus galloprovinciallis*	0.21	Ar	*Gammarus chevreuxi*	0.13				Ar	Ostracoda	0.22
			Ar	*Paleamon serratus*	0.21	Ar	Gnathiidae	0.13				Ar	Baetidae	0.14
			M	*Scrobicularia plana*	0.21	Ar	Hirudinea	0.13				Ar	Tipulidae	0.14
						Ar	Hydroptilidae	0.13				Ar	*Janira maculosa*	0.11
						Ar	*Monocorophium insidiosum*	0.13				Ar	Anthomyidae	0.08
						Ar	Ostracoda	0.13				M	*Peringia ulvae*	0.08
						Ar	Simuliidae	0.13				Ar	Hydropsychidae	0.06
												Ar	Branchycentridae	0.03
												Ar	Ceratopogonidae	0.03
												M	*Ancylus lacustris*	0.03
												Ar	Ecnomidae	0.03
												Ar	Gyrinidae	0.03
												Ar	Pephydridae	0.03
												Ar	Sciomyzidae	0.03
												Ar	Trichoptera sp.1	0.03

Table 4.D Relative abundance (A) per taxon of the measured specimens collected by 5 mm leaf-bags with *Phragmites australis* leaves along the full salinity gradient (area 1 to 5) in the Mira Channel ranked from highest to lowest. Area 1 - euhaline; area 2 - polyhaline; area 3 - mesohaline; area 4 - oligohaline; and area 5 - limnetic. The letters before the species/taxon name refer to the Phylum: A - Annelids; Ar - Arthropods; M – Molluscs ans O – Others.

Area 1			Area 2			Area 3			Area 4			Area 5		
Taxon		A%	Taxon		A%	Taxon		A%	Taxon		A%		Taxon	A%
Ar	*Melita palmata*	36.00	Ar	*Gammarus insensibilis*	76.79	Ar	*Melita palmata*	79.68	Ar	*Gammarus chevreuxi*	57.06	Ar	Chironomidae	35.34
Ar	*Gammarus insensibilis*	27.00	Ar	*Melita palmata*	14.07	Ar	*Gammarus insensibilis*	8.29	Ar	*Leptocheirus pilosus*	37.18	Ar	Caenidae	32.07
Ar	*Carcinus maenas*	9.50	Ar	*Microdeutopus gryllotalpa*	2.72	M	*Peringia ulvae*	5.33	Ar	*Lekanesphaera hookeri*	1.77	M	*Physa acuta*	9.72
Ar	*Gammarus chevreuxi*	6.50	Ar	*Idotea sp.*	2.22	Ar	*Idotea sp.*	2.84	Ar	Chironomidae	1.46	Ar	Cladocera	5.59
Ar	*Heterotanais oerstedii*	4.50	M	*Peringia ulvae*	1.73	Ar	*Carcinus maenas*	0.79	Ar	*Corophium multisetosum*	0.81	Ar	Empididae	5.33
M	*Scrobicularia plana*	4.50	Ar	*Carcinus maenas*	0.99	Ar	*Lekanesphaera levii*	0.68	Ar	*Melita palmata*	0.45	Ar	Hydroptilidae	3.96
Ar	*Microdeutopus gryllotalpa*	3.00	M	*Cerastoderma edule*	0.25	M	*Scrobicularia plana*	0.68	A	*Streblospio shrubsolii*	0.45	M	*Corbicula fluminea*	1.46
M	*Cerastoderma edule*	2.00	Ar	*Corophium acherusicum*	0.25	Ar	*Gammarus chevreuxi*	0.45	Ar	*Cyathura carinata*	0.20	O	Anthozoa	1.20
Ar	Chironomidae	2.00	A	*Eteone picta*	0.25	Ar	*Leptocheirus pilosus*	0.45	Ar	*Heterotanais oerstedii*	0.15	O	Turbellaria	0.95
M	*Peringia ulvae*	1.00	M	*Physa acuta*	0.25	Ar	Chloroperlidae	0.23	Ar	*Idotea* sp.	0.15	Ar	Gyrinidae	0.60
A	*Prionospio* sp.	1.00	Ar	*Praunus flexuosus*	0.25	M	*Planorbiscorneus*	0.23	Ar	Hydroptilidae	0.10	Ar	Hirudinea	0.52
Ar	*Cyathura carinata*	0.50	A	*Tharyx marioni*	0.25	Ar	Caenidae	0.11	Ar	*Carcinus maenas*	0.05	Ar	Hydropsychidae	0.52
A	*Glycera tridactyla*	0.50				Ar	Gnathiidae	0.11	M	*Peringia ulvae*	0.05	Ar	Anthomyidae	0.43
Ar	*Idotea* sp.	0.50				Ar	*Heterotanais oerstedii*	0.11	M	Physa acuta	0.05	M	*Planorbis corneus*	0.43
A	*Spio filicornis*	0.50							Ar	*Simuliidae*	0.05	Ar	*Idotea* sp.	0.34
A	*Tharyx marioni*	0.50										Ar	Tipulidae	0.26
M	*Venerupis pullastra*	0.50										Ar	Elmidae	0.17
												M	*Peringia ulvae*	0.17
												Ar	Baetidae	0.09
												M	*Ancylus lacustris*	0.09
												Ar	Coenagrionidae	0.09
												Ar	Coriixidae	0.09
												Ar	*Diptera* sp.	0.09
												Ar	*Gammarus chevreuxi*	0.09
												Ar	Haliplidae	0.09
												Ar	Janira maculosa	0.09
												M	*Potamopyrgus jenskei*	0.09
												Ar	Psychodidae	0.09
												Ar	Simuliidae	0.09

Table 4.E Relative abundance (A) per taxon of the measured specimens collected by the sediment samples along the full salinity gradient (area 1 to 5) in the Mira Channel ranked from highest to lowest. Area 1 - euhaline; area 2 - polyhaline; area 3 - mesohaline; area 4 - oligohaline; and area 5 - limnetic. The letters before the species/taxon name refer to the Phylum: A - Annelids; Ar - Arthropods; M – Molluscs ans O – Others.

Area 1			Area 2			Area 3			Area 4			Area 5		
Taxon		A%	Taxon		A%	Taxon		A%	Taxon		A%	Taxon		A%
A	*Mediomastus fragilis*	52.3	A	*Mediomastus fragilis*	30.0	A	*Streblospio shrubsolii*	37.5	Ar	*Corophium multisetosum*	63.2	Ar	Chironomidae	94.7
M	*Angulus tenuis*	25.8	Ar	*Bathyporeia sarsi*	10.0	Ar	*Amphibalanus improvisus*	12.5	A	*Streblospio shrubsolii*	22.7	M	*Corbicula fluminea*	2.9
A	*Streblospio shrubsolii*	7.9	Ar	*Corophium multisetosum*	10.0	Ar	*Corophium multisetosum*	12.5	Ar	*Cyathura carinata*	11.7	Ar	Cladocera	1.2
M	*Scrobicularia plana*	2.6	M	*Scrobicularia plana*	10.0	A	*Scoloplos armiger*	12.5	Ar	*Lekanesphaera hookeri*	1.2	Ar	Caenidae	0.3
Ar	*Urothoe brevicornis*	2.0	O	Anthozoa	5.0	M	*Scrobicularia plana*	12.5	Ar	*Carcinus maenas*	0.6	M	*Physa acuta*	0.3
O	Anthozoa	1.3	M	*Cerastoderma edule*	5.0	Ar	*Melita palmata*	6.3	Ar	*Crangon crangon*	0.6	Ar	Baetidae	0.2
A	*Glycera tridactyla*	1.3	Ar	*Cyathura carinata*	5.0	M	*Peringia ulvae*	6.3				Ar	*Cyathura carinata*	0.2
A	*Nephtys hombergii*	1.3	A	*Glycera tridactyla*	5.0							Ar	Empididae	0.2
M	*Cerastoderma edule*	0.7	M	*Kurtiella bidentata*	5.0									
A	*Heteromastus filiformis*	0.7	Ar	*Melita palmata*	5.0									
M	*Kurtiella bidentata*	0.7	Ar	*Paramysis bacescoi*	5.0									
A	*Nephtys cirrosa*	0.7	A	*Scoloplos armiger*	5.0									
M	*Peringia ulvae*	0.7												
A	*Syllidia armata*	0.7												
O	Turbellaria	0.7												
M	*Venerupis decussata*	0.7												

Chapter 5. Variability of taxonomic and non-taxonomic biotic indices applied to macroinvertebrates sampled by leaf-bags and corers

5.1 Abstract

The variability of taxonomic and non-taxonomic indices was studied along a full salinity gradient in the most pristine channel of the Ria the Aveiro, the Mira Channel, using different sampling techniques, the leaf-bag technique with *Phragmites australis* and two mesh-sizes (1 mm and 5 mm) and a hand-held corer sediment samples. The AMBI and M-AMBI indices were applied to the original sampled data and to the sub-set of specimens and taxa that could be measured and used for the ISS index calculation.

No significant differences were found in the AMBI, M-AMBI and ISS values along the salinity gradient for all the samplers, considring all the data set and the sub-set of measured specimens. When the AMBI and M-AMBI calculated with the original data and measured specimens were compared in each salinity area, no significant differences were found for all samplers in each area, indicating that the exclusion of some specimens and even some taxa from the original data set did not affect the AMBI and M-AMBI results. The environmental quality of the 1mm and 5 mm leaf-bags samples along the salinity gradient was the same when all the macrobethic sampled was used. Considering only the sub-set of measured specimens the environmental quality of the 5 mm leaf-bags decreased. The environmental quality of the corer samples was lower than that of the leaf-bags.

The ecological quality obtained with the ISS was higher than that obtained with the M-AMBI index in the leaf-bags. An opposite trend was observed in the corer samples which could be related to the low taxa richness and abundance of the corer samples. The inability of the M-AMBI to classify the Mira Channel in such a good ecological quality like the ISS could be related to the fact that M-AMBI has been developed to coastal environments, in which the presence of species that naturally occur in transitional systems is indicative of environmental problems. It could also be related to the ISS being relatively recent, needing a better intercalibration with other indices.

Keywords: Taxonomic indices; Non-taxonomic indices; Leaf-bag technique; Corer samples; Salinity gradient; Ria de Aveiro.

5.2 Introduction

The interest by the bio-assessment of human impact on littoral ecosystems has been intensified since the implementation of the European Water Framework Directive (WFD) in 2000 (Directive 2000/60/EC, 2000; Borja, 2005; Borja and Heinrich, 2005; Dauvin et al., 2007). The monitoring of water bodies quality is carried out by a set of physico-chemical and biological quality elements defined in the Annex V of the WFD. Considered as potentially powerful indicators of aquatic ecosystems health, benthic macroinvertebrates are among the biological quality elements to consider in the assessment of the ecological quality statement (EQS) in transitional systems (Beukema and Cadée, 1986; Dauvin, 1993) and, consequently, numerous taxonomic based indices have been developed or adapted to the WFD requirements (AMBI, Borja et al., 2000, BENTIX, Simboura and Zenetos, 2002, BQI, Rosenberg et al., 2004, M-AMBI, Borja et al., 2003; Muxika et al., 2007; BOPA, Dauvin and Ruellet, 2007). The AMBI index is based on the species sensitivity/tolerance to organic enrichment and the multimetric M-AMBI index combines the species richness, the Shannon-Wiener diversity and the AMBI index. However, due to the high dynamism of the physico-chemical and hydro-morphologic conditions, transitional systems are composed by a mosaic of habitats characterized by species particularly well adapted to variability and with high levels of tolerance to changes, being more difficult to develop suitable and coherent quality indicators based on species composition and sensitivity to disturbance (Elliott and Quintino, 2007; Blanchet et al., 2008). In an attempt to overcome this difficulty, non-taxonomic indices based on functional traits, such as biomass, were developed. These indices are based on overall biomass distribution (Gray and Mirza, 1979; Clarke, 1990; De Eyto and Irvine, 2007) as well as on size distribution metrics, such as skewness (ISD) (Reizopoulou and Nicolaidou, 2007), size diversity (Quintana et al., 2008), proportion of large individuals or size-classes (Basset et al., 2008) and measure of macroinvertebrate size classes sensitivity to stress (ISS) (Basset et al., 2012). The development of these indices are based on the fact that large species are more sensitive to disturbance than small ones (Pearson and Rosenberg, 1978) and relationship between the size class distribution and the energy availability (Brown et al., 2004; Basset et al., 2008) and anthropogenic impact (Queiros et al., 2006).

This study was carried out in Mira Channel, one of the most pristine channels in Ria de Aveiro (Castro et al., 2006). This channel presents a full salinity gradient, from marine to freshwater, which makes from it a favorable area to study the variability patterns of taxonomic (AMBI and M-AMBI) and non-taxonomic (ISS) indices along a full salinity gradient, in the same system, using different sample techniques, the leaf-bag technique with 1 mm and 5 mm mesh-size leaf-bags with the macrophyte *Phragmites australis* and corer samples.

5.3 Material and methods

5.3.1 *Study area*

Ria de Aveiro is located on the Northwestern coast of Portugal, between 40°38`N and 40°57`N. With a maximum width and length of 10 and 45 km, covers an area of 83 km^2 and 66 km^2 at high and low spring tide, respectively. This transitional system is separated from the ocean by a sand bar and is formed by four main channels, Mira, Ílhavo, Espinheiro and São Jacinto, all connect with the sea by an artificial inlet and receiving freshwater inputs (Figure 6.1). This study was conducted in the Mira Channel, a narrow 20 Km long, running south from the entrance. This channel presents a full salinity gradient, ranging from fully marine at the mouth to freshwater at the head and is considered as one of the most pristine channels in Ria de Aveiro (Moreira et al., 1993; Castro et al., 2006).

5.3.2 *Field and laboratory procedures*

This study was based on an experimental field study of the decay of *Phragmites australis* leaves undertaken in the same sites, using the leaf-bag technique (Petersen and Cummins, 1974, Quintino et al., 2009). The study was carried out during winter 2008 (January-March), in a total of fifteen sampling sites distributed in five areas (area 1 to area 5), three sites per area, along the Mira Channel (Figure 5.1). The five areas cover all levels of the salinity gradient according to the Venice System (1959) for the Classification of Estuarine Waters: euhaline (area 1); polyhaline (area 2); mesohaline (area 3); oligohaline (area 4) and limnetic (area 5) (Figure 6.1). The benthic macrofauna was sampled using the leaf-bag technique with *Phragmites australis* leaves and two mesh sizes (1 mm and 5 mm). At the beginning of the experiment (day 0), all the leaf-bags were placed in the field sites, at the bottom, in the subtidal, and replicates were collected over time, at days 3, 7, 15, 30 and day 60. At each sampling time, four replicates of the leaf-bags were collected per site and placed in separate plastic containers. Four sediment samples were also collected per site with a 0.01 m^2 hand-held corer for the study of the benthic community. The leaf-bags macrofauna samples were brought to the laboratory, washed through a 0.5 mm mesh-sieve and the residue preserved in 70% ethanol. The sediment samples were sieved through 1 mm mesh-sieve and the material retained in the sieve was fixed in 4% buffered formalin and stained with Rose Bengal. In the laboratory, all samples were individually hand-sorted, identified using binocular stereoscopic and optical microscopes to species level whenever possible, measured and weighed. The measurements of length and weight were done according to the procedures described in sub-section 4.3.2 of Chapter 4. At the end of the experiment, was elaborated the biological data matrix including the macroinvertebrate species/taxa and their abundance per replicate and per site for the corer samples and also per sampling time for the 1 mm and 5 mm leaf-bags. The length and weight data matrix was also organized per replicate and per site for thecorer samples and also per sampling time for the 1 mm and 5 mm leaf-bags.

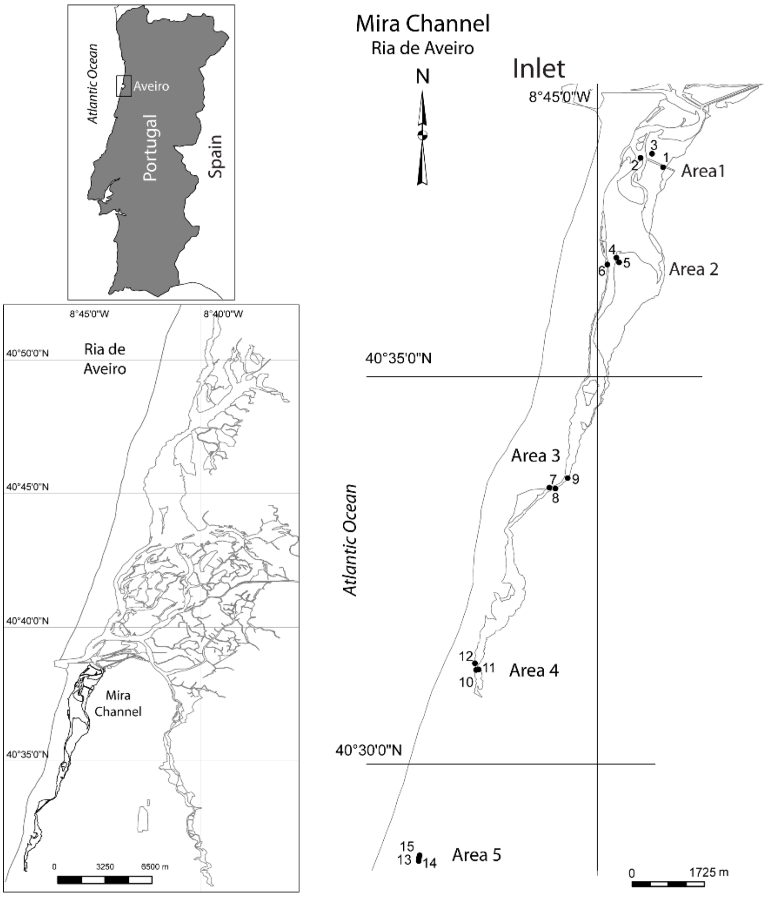

Figure 5.1 Positioning of the study sites in the Mira Channel, Ria de Aveiro, Western Portugal, for the study of the taxonomic and non-taxonomic indices using benthic macroinvertebrates obtained with the 1 mm and 5 mm leaf-bags and the sediment samples (sites 1 to 15, nested in areas 1 to 5).

5.3.3 Data analysis

Considering the lower abundance and taxa richness of the samples in each sampling moment (days 3, 7, 15, 30 and 60), the macrobenthic data was analyzed considering the composite sample of all the sampling moments per replicate. The AMBI index (Borja et al., 2000) is a frequent component of multimetric indices under scrutiny within the Ecological Quality Assessment of superficial waters in the European Union's Water Framework Directive (WFD). The multimetric M-AMBI index combines the species richness, the Shannon-Wiener diversity and the AMBI indices (Borja et al., 2003; Muxika et al., 2007), using the software AMBI v5.0 (www.azti.es). The AMBI and the M-AMBI indices were calculated at the replicate level for the 1 mm and 5 mm leaf-bags and the corer samples, considering all the individuals sampled and only the sub-set of specimens and taxa that could be measured. Data from the limnetic area (area 5) was excluded due to the fact that the majority of the taxa from this area are not included in the AMBI catalogue of species. The threshold values for the M-AMBI classification are based upon the European intercalibration (Borja et al., 2007, 2009): 0 ≤ Bad ≤ 0.20 < Poor ≤ 0.38 < Moderate ≤ 0.53 < Good ≤ 0.77 < High > 1. The variability of the AMBI and M-AMBI values considering the original data and the sub-set of specimens and taxa measured were analyzed, separately, in an experimental design with two factors, using areas as fixed factor and sites as a random factor nested in areas under the null hypotheses of no significant differences in the AMBI and M-AMBI values along the salinity gradient for the 1 mm and the 5 mm leaf-bags and the corer samples. The AMBI and M-AMBI values obtained for the original data and the sub-set of taxa and specimens measured in each salinity area were tested in an experimental design with three factors, using data (original and measured data) and areas as fixed factors and sites as a random factor nested in areas under the null hypothesis of no significant differences in the AMBI and M-AMBI values obtained with the original data and the measured specimens in each salinity area. This null hypothesis was tested separately for the 1 mm and 5 mm leaf-bags and the corer samples. The resemblance matrix between samples was obtained with the normalized Euclidean distance.

The multimetric index ISS (Basset et al., 2012) was calculated per replicate in each salinity area for the 1 mm and 5 mm leaf-bags and the sediment samples. The index ISS is based on the sensitivity/tolerance of body-size (by weight) macroinvertebrate classes as described by Basset et al. (2012). This index was calculated using the correction factor values suggested by Basset el al. (2012): 1 for all samples with more than 5 taxa, 0.8 for 4 and 5 taxa, 0.4 for 2 and 3 taxa, and 0.2 for samples with 1 taxon. The Ecological Quality was assessed according to the boundaries established by Basset et al. (2013) considering the Ria the Aveiro as a large ecosystem, with an average tidal > 0.5 m and a salinity ≥ 30 in the euhaline and polyhaline areas and a salinity < 30 in the mesohaline, oligohaline and limnetic areas. Thus, for the euhaline and polyhaline areas the Ecological Quality Boundaries were Azoic = 0 < Bad < 0.8 ≤ Poor < 1.4 ≤ Moderate < 1.8 ≤ Good < 2.5 ≤ High ≤ 6.0 and for the mesohaline, oligohaline and limnetic areas the Ecological Quality Boundaries were Azoic = 0 < Bad < 0.8 ≤ Poor < 1.5 ≤ Moderate < 1.9 ≤ Good < 2.8 ≤ High ≤ 6.0. The ISS values were analyzed with the experimental design described before for the AMBI and M-AMBI indices and the resemblance matrix between samples was obtained with the normalized Euclidean distance under the null hypothesis of no significant differences in the ISS values along the full salinity gradient for the leaf-bag and corer samples.

Hypothesis testing was performed by Permutation Multivariate Analysis of Variance (Anderson, 2001), using the software PRIMER v6 (Clarke and Gorley, 2006), with the add-on PERMANOVA+ (Anderson et al., 2008). This method allows partitioning the variability from a resemblance matrix and test individual terms, including interactions, using permutations (Anderson and ter Braak, 2003). To run the PERMANOVA tests we considered 9999 Monte Carlo permutations (Anderson et al., 2008). The *F*-values in the main tests and the t-statistic in the pairwise comparisons were evaluated in terms of the significance among levels of the tested factor. Values of $p \leq 0.05$ reveal that the groups differ significantly.

5.4 Results

5.4.1 Taxonomic indices - AMBI and M-AMBI

The original benthic data sampled with the 1 mm and 5 mm leaf-bags and the corer samples were used to calculate the AMBI and M-AMBI indices per salinity area and the mean values of which are shown in Table 5.1. The AMBI and M-AMBI values were tested under the null hypothesis of no significant differences in these indices along the salinity gradient and the null hypothesis was accepted for all samplers (AMBI - 1 mm: pseudo-*F* = 0.790, p = 0.529; 5 mm: pseudo-*F* = 2.794, *p* = 0.1061; corer samples: pseudo-*F* = 2.585, *p* = 0.131; M-AMBI - 1 mm: pseudo-*F* = 0.315, *p* = 0.807; 5 mm: pseudo-*F* = 3.982, *p* = 0.054; corer samples: pseudo-*F* = 2.111, *p* = 0.173). When translating the M-AMBI values into an environmental quality statement, the 1 mm and 5 mm leaf-bag samples were classified as ‘Good’ in all areas, excepting in the mesohaline area, classified as ‘Moderate’ with the 1 mm leaf-bags. For the corer samples, the euhaline area was classified as ‘Good’, and the other areas as ‘Moderate’ (Table 5.1).

The mean values of the AMBI and M-AMBI indices calculated for the measured specimens in the 1 mm and 5 mm leaf-bags and the corer samples are shown in Table 5.1. In this case, no significant differences were found in the AMBI and M-AMBI values along the salinity gradient for the 1 mm leaf-bags and corer samples (AMBI - 1 mm: pseudo-F = 4.029, p = 0.053; corer samples: pseudo-F = 0.9687, p = 0.450; M-AMBI - 1 mm: pseudo-F = 0.907, p = 0.488; corer samples: pseudo-F = 2.734, p = 0.108). In the 5 mm leaf-bags, contrarily to the M-AMBI (pseudo-F = 2.089, p =0.180), significant differences were found in the AMBI values (pseudo-F = 4.619, p =0.007) between the oligohaline and the poly- and mesohaline areas (2,4: t = 5.228, p = 0.005; 3,4: t = 5.287; p = 0.006). The AMBI value was higher in the oligohaline than in the poly- and mesohaline areas. When translating the M-AMBI values into an environmental quality statement (cf. Table 5.1), for the 1 mm leaf-bags, the ecological quality of the salinity areas was the same for the all mabrobenthic fauna sampled and the sub-set of measured specimens. For the 5 mm leaf-bags, the ecological quality decreased when considering only the sub-set of measured specimens. With all the macroinvertebrates samples, all the areas were classified as ‘Good’ and with the measured individuasl as ‘Moderate’. For the corer samples, the euhaline and oligohaline areas were classified as ‘Good’ and the polyhaline and mesohaline as ‘Moderate’ (cf. Table 5.1).

The null hypothesis of no significant differences in the AMBI and M-AMBI values between the original data and the sub-set of specimens and taxa measured in each salinity area was accepted for the 1 mm leaf-bags and the corer samples ([area x data] 1mm - AMBI: pseudo-F = 0.893, p = 0.478; M-AMBI: pseudo-F = 0.386, p = 0.777; corer samples - AMBI: pseudo-F = 2.943, p = 0.096; M-AMBI pseudo-F = 2.151, p = 0.174). For the 5 mm leaf-bags, no significant differences were found between the AMBI values calculated with the original and the measured specimens in all the salinity areas ([area x data] pseudo-F = 1.388, p = 0.317) but significant differences were found in the M-AMBI values ([area x data] pseudo-F = 5.272, p = 0.028) in the mesohaline area (t = 12.500, p = 0.006).

Table 5.1 Mean values per area of the AMBI and M-AMBI indices calculated for the original data and the sub-set of specimens and taxa measured in the 1 mm and 5 mm leaf-bag and corer samples along the salinity gradient (areas 1 to 4). EQS - Ecological Quality Statement.

		Original				**Measured**			
		1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
1 mm	AMBI	2.031	2.062	1.138	2.653	0.968	1.086	0.845	2.322
	M-AMBI	0.597	0.541	0.519	0.572	0.595	0.519	0.516	0.604
	EQS	Good	Good	Moderate	Good	Good	Good	Moderate	Good
5 mm	AMBI	2.177	0.590	0.422	1.815	1.230	0.296	0.345	1.383
	M-AMBI	0.644	0.572	0.568	0.555	0.519	0.426	0.488	0.513
	EQS	Good	Good	Good	Good	Moderate	Moderate	Moderate	Moderate
Corer samples	AMBI	2.552	3.867	3.281	3.400	2.004	2.693	2.929	2.946
	M-AMBI	0.643	0.409	0.429	0.465	0.600	0.432	0.427	0.587
	EQS	Good	Moderate	Moderate	Moderate	Good	Moderate	Moderate	Good

5.4.2 Non-taxonomic indices - ISS

The statistical analysis of the ISS values along the salinity gradient accepted the null hypothesis for all the samplers (1 mm: pseudo-*F* = 1.790, *p* = 0.198; 5 mm: pseudo-*F* = 1.169, *p* = 0.385; corer samples: pseudo-*F* = 1.401, *p* = 0.300). The ecological quality assessment along the salinity gradient considering the mean ISS value per salinity area and all the samplers is shown in Table 5.2. The ecological quality of the 1 mm and 5 mm leaf-bag samples was 'High' along the salinity gradient. For the corer samples, the euhaline area was classified as 'Good', the polyhaline as 'Poor' and the other areas as 'Moderate' (cf. Table 5.2).

Table 5.2 Mean values per area of the ISS indicex calculated for the sub-set of specimens and taxa measured in the 1 mm and 5 mm leaf-bag and corer samples along the salinity gradient (areas 1 to 5). Area 1 - euhaline, Area 2 – polyhaline, Area 3 – mesohaline, Area 4 – oligohaline, Area 5 - limnetic. EQS - Ecological Quality Statement.

		1	2	3	4	5
1 mm	ISS	2.653	3.119	3.293	3.135	3.059
	EcoQ	High	High	High	High	High
5 mm	ISS	3.097	2.898	3.530	3.633	2.996
	EcoQ	High	High	High	High	High
Corer samples	ISS	2.027	1.094	1.501	1.718	1.696
	EQS	Good	Poor	Moderate	Moderate	Moderate

5.5 Discussion

Estuaries are complex and naturally stressed ecosystems characterized by low species richness and dominance of few tolerant species. The use of biotic indices to assess the ecological status of these particular ecosystems has created controversy and uncertainty in what concerns to their efficiency and reliability. Water salinity is one of the most important factors affecting species richness and distribution (Quintino and Rodrigues, 1989; Rodrigues and Quintino, 1993; Basset et al., 2006; Rodrigues et al., 2006; 2011; Uwadiae, 2009; Whitfield et al., 2012) and the influence of salinity in the distribution of species along the Mira Channel was already observed by Quintino et al. (2011) and Lopes et al. (2013). A previous study described in Chapter 4 showed that the sub-set of taxa and specimens measured from the 1 mm and 5 mm leaf-bags and corer samples were able to show equally well the benthic succession from the marine to the freshwater areas with significant differences between all the salinity areas excepting between the euhaline and the polyhaline areas. However, in the case of the 1 mm leaf-bags and corer samples, some dominant taxa were not measured, changing the community structure. The body size abundance distribution of the benthic macroinvertebrates by the ISS size classes along the salinity gradient did not show any particular trend from the marine to the freshwater environment for the leaf-bags and corer samples, indicating that a salinity range from 0 to 35 will not condition the ISS response in areas affceted by anthropogenic stresses. Considering these previous knowledges, taxonomic (AMBI and M-AMBI) and non-taxonomic (ISS) indices were applied to the original data and to the sub-set measured specimens in order to understand if both indices show the same response along the salinity gradient.

In general, no significant differences were found in the AMBI, M-AMBI and ISS values along the salinity gradient for the 1 mm and 5 mm leaf-bags and corer samples, considering all the data set and the sub-set of specimens measured. These results were not in accordance with those obtained by Basset et al. (2013) in Mediterranean and Black Sea lagoons used as reference environments. These authors showed that M-AMBI and ISS were significantly affected by salinity, being higher in euhaline than in polyhaline, mesohaline and oligohaline conditions.

When the AMBI and M-AMBI calculated with the original data and measured specimens were compared in each salinity area, no significant differences were found for all samplers in each area, indicating that the exclusion of some specimens and even some taxa from the original data set did not affect the AMBI and M-AMBI results. When the M-AMBI calculated with all the macroinvertebrates sampled was translated into an environmental quality statement, the 1 mm and 5 mm leaf-bags classified all the salinity areas in the same way, with the exception of the mesohaline areas for the 1 mm leaf-bags, indicating that both mesh sizes were good samplers to classify the environmental quality of the Mira Channel along the salinity gradient. However, when the M-AMBI was calculated with the sub-set of measured specimens, the environmental quality assessment in the 5 mm leaf-bags decreased, indicating that the taxa and specimens excluded influenced the index response. For the corer samples, the original data classified the polyhaline, mesohaline and oligohaline areas in a worst environmental quality than the leaf-bags. This difference was the reflex of the difference between the dominant species in both types of samples, Annelids in the corers and Arthropods in the leaf-bags. When the sub-set of measured specimens was considered, the environmental quality of all areas remains the same, excepting in the oligohaline area. In this case the environmental quality increased from ‘Moderate’ to ‘Good’ probably due to the exclusion of species of Annelid that were considered by the AMBI as indicative of stress.

For the non-taxonomic index ISS, no significant differences were found along the salinity gradient for all the samplers and all the salinity areas were classified with a high ecological quality for the 1 mm and 5 mm leaf-bags. For the corer samples, a lower ecological quality was obtained even when compared with the classification attributed by the M-AMBI calculated using the measured specimens. However, according to Borja et al. (2011), the calculation of the ISS with abundances lower than 25 individuals should be made with some precautions. In this case, corer samples were poor with low taxa richness and abundance, indicating that these results should be considered with some precautions. The inability of the M-AMBI to classify the Mira Channel in such a good environmental quality like the non-taxonomic index ISS was related with the fact that the taxonomic index M-AMBI had been developed to coastal environments, in which the presence of species that naturally occur in transitional systems is indicative of arthropogenic stress. Estuaries are naturally stressed environments due to the high degree of variability in their physico-chemical and hydro-morphological characteristics. However, their biota is well-adapted to cope with that stress absorbing stress without adverse effects (Environmental Homeostasis) (Elliott and Quintino, 2007). Thus, transitional systems may only be regarded as stressful for marine or freshwater-adapted organisms and not for the organisms that naturally live in these stressed ecosystems. Elliott and Quintino (2007) showed that the characteristics of natural stress in estuaries are similar to those for anthropogenic stress, making the detection of the anthropogenic stress more difficult (Estuarine Quality Paradox). Considering this, the use of non-taxonomic indices instead of taxonomic based ones should be considered in detecting environmental perturbations in estuaries.

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Chapter 6. Ecological effects of contaminated sediments following a decade of no industrial effluents emissions: the Sediment Quality Triad approach

6.1 Abstract

Sediments contaminated by industrial effluents a decade after the emissions were stopped were statistically compared to sediments from reference channels, using the Sediment Quality Triad approach. The metals and metalloid concentrations, mainly Hg and As, increased towards the upper part of a contaminated channel, where the industrial discharge was located. A bioaccumulation assay with *Scrobicularia plana* showed the highest bioaccumulation and mortality in the most contaminated sediments and bioaccumulation strongly correlated with the sediments metals and metalloid concentrations. The resident macroinvertebrate community also showed significant differences between the contaminated and reference channels, in the upper areas, where the community was most affected. All three elements of the quality triad rejected the null hypothesis and indicated that despite the emissions ceasing in 2004, sediments remain contaminated by high levels of metals and metalloid, leading to bioaccumulation and with severe community level consequences.

Keywords: Heavy metals; Bioaccumulation; Sediment toxicity; *Scrobicularia plana*; Benthic macrofauna; Ria de Aveiro.

6.2 Introduction

Estuaries are among the most productive ecosystems on earth. They are important for many species and constitute preferential areas for the reproduction and growth of several aquatic species, some with high economic value (among others, McLusky and Elliott, 2004). They have long been the sites of large urban and industrial settlements and intensive agriculture upstream, all of which are sources of pressures to surface water bodies (Ferreira et al., 2004; Chapman et al., 2013). The Ria de Aveiro, Western Portugal, is no exception since it has received industrial discharges from the Estarreja Chemical Complex (CQE), which began operating in the 1930s with the production of fertilizers, mineral acids, chlorine and soda, plastics, aromatics and other products. Until 1975, industrial effluents containing organic compound, metals and metalloid, such as mercury, arsenic, zinc, lead, aluminum, cadmium, copper, vanadium and iron were discharged into artificial channels and then into the Estarreja Channel. This produced highly contaminated superficial sediments in the upper areas of the Estarreja Channel (Costa and Jesus-Rydin, 2001; Lucas et al., 1986; Hall et al., 1985; Monterroso et al., 2003; Pereira et al., 1998a, 2009). After 1975, the effluents were discharged directly by pipes into the Estarreja Channel. In 1994, mercury emissions were reduced to regulatory levels (50 µg L^{-1}, the limit value for discharges from chlor-alkali electrolysis industry in accordance with the European Directive 82/176/EEC, 1982) and then emissions ceased in 2002. In 2004 the effluents discharged into the Estarreja Channel were channelled to a multi-municipality sanitation system (SIMRIA) and ocean outfall, thus removing all sewage and industrial effluent discharges from the Ria de Aveiro.

Sediments represent, quantitatively, the major compartment for metal storage in aquatic environments and, consequently, a potential source to interstitial waters and the infauna living in direct contact with the sediments (Chapman et al., 1998; Simpson and Batley, 2007; Luoma and Rainbow, 2011). Macrobenthic organisms and communities are a key component of coastal ecosystems, playing an important role in detritus decomposition, nutrient cycling, and energy flow to higher trophic levels and due to their life-history characteristics, have frequently been used to evaluate the impact of both anthropogenic and natural disturbances in the estuarine environment (Gray and Elliott, 2009). Due to their relatively long lifespan, sedentary lifestyle and consequent incapacity to avoid unfavorable conditions, benthic communities integrate contaminant impacts over time and permit discriminating between episodic and chronic disturbances (Reiss and Kröncke, 2005; Reiss et al., 2006; Dauvin et al., 2007; Elliott and Quintino, 2007; Ward et al., 2013). Their taxonomic diversity produces communities with organisms having a wide range of response/tolerance to environmental stressors (Dauer, 1993; McLusky and Elliott, 2004).

Although the total metal concentrations in sediments indicate contamination, such measures do not necessarily predict the toxicity of these contaminants to aquatic organisms. The ecotoxicological risk induced by contaminated sediments will depend on metal availability as well as of the ability of living organisms to assimilate those (Amiard et al., 2007; Campana et al., 2012). The relationship between metal accumulation and the feeding behavior of the benthic organisms influences the bioavailability of different metals in the aquatic environment (Monperrus et al., 2005).

This study uses the Sediment Quality Triad (SQT) approach (Long and Chapman, 1985) to analyze the ecological effects of contaminated sediments associated with the industrial chemical effluents discharged in the Estarreja Channel, a decade after the emissions ceased. This approach has been used in marine, estuarine and freshwater environments (e.g., Chapman et al., 1987; Quintino et al., 1995; 2001; Canfield et al., 1996; Delvalls et al., 1998; Carr et al., 2000; Hollert et al., 2002; Khim and Hong, 2014) and integrates the sediment chemistry, sediment toxicity or bioaccumulation assays and resident benthic communities (Chapman, 1986; 1989; 1990). This study compared SQT descriptors analyzed in samples from the impacted (Estarreja) and reference channels (Salreu and Canelas), under the null hypothesis of no significant differences associated with the sediment contamination. The SQT descriptors included sediment metal and metalloid concentrations, their bioaccumulation in the bivalve *Scrobicularia plana* exposed to the test sediments under controlled laboratory conditions and the resident macrofauna benthic community species composition and abundance.

6.3 Material and methods

6.3.1 Study area

The Ria de Aveiro is located on the NW coast of Portugal, between 40º38`N and 40º57`N, and is characterized by extensive intertidal mud and sand flats, salt marshes and islands. The study was conducted in three small channels, Estarreja, Canelas and Salreu, discharging to the Laranjo Bay, in the central area of the Ria (Figure 6.1). The Estarreja Channel received continuous industrial discharges for decades which produced an environmental contamination gradient (Pereira et al., 1998a, 1998b). The Canelas and Salreu Channels were reference sites, representing largely uncontaminated channels. For the bioaccumulation assays, control sediment was also collected at the mouth of the Mira Channel, from where the specimens of *Scrobicularia plana* used in the assay were also collected (cf. Figure 6.1).

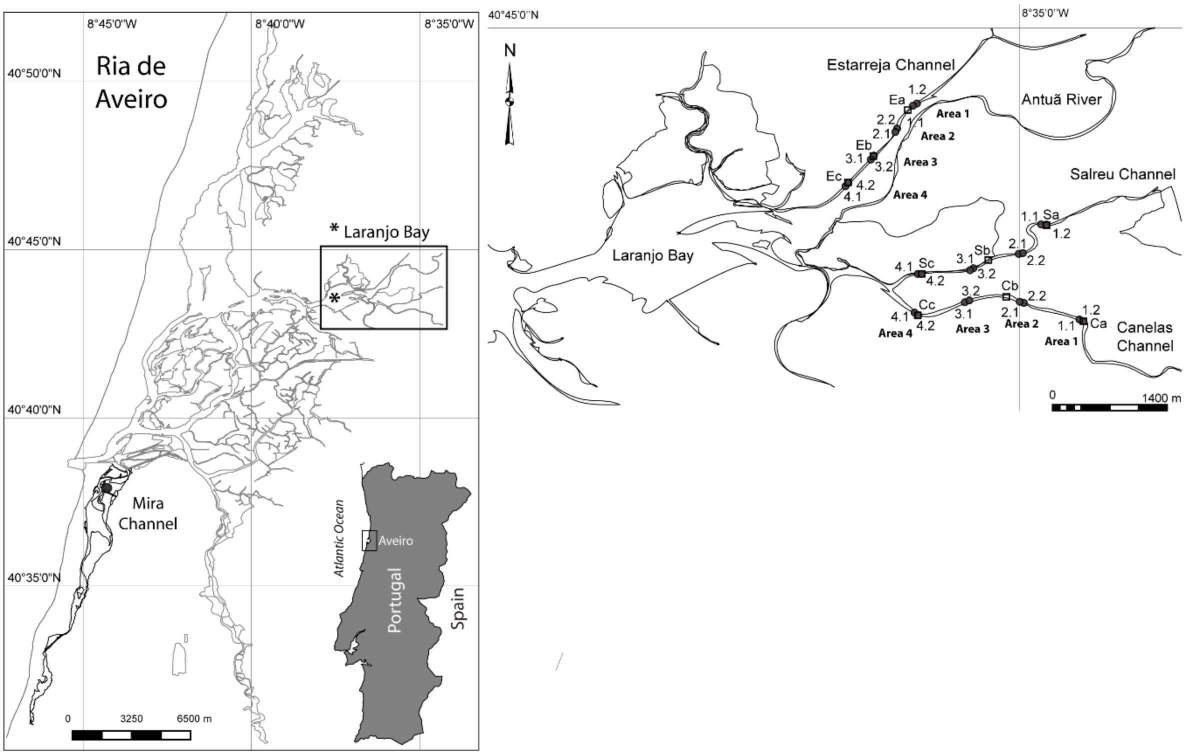

Figure 6.1 The sediment sampling sites in Mira, Estarreja, Salreu and Canelas Channels in Ria de Aveiro, Western Portugal. Also shown the sites for the study of bottom water salinity in Estarreja, Salreu and Canelas (Ea to Ec, Sa to Sc and Ca to Cc, respectively).

6.3.2 Field and laboratory procedures

The sediment samples for this study were collected in 2011 and 2013. In 2011, the sediment concentrations of Hg, Cr, Ni, Cu, As, Cd, Zn and Pb and the benthic macroinvertebrate communities were studied along the impacted and the reference channels. In 2013 the sediment contamination analyses and the macrofauna study were repeated and a bioaccumulation assay with the bivalve *Scrobicularia plana* was set up in order to assess the bioavailability and accumulation of the sediment contaminants. In view of the results obtained in 2011, the sampling effort in 2013 was concentrated in the four areas located along the impacted channel and in the upstream areas of the reference channels.

6.3.3 Sampling, sediment baseline and contamination descriptors and macrofauna communities

Sediment samples for the macrofauna study in the Estarreja, Salreu and Canelas Channels in April 2011 were collected in four areas per channel, with two sites per area and four replicates per site, totaling 96 replicates, 32 per channel (Figure 6.1). Each sample was collected with a 0.01 m^2 hand-held corer and placed separately in a plastic container, brought to the laboratory, washed through a 0.5 mm mesh sieve and the residue preserved in 70% ethanol. Benthic macroinvertebrates were hand-sorted and identified using binocular stereoscopic and optical microscopes to species level whenever possible. For each site a species/taxa list with the respective abundance was prepared.

An extra sediment sample per site was collected for grain-size, organic matter and metal and metalloid analyses. The sediment grain-size was analyzed by wet and dry sieving, following Quintino et al. (1989). The fines fraction (particles with diameter <0.063 mm) was wet sieved through a 0.063 mm mesh screen. The sand (particles with diameter from 0.063 mm to 2 mm) and the gravel fractions (particles with diameter above 2 mm) were dry sieved through a nest of sieves spaced at 1ϕ interval ($\phi = -\log_2$ the particle diameter expressed in mm).

The sediment samples for the total organic matter analysis were kept at -20°C. After thawing, the samples were oven dried at 60°C for 24 hours and ground. Total organic matter concentration (TOM) was obtained by loss on ignition, as the percent weight loss in 1 g of dried sediment, after combustion at 450 °C for 5 hours (Kristensen and Anderson, 1987).

Total mercury (Hg), chromium (Cr), nickel (Ni), copper (Cu), arsenic (As), cadmium (Cd), zinc (Zn) and lead (Pb) were analysed by an accredited laboratory. For Hg, Cr, Ni, Cu, As, Cd, Zn and Pb quantification, 2 g of homogenised air dried sediment were digested overnight (±18 h) at 115 °C with 10 ml of 65% HNO_3 (Suprapur, Merck) in digestion Teflon bombs (sealed chambers). To prevent the loss of metals and metalloid by volatilisation, chambers were only opened when completely cooled. The cooled digest was made up to 25 ml with1M HNO_3. After a 20x dilution, the concentrations of Cr, Ni, Cu, As, Cd, Pb and Hg were determined by inductively coupled plasma mass spectrometry (*ICP-MS*) on a Thermo ICP-MS X-Series equipped with a auto sampler CETAX ASX-510, Peltier Nebulizing Camera Burgener nebulizer, nickel cones, the CeO^+/Ce^+ ratio was optimized at <2% and internal standard: indium (In) and terbium (Tb). The concentration of Zn was measured by inductively coupled plasma atomic emission spectroscopy (*ICP-AES*) Jobin Yvon Activa M equipped with auto sampler JY-AS500 and Burgener Mira Mist nebulizer.

In order to measure salinity over a tidal cycle, bottom water samples were collected simultaneously every 30 minutes during a period of 12 hours, at three sites per channel, (sites Ea to Ec in Estarreja, Ca to Cc in Canelas and Sa to Sc in Salreu, Figure 6.1).

In April 2013, sediment metal and metalloid analyses were repeated and a bioaccumulation assay was carried out in order to test the bioavailability of the sediment contaminants and their effect in the survival of the test bivalve *Scrobicularia plana*. Taking into account the results from 2011, the sampling effort in 2013 was concentrated to the four areas of the Estarreja Channel and the most upstream area of the reference channels, Canelas and Salreu. The number of replicate samples per site was increased in order to increase statistical hypothesis power. For the bioaccumulation assay, a control sediment was also sampled in the Mira Channel, near the mouth of the Ria de Aveiro, from where the specimens of *S. plana* used in the assay were collected (Figure 6.1). This bivalve is a long-lived facultative surface deposit feeder and suspension feeder (Kamermans, 1994). The natural habitat of this species ranges from muddy to sandy sediments, with a wide salinity range (4-30) and geographical distribution (Byrne and O'Halloran, 2001). It has a lifespan of 5 years, annual recruitment and growth rate of approx. 1cm y^{-1} in S Europe (Verdelhos et al., 2005). This species has been previously used both in biomonitoring programs (Cheggour et al., 2005; Anajjar et al., 2008) and toxicity tests (Byrne and O'Halloran, 2001; García-Luque et al., 2004; Pérez et al., 2004; Riba et al., 2004).

For the bioaccumulation assay, four sediment replicates per area were collected with a plastic spatula, placed in separate plastic containers and brought to the laboratory. Four sediment replicate samples per area were also collected for the study of the resident benthic macrofauna communities in 2013, using a 0.01m^2 hand-held corer. The same number of sediment replicates was collected for grain-size and total organic matter analyses and to quantify the sediment total Hg, Cr, Ni, Cu, As, Cd, Zn and Pb, following the procedures described above.

6.3.4 Bioaccumulation assay

The bioaccumulation assay was performed according to EPA (1993). Specimens of *S. plana* were collected in April 2013 in the Mira Channel, where the species occurs naturally, during low tide. The total length of the individuals used in this experiment ranged from 3.4 to 4.2 cm, corresponding to individuals 3-4 years old. The specimens were transported to the laboratory and placed in a walk-in climatic chamber at a temperature of 18 °C and a 14:10 hours day:night photoperiod. A 6 days acclimation period was established in containers without sediment. During this period, salinity was gradually reduced from 30 to 12, in order to run the bioaccumulation assay with salinity closer to that observed in the test and reference channels (Lopes et al., 2013). No food was added during acclimation and continuous aeration was kept by gently air-bubbling the water.

Four replicate test sediment samples were collected at each of seven areas, four in the impacted channel (Estarreja) and in one area from each of the reference (Canelas and Salreu) and the control (Mira) channels (Figure 6.1). In the laboratory, each sediment replicate was individually homogenized to reduce the natural heterogeneity and to remove the resident macrofauna as much as possible. The sediment from each replicate sample was distributed into six glass containers (±1 L and Ø10 cm), creating an approx. 8 cm sediment column (about ⅓ of the height of the container). Prior to use, the glass containers were rinsed with filtered water, dipped in 10% nitric acid for two hours and again rinsed with filtered water. The containers with the sediment were filled with filtered water with salinity 12 and left to stabilize for 24 hours. After this period, each container was individually aerated and one specimen of *S. plana* was placed per container, to give a total of 168 containers (7 areas x 4 sites x 6 specimens) which were randomly distributed in the walk-in chamber. About ⅓ of the water of each container was renewed weekly and neither sediment nor food was added during the 28 days of exposure. After this period, the surviving organisms were recovered, rinsed in water at salinity 12 and placed in constantly aerated clean glass containers with saltwater for 48 hours to allow depuration. At the beginning of the experiment, when the organisms were exposed to the test sediments (T_0), 16 individuals were randomly taken from the overall sample and measured (shell total length). Their soft tissue was frozen, lyophilized and weighted and sent for metal and metalloid analysis. Following the exposure period (T_{28}) and 48h depuration, all the shells were measured. The surviving specimen soft tissue was frozen, lyophilized and sent for metal and metalloid analysis. Mortality was calculated per site in each area. The shells were measured with a Vernier caliper to the nearest 0.05 mm and the individuals weighed on an analytical balance with an associated error of ±1 mg.

Each individual *S. plana* was analyzed for total Hg, Cr, Ni, Cu, As, Cd, Zn and Pb in an accredited laboratory by digesting overnight (±18 h) at 115 °C with 2 ml of 65% HNO3 (Suprapur, Merck) then the cooled digest was made up to 5 ml using 1 M HNO3. The metals were then analyzed by methods similar to the sediment.

6.4 Data analysis

6.4.1 Environmental characterization

The grain-size fractions were expressed as a percentage of the whole sediment, dry weight and the data used to calculate the median particle diameter value, P50, in phi (ϕ) units. The median and the % fines were used to classify the sediment, according to the Wentworth scale: very fine sand (median from 3 to 4 ϕ); fine sand (2 - 3 ϕ); medium sand (1 - 2 ϕ); coarse sand (0 - 1 ϕ); very coarse sand (-1 to 0 ϕ). The final classification adopted the description 'clean', 'silty' or 'very silty' when the silt and clay fraction ranged from 0% to 5%, from 5% to 25% and from 25% to 50%, respectively, of the total sediment, dry weight (Doeglas, 1968; Larsonneur, 1977). Samples with <50% fines content were classified as mud.

The experimental design for the samples taken in 2011 considered a data analysis according to three factors: contamination, fixed with two levels (the impacted, Estarreja and the reference channels, Salreu and Canelas), channels, random and nested in the contamination (only the reference channels were replicated) and areas, random and nested in the channels, with four levels. The strong contamination gradient that was observed from the upstream to the downstream areas in the Estarreja Channel, masked the differences between the impacted and the reference channels, precluding analyzing the data according to this model. The sediment contamination was then analyzed separately for each of the four areas per channel in an experimental design with two factors, contamination, fixed, and channels, random and nested in the contamination. The difference among areas within the contaminated channel was tested using an experimental design with areas as a fixed factor. The resemblance matrix between samples was obtained with the Euclidean distance. In 2013, for the reference channels only the upstream areas were sampled and compared to the areas of the impacted channel. The sediment contamination data was analyzed under the null hypotheses of no significant differences between the impacted and the reference channels and no significant differences among areas along the contaminated channel.

The sediment contamination from 2011 and 2013 data were also represented in ordination analyses, using Principal Component Analysis (PCA), following normalization of the raw data (each variable was reduced to mean zero and unit standard deviation).

6.4.2 Bioaccumulation assay

The bioaccumulation data were analyzed using the same model as the sediments data sampled in 2013, under the null hypotheses of no significant differences in the bioaccumulation of Hg, Cr, Ni, Cu, As, Cd, Zn and Pb between the control (Mira), the reference (Salreu and Canelas) and the contaminated (Estarreja) channels. The null hypothesis of no significant differences in the bioaccumulation values between T_0 and T_{28} was also tested with the data from the organisms exposed to the control sediment. The bioaccumulation data were also subjected to ordination analyses, using Principal Component Analysis (PCA), following normalization of the raw data, as previously.

At the end of the bioaccumulation assay, the shell length and the weight of the specimens exposed to the control sediment (Mira Channel) were compared to those from the 16 individuals measured and weighed at the beginning of the experiment (T_0), under the null hypothesis of no significant differences in the length and weight of the specimens between T_0 and T_{28}. The shell length and weight at T_{28} were also tested, under the null hypothesis of no significant differences in these descriptors in the specimens exposed to the contaminated, the control and the reference sediments.

6.4.3 Benthic macrofauna

The benthic macrofauna data were represented by the abundance of species per replicate and per site for the Estarreja, Salreu and Canelas Channels in 2011 and 2013. Primary and derived biological descriptors, namely the number of species/taxa, the abundance of specimens, the Margalef richness index (*d*) and the Shannon-Wiener diversity index (*H'*,

$\log_2$), were calculated per channel and per area in each channel (considering the individual replicates and the composite sample). Given the low abundance and species richness in each replicate corer sample, hypothesis testing was based on the composite sample of the four corer replicates per site, and so representing each area by two samples, one from each site. The benthic macrofauna data were analyzed using a model with two factors, contamination, fixed with two levels, and channels random and nested in the contamination, under the null hypothesis of no significant differences in the resident benthic community between the contaminated and the reference channels. Also knowing from the sediment analysis that the upstream areas of the impacted channel, areas 1 and 2, were more contaminated than the downstream areas, 3 and 4, the macrofauna data analysis paired the data from the areas (1 and 2) and (3 and 4), representing two groups, one the more contaminated part of the channel and the other the less contaminated part. The model was run separately for the upstream and downstream parts of the channels. The resemblance matrix between samples was obtained with the Bray-Curtis similarity coefficient, following standardization and a dispersion weighting for the contamination factor of the original data (Clarke et al., 2006b).

Hypothesis testing was performed by Permutation Multivariate Analysis of Variance (Anderson, 2001), using the software PRIMER v6 (Clarke and Gorley, 2006), with the add-on PERMANOVA+ (Anderson et al., 2008). This method allows partitioning the variability from a resemblance matrix and test individual terms, including interactions, using permutations (Anderson and ter Braak, 2003). To run the PERMANOVA tests, we considered 9999 Monte Carlo permutations. The pseudo-F values in the main tests and the t-statistic in the pairwise comparisons were evaluated in terms of the significance among levels of the tested factor. Values of $p \leq 0.05$ revealed that the groups differed significantly.

6.5 Results

6.5.1 Environmental characterization

Figure 6.A (see supporting information) shows the evolution of bottom water salinity along a complete tidal cycle in three sites per channel. The mean salinity and the salinity range

per site are shown in Table 6.1. According to the Venice System (1959) classification of brackish waters, the whole study area was classified as mesohaline, although salinity variation reached polyhaline conditions in the downstream part of the Estarreja Channel (site Ec, see Figure 6.1 and Table 6.1). No significant differences were found among channels at low tide (Estarreja vs Salreu, Canelas: *pseudo-F* = 18.7500, *p* = 0.139; Salreu vs Canelas: *pseudo-F* = 0.0188, *p* = 0.893). At high tide, the salinity in the Estarreja Channel was higher, especially in the upstream site when compared to the upstream sites in Salreu and Canelas (Ea, Sa and Ca, see Table 6.1). These differences are due to the upstream areas of the Salreu and Canelas Channels being further from the saltwater source (see Figure 6.1). The sediments sampled in the three channels in 2011 were classified as mud, except some replicates in the Canelas Channel (2.2, 3.1 and 4.2), which had a higher proportion of the sand fraction. In most cases the percentage of fines (<0.063mm) was above 80-90%. The mean total organic matter concentration (TOM) from areas 1 to 4 in the Estarreja Channel was 9.2%, 10.1%, 8.4% and 7.5%, respectively. In Salreu and Canelas Channels, TOM from areas 1 to 4 was 10.4%, 12.0%, 12.1%, 11.4% and 10.2%, 4.8%, 8.9%, 5.5%, respectively, the values from Canelas being slightly lower and in agreement with the higher proportion of the sand fraction in this channel. The sediments collected in 2013 in the Estarreja, Canelas and Salreu channels and used in the bioaccumulation assay were all classified as mud, with a percentage of fines (<0.063mm) >80-90%. The control sediment, from the Mira Channel, where the *Scrobicularia plana* specimens were collected, was characterized as medium sand, with mean organic matter concentration lower than in the other areas (1.0%). In the reference areas (area 1 of Salreu and Canelas), TOM reached 12.1% and 6.5%, respectively. Along the contaminated channel (Estarreja), TOM concentrations in the areas 1 to 4 were, respectively, 12.0%, 9.1%, 7.2% and 5.9%.

Table 6.1 Mean salinity (± standard deviation) and salinity range during a tidal cycle in the three sites in the Estarreja, Canelas and Salreu Channels. n = 26. See figure 1 for the positioning of areas Ea to Ec (Estarreja), Sa to Sc (Salreu) and Ca to Cc (Canelas).

	Estarreja Channel		Salreu Channel		Canelas Channel	
Area	Mean salinity (±sd)	Salinity range	Mean salinity (±sd)	Salinity range	Mean salinity (±sd)	Salinity range
a	16.8 (±5.67)	6 - 22	11.2 (±3.14)	8 - 18	9.8 (±1.54)	6 - 12
b	19.0 (±5.22)	7 - 24	12.4 (±2.97)	9 - 20	11.2 (±1.92)	10 - 15
c	21 .0(±4.09)	11 - 26	17.6 (±4.68)	10 - 23	17.3 (±4.65)	10 - 24

Figure 6.2A gives the principal components ordination analysis of the sediment metals and metalloid concentrations in the samples collected in 2011. Axis 1 explained almost 80% of the total variance and showed a contamination gradient along the Estarreja Channel, with significant differences between areas 1 and 3, 1 and 4 and 3 and 4 (1-3: t = 15.188, p = 0.002; 1-4: t = 32.543, p = 0.001; 3-4: t = 4.475, p = 0.024). In the Canelas and Salreu Channels such a gradient was not observed, and the highest contamination levels were in areas 3 and 4 in Salreu and in area 3 in Canelas, closer to the downstream rather than the upstream part of the channels. The contamination gradient in the Estarreja Channel was also observed for each individual metal and metalloid, with decreasing concentrations from the upstream (area 1) to the downstream part of the channel (area 4) (Figure 6.2B). The null hypothesis of no significant differences between the contaminated (Estarreja) and the reference channels (Salreu and Canelas) was rejected for Ni, Zn, As and Hg in area 1, and for Hg in areas 1, 2 and 3. For area 4, the data for none of the metals and metalloid led to rejection of the null hypothesis (Table 6.A, given as supporting information). This confirmed that metal and metalloid concentrations were not homogenous along the Estarreja Channel and that the most contaminated areas were located upstream. The analysis for the whole set of metals and metalloid together, showed significant differences only between the upstream area of the contaminated channel (area 1) and the reference channels (Salreu and Canelas) (cf. Table 6.A, supporting information). On the basis of these results, the detailed metal and metalloid analysis and bioaccumulation studies conducted in 2013, focused on the four sampling areas along the Estarreja Channel and were reduced to area 1 in the reference channels. The metals and metalloid concentrations in the sediments collected in 2013, which were used in the bioaccumulation assay, also detected the increasing contamination from the downstream to the upstream part of the Estarreja Channel, as shown in Figure 6.3A ordination analysis. Along this gradient, significant

differences were found between all the areas, except between areas 1 and 2, the most upstream, and between areas 3 and 4, the most downstream (1-3: t = 4.0058 , p = 0.001; 1-4: t = 2.6661, p = 0.020; 2-3: t = 7.0734, p = 0.0001; 2-4: t = 3.7242, p = 0.005). Considering the reference channels, Salreu showed higher contamination than Canelas, in particular concerning Cr, which explains why the samples from these two channels were placed away from each other in the ordination diagram (Figure 6.3A). The control area in the Mira Channel was the least contaminated (Figure 6.3A and 6.3B). Despite areas 1 and 2 of the Estarreja Channel showing the highest levels of each contaminant (Figure 6.3B), there were only significant differences for Hg between areas 1 and 2 of the contaminated channel and the reference areas (E1 - (S,C): pseudo-F = 10.075; p = 0.018; E2 - (S,C): pseudo-F = 52.348; p = 0.023), due to the large difference between the two reference locations. These results confirmed those obtained in 2011.

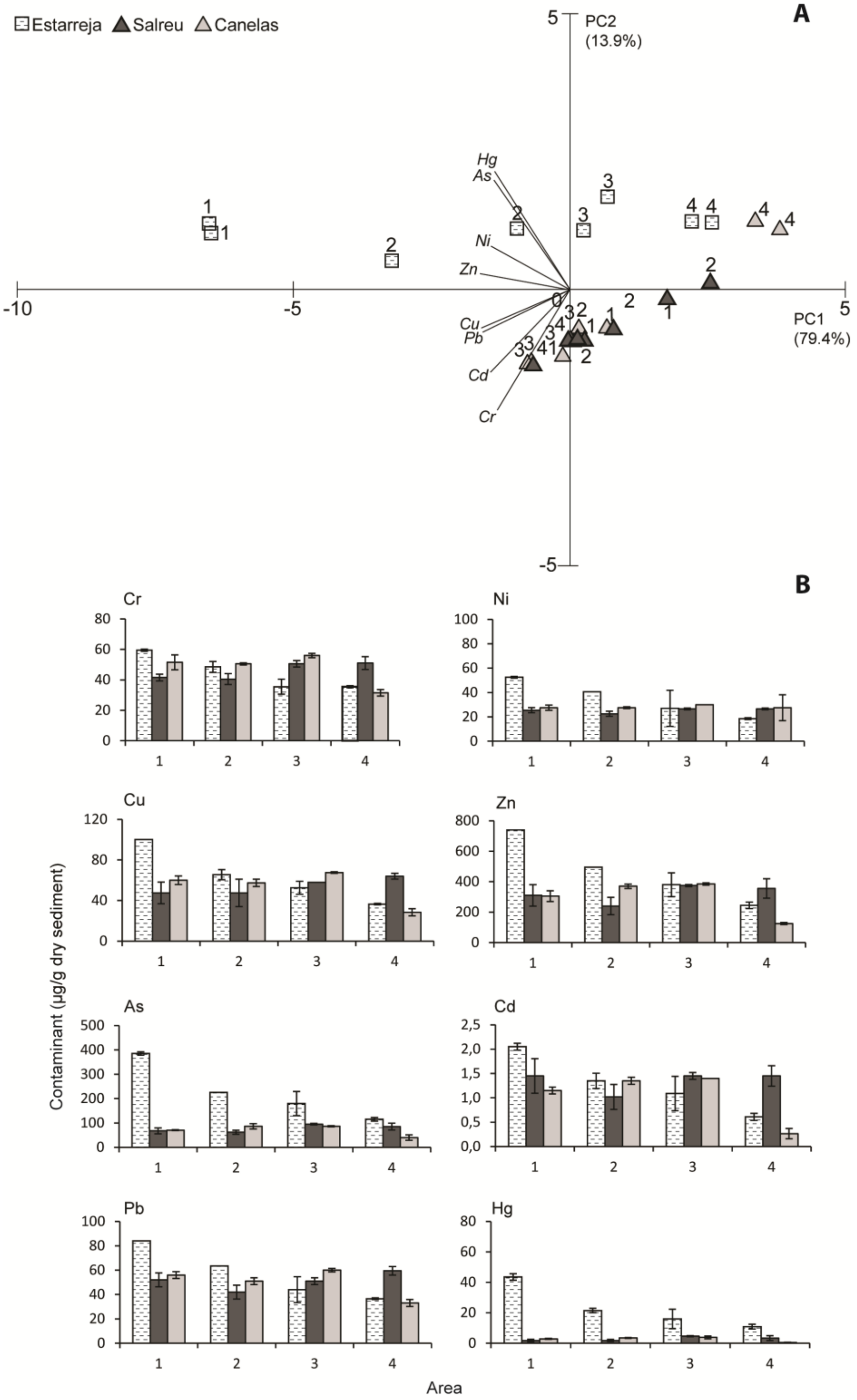

Figure 6.2 Principal component analysis (A) and bar graph (B) based on the concentrations of Cr, Ni, Cu, Zn, As, Cd, Pb and Hg in the sediment samples collected in areas 1 to 4 of the Estarreja (E), Salreu (S) and Canelas (C) channels in 2011. Bars indicate the standard deviation around the mean.

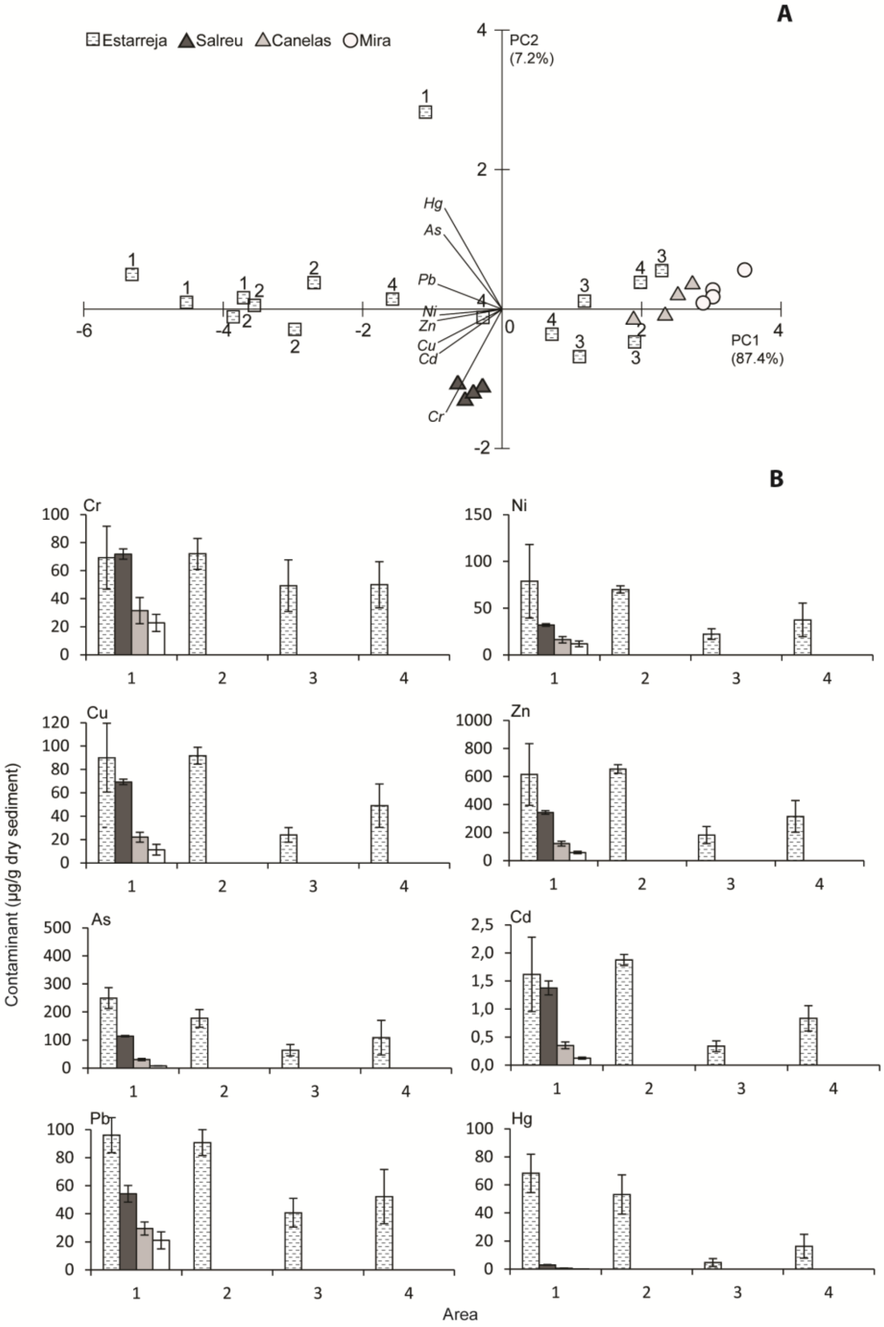

Figure 6.3 Principal component analysis (A) and graphic bars (B) based on the concentrations of Cr, Ni, Cu, Zn, As, Cd, Pb and Hg in the sediment samples collected in areas 1 to 4 of the contaminated channel, Estarreja (E) and in the areas of the reference channels, Salreu (S) and Canelas (C) and the control channel, Mira (M), in 2013. Bars indicate the standard deviation around the mean.

6.5.2 Mortality and bioaccumulation assay

The mean individual shell length of the specimens of *S. plana* as well as the mean individual weight at the beginning and at the end of the bioaccumulation assay (Table 6.B, given as supporting information) showed no significant differences between the various sediments (contaminated, reference and control) (pseudo-$F = 0.315$; $p = 0.93$) and the weight loss in the control sediment from day 0 to day 28 was not significant ($t = 1.9629$; $p = 0.06$). Also, at day 28, there were no significant differences in the weight of the specimens that survived in the various sediments (pseudo-$F = 0.6092$; $p = 0.7306$).

The mortality of *S. plana* after 28 days of exposure differed between the control, the reference and the contaminated sediments. The lowest mortality was registered in the control sediment (8%). Mortality in the reference sediments was higher in Salreu, 42%, than in Canelas, 21%. The highest mortality occurred in sediments from the most upstream areas of the contaminated channel, areas 1 and 2, respectively 58% and 75%. Sediments from areas 3 and 4 of this channel showed a lower mortality, 38% and 54%, respectively. The mortality of *S. plana* was directly related to the metals and metalloid concentrations, individually or together (Figure 6.4). The Spearman rank correlation between mortality and sediment contamination was high for all the metals and metalloid, almost always higher than 0.8 (Figure 6.4, significant at $p < 0.05$ for all cases).

The metals and metalloid concentrations were analyzed in 16 organisms at the beginning of the experiment (T_0) and after 28 days of exposure (T_{28}), in the control sediment. The values were generally higher at T_{28} than at T_0, except for Hg. The differences were significant for Cr, Ni, As, Cd and Hg (Cr: pseudo-$F = 11.2180$, $p = 0.002$; Ni: pseudo-$F = 7.0848$, $p = 0.009$; As: pseudo-$F = 6.7679$, $p = 0.013$; Cd: pseudo-$F = 5.2035$, $p = 0.030$; Hg: pseudo-$F = 12.5070$, $p = 0.001$).

Metals and metalloid concentrations were analyzed at T_{28} in all the individual organisms that survived the bioaccumulation assay. The organisms exposed to the sediments collected in areas 1 and 2 of the Estarreja Channel differed from the others, mainly due to the high bioaccumulation values of Hg and As, as shown in the ordination analysis in Figure 6.5A. The null hypothesis of no significant differences in the bioaccumulation data in the organisms exposed to the sediments from the four areas of the contaminated channel was rejected when all metals and metalloid were considered (pseudo-$F = 5.7744$, $p < 0.0001$). The pairwise comparisons between areas indicated that the null hypothesis was not rejected only when comparing areas 3 and 4, the most downstream, distributing the sampling areas of the contaminated channel into three coherent groups: area E1, the most upstream and with the highest bioaccumulations, area E2, and the group including areas E3 and E4, the most downstream and the lowest bioaccumulation. In the ordination diagram (Figure 6.5A), areas E3 and E4 appear close to the reference areas (Salreu and Canelas) and the control sediment (Mira). In general, the highest bioaccumulation for each metal and metalloid was observed in the more contaminated areas of the Estarreja Channel (areas 1 and 2). The test results of the null hypothesis of no significant differences in the bioaccumulation at T_{28} of the bivalves exposed to the control, the reference and the contaminated sediments is shown in Table 6.C (supporting information). There were no significant differences between the control (Mira) and the reference sediments (Salreu and Canelas), for the metals and metalloid data together or individually. Between the reference and each area of the contaminated channel, the null hypothesis was rejected for areas E1 and E2 when considering all the metals and metalloid, and individually for Hg and Pb (cf. Table 6.C, supporting information). For Cr, Zn and Ni, the null hypothesis was only rejected in the comparisons between the reference sediments and the area E1, while for As the null hypothesis was rejected in comparing the reference sediments and all the areas of the contaminated sediments, except E3. For the metals Cu and Cd, the null hypothesis was not rejected (cf. Table 6.C, supporting information). Figure 6.5B represents the relationship between the sediment contamination and bioaccumulation, while showing the associated *S. plana* mortality. Sites E1 and E2 are identified in all the diagrams as they usually are separated from the others. In Figure 6.5B, the graph representing the whole metals and metalloid data illustrates well the overall distinction between these two sites and the remaining ones. This graph also shows a non-linear relationship between sediment contamination and bioaccumulation. Elevated bioaccumulation only occurred when sediment concentrations went above some threshold values. For individual metals and metalloid this was especially clear for Hg, but also for As, Pb and Cd. Cu, Cr, Zn and Ni, did not show such a relationship between sediment contamination, bivalve bioaccumulation and mortality, suggesting that Hg, As, Pb and Cd were mostly responsible for the mortality values observed in the bioaccumulation assay.

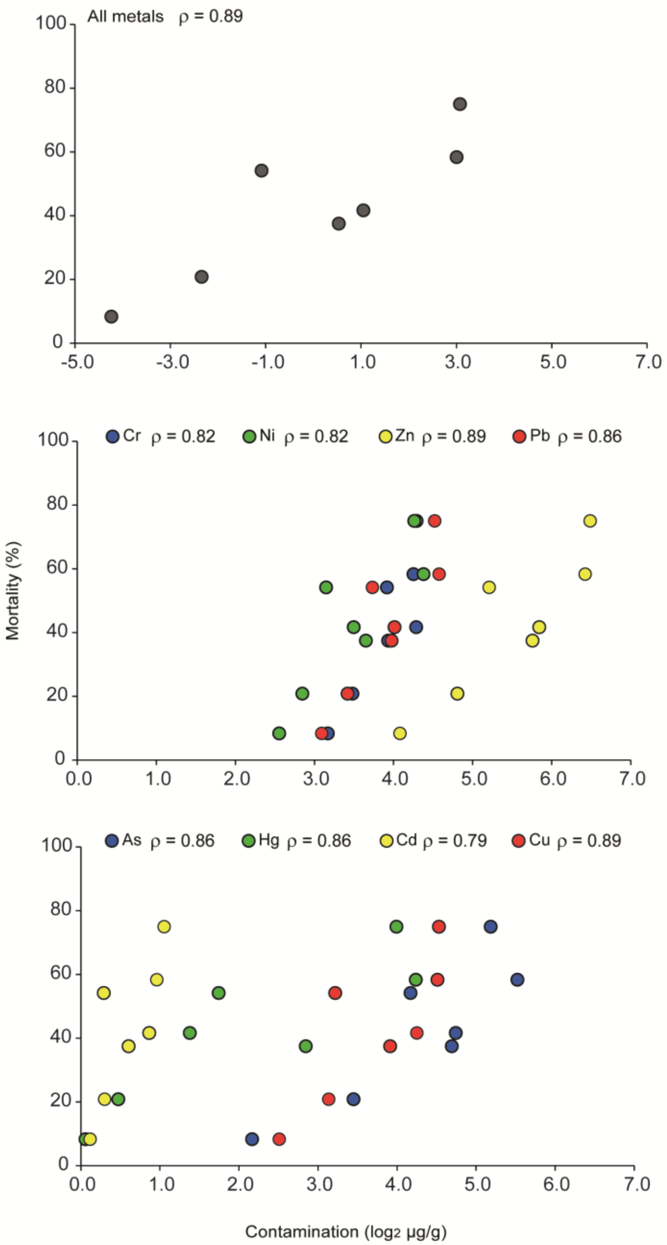

Figure 6.4 Relationship between the mortality of *S. plana* in the bioaccumulation assay and the sediment metal/metalloid contamination (Cr, Ni, Cu, Zn, As, Cd, Pb and Hg, individually and summed concentrations). For the whole set of contaminants, the reported concentrations (x-axis) correspond to the site scores of PCA axis 1. The Spearman rank correlations (ρ) are shown (significant at $p < 0.05$ for all cases).

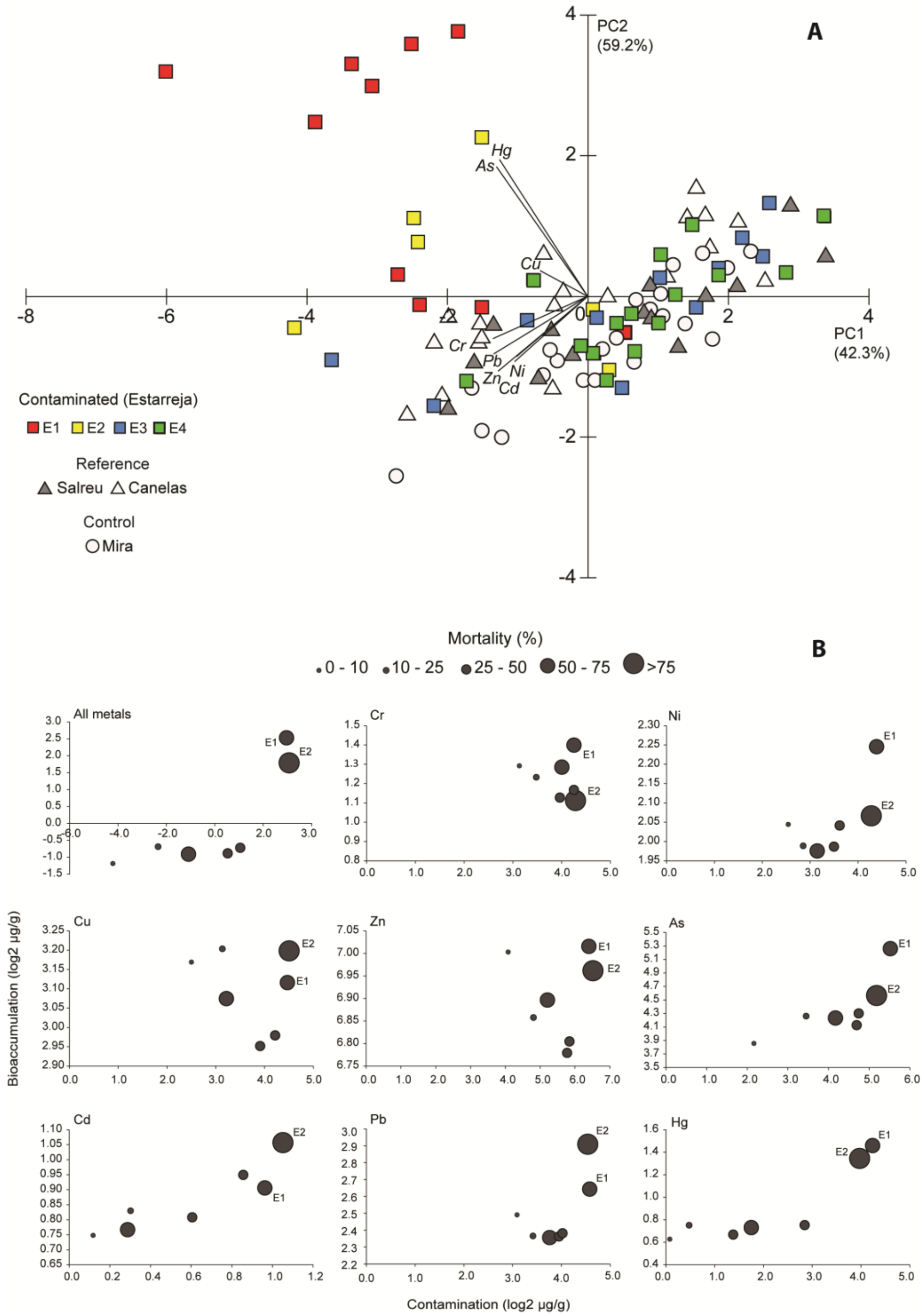

Figure 6.5 Principal component analysis of the accumulated metal/metalloid in *S. plana* at the end of the bioaccumulation assay (A) and relation between the sediment contamination and bioaccumulation with the associated *S. plana* mortality (B), exposed to the sediment samples collected in 2013 in the contaminated (Estarreja, E1 to E4), the reference (Salreu, S and Canelas, C), and the control (Mira, M) channels.

6.5.3 Resident benthic macrofauna

In 2011, the mean benthic macrofauna abundance in the Estarreja, Salreu and Canelas Channels was of 201.6, 211.3 and 241.4 individuals per 0.01m^2, distributed among 12, 16 and 17 taxa, respectively (Table 6.2). In the Estarreja Channel, the highest mean abundances were found in the upstream areas (areas 1 and 2), with the highest contamination levels. In the reference channels, the upstream areas presented the lowest mean abundances. The taxa richness, Shannon-Wiener diversity and Margalef richness were calculated considering the replicate samples and the composite sample per area, which combined the individual replicates in a single sample. When considering the individual replicate sample, mean taxa richness was lower in the contaminated channel (Estarreja) than in the reference channels (Salreu and Canelas). In the Estarreja Channel, the mean taxa richness also increased from the more contaminated (upstream) to the less contaminated areas (downstream). In the Salreu channels the mean taxa richness decreased from the upstream to the downstream areas. In the Canelas Channel, the mean taxa richness did not show any particular pattern. Considering the composite sample, the mean taxa richness was much higher and the patterns observed for the individual sample were more evident. The indices related to species richness and diversity (*H’* and *d* Table 6.2)) showed higher values in the reference channels (Salreu and Canelas) than in the contaminated channel (Estarreja). In this channel, *H’* and d also increased from the more to the less contaminated areas, upstream to downstream. Such a trend, upstream to downstream, was not observed in the reference channels. In Salreu, *H’* and d were lower downstream and in Canelas, *H’* and d did not show any particular spatial pattern.

The null hypothesis of no significant differences between the contaminated and the reference channels was rejected for the upstream part of the channels, areas 1 and 2 (pseudo-F = 14.388, p = 0.04), but not for the downstream part, areas 3 and 4 (pseudo-F = 2.9587, p = 0.19), indicating that the disturbance effects were not homogeneous along the contaminated channel, as in previous descriptors.

The annelids *Alkmaria romijni*, *Streblospio shrubsolii*, *Hediste diversicolor* and Oligochaeta were dominant in all areas of the contaminated and the reference channels, except in areas 3 and 4 of the Estarreja Channel, where the Oligochaeta was replaced by the arthropod *Melita palmata* (area 3) or by the annelid *Capitella* spp. (area 4). In the contaminated channel, the arthropods were absent or strongly reduced in the upstream, most contaminated areas (1 and 2), both in 2011 and 2013. This was not observed in the reference channels (Table 6.2, Figure 6.6 and

Table 6.D, given as supporting information). The macrofauna benthic community in 2013 was dominated by the same taxa as in 2011, but with lower abundance. Taxa richness and diversity were also lower in 2013 (cf. Table 6.D, supporting information). Nevertheless, the overall distribution pattern of the taxa was similar along the contaminated channel (Estarreja), without arthropods in the most contaminated areas (areas 1 and 2) (cf. Figure 6.6).

Table 6.2 Abundance (*A*), Taxa richness (*S*), Shannon-Wiener diversity (*H'*, log$_2$) and Margalef richness (d) of the replicate samples and the composite sample per area (n=8) collected in 2011 in the areas 1 to 4 in the Estarreja (E), Salreu (S) and Canelas (C) Channels. The value in brackets represents the composite sample per channel (n=32). The letters before the species/taxon name indicate the Phylum: An - Annelids; Ar - Arthropods; M – Molluscs; N – Nemertea. The taxa are ranked from highest to lowest total abundance and the four dominant taxa in each area are highlighted, in light gray.

		Abundance*											
	Taxa	1E	2E	3E	4E	1S	2S	3S	4S	1C	2C	3C	4C
An	*Alkmaria romijni*	1393	1799	1287	1121	697	1301	1387	692	524	1441	1925	346
An	*Streblospio shrubsolii*	159	50	77	110	157	372	243	570	113	773	232	927
An	*Hediste diversicolor*	69	75	138	69	63	279	197	324	130	229	193	226
An	Oligochaeta	9	13		3	186	131	32	54	31	150	47	146
An	*Capitella* spp.				36		5	1	9	4	10		89
Ar	*Corophium multisetosum*					3	1			20	59	4	
Ar	*Melita palmata*			23	1	10	1	1					18
Ar	*Gammarus chevreuxi*					7	1	1		6	6	8	19
Ar	*Cyathura carinata*		3	2	2	13	3			1	2		3
Ar	*Leptocheirus pilosus*			1		1					4	1	7
Ar	Chironomidae					8	1	1			1		2
M	*Scrobicularia plana*				3			1				7	1
Ar	*Heterotanais oerstedtii*										3		6
N	Nemertini ni				5								3
Ar	Dolichopodidae							5		2	1		
Ar	*Lekanesphaera hookeri*						1	1					1
M	*Peringia ulvae*			2									
Ar	*Carcinus maenas*			2									
Ar	*Neomysis integer*											2	
An	*Heteromastus* sp.								1				
An	*Autolytus brachichephalus*					1							
A	Individual sample ($0.01m^2$)	203.75	242.50	191.50	168.75	143.25	262.00	233.75	206.25	103.88	334.88	302.38	224.25
	Composite sample	(201.63)				(211.31)				(241.34)			
S	Individual sample ($0.01m^2$)	3.25	3.50	4.38	4.13	6.50	5.00	4.63	4.25	5.75	6.88	5.38	7.75
	Composite sample	4	5	8	9	11	11	11	6	9	12	9	14
		(12)				(16)				(17)			
d	Individual sample ($0.01m^2$)	0.42	0.45	0.65	0.63	1.23	0.74	0.72	0.61	1.07	1.05	0.78	1.27
	Composite sample	0.56	0.73	1.33	1.56	2.01	1.80	1.83	0.94	1.72	1.89	1.40	2.40
		(2.07)				(2.80)				(2.92)			
H' (log2)	Individual sample ($0.01m^2$)	0.75	0.45	0.84	0.86	1.81	1.49	1.26	1.66	1.49	1.74	0.99	1.93
	Composite sample	0.76	0.48	0.88	0.97	1.75	1.57	1.20	1.73	1.66	1.75	1.07	2.08
		(0.79)				(1.63)				(1.80)			

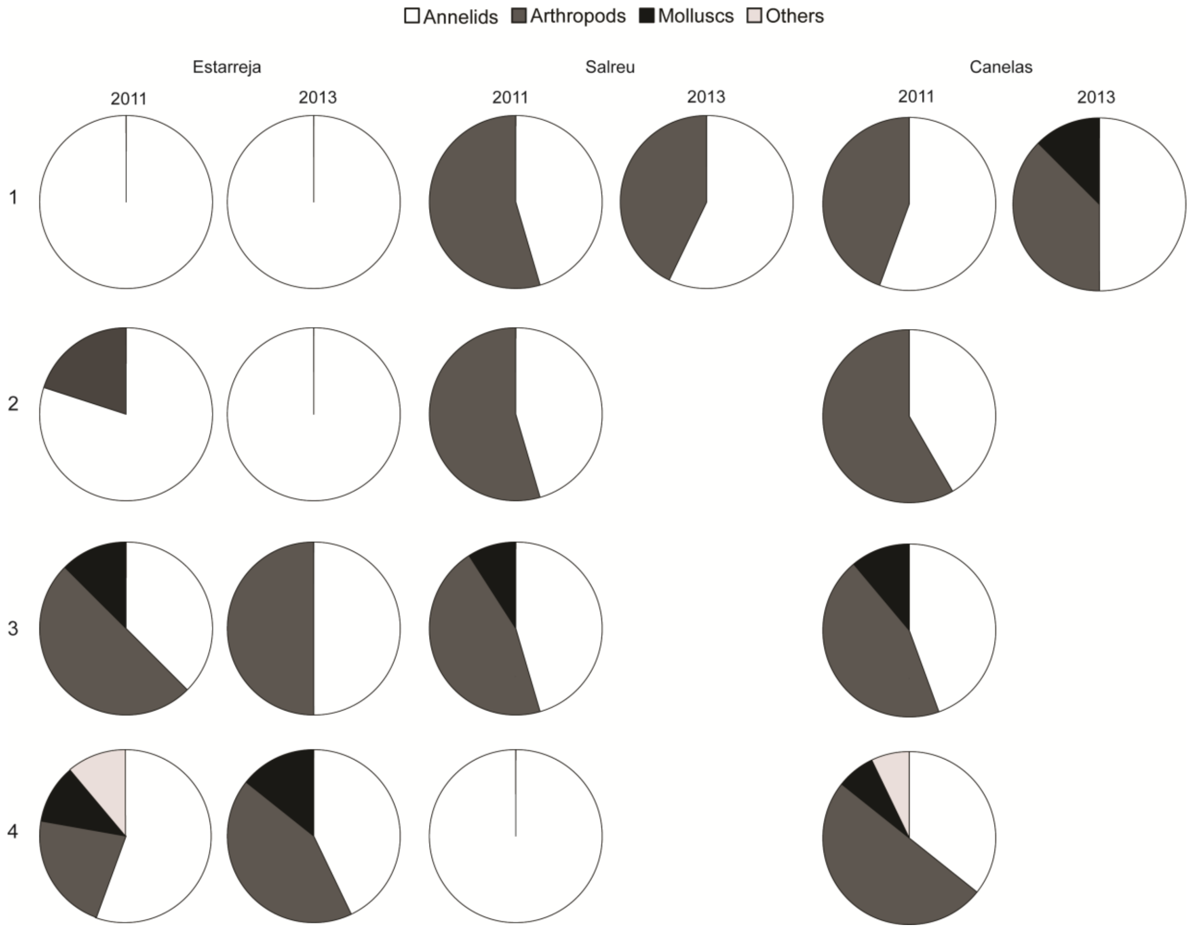

Figure 6.6 Pie charts representing the proportion of Annelids, Molluscs, Arthropods and other groups in the sediment samples collected in the study areas 1 to 4 of the Estarreja (E), Salreu (S) and Canelas (C) Channels in 2011 and 2013.

6.6 Discussion

Sediment contamination by metals and metalloid has been shown to negatively affect the aquatic environment worldwide (e.g. Stark, 1998; Blasco et al., 1999; Warwick, 2001). A decade after stopping the discharge of contaminated industrial effluents, the sediments in the Estarreja Channel still presented high levels of contaminants, such as Hg, As, Ni and Zn, the metals and metalloid for which significant differences between the contaminated and the reference channels were found. Mercury is one of the most hazardous contaminants in the aquatic environment, widely considered to be among the highest priority environmental pollutants in the scope of the European Water Framework Directive (WFD) (Decision 2455/2001/EC). Despite the restrictions on anthropogenic sources of mercury, historically contaminated sediments may still be a source of mercury to the aquatic environment, and available to aquatic organisms, as shown in this study (see also Elliott and Griffiths, 1986; Coelho et al., 2006; Cardoso et al., 2009). For several decades, a chlor-alkali plant discharged an effluent rich in mercury into the Estarreja Channel and most of the mercury was retained in the sediments of a small area of around 1.7 km^2 which corresponds to the Estarreja Channel and the Laranjo Bay, located downstream. The discharges induced an environmental contamination gradient inside Ria de Aveiro and resulted in an estimated accumulation of 33 tonnes of mercury, 77% of which could be stored in the Estarreja Channel and Laranjo Bay (Pereira et al., 1998a; 2009). Although the plant altered the production process in 1994, decreasing the industrial discharges to regulatory levels (50 µg L^{-1}, European Directive 82/176/EEC, 1982) and the use of mercury completely ceased in 2002, the mercury emitted from the plant still remains significantly high in the surrounding environment. In this study, nine and eleven years after the cessation of the mercury emissions to the environment, there were high levels in the more contaminated areas of the Estarreja Channel, ranging from 15-45 mg/kg in 2011 and from 40-82 mg/kg in 2013. The vertical variation of mercury in the sediments from the Laranjo Bay showed higher concentrations with increasing depth (Pereira et al., 1998a). This suggests that severe winters, like that of 2013, could wash out the upper sediments layers, mainly in the upstream areas, and expose the sub-superficial sediments, more contaminated, supporting the higher contamination values noted in 2013.

The Ria de Aveiro has a semi-diurnal tidal rhythm with an amplitude of 1.4 m at spring tides and currents play a major influence on the dynamics of the suspended particles where the mercury is predominantly stored (Pereira et al., 1998b). The entrainment of the suspended particles by the currents, disperse the mercury from the more contaminated areas to the rest of the lagoon, as was observed by Hall et al. (1985), Lucas et al. (1986), Pereira et al. (1998b) and Pato et al. (2010). In our study, the Canelas and Salreu Channels (the reference channels) showed the highest contamination levels in the downstream areas, located nearest to the Laranjo Bay, suggesting that the contamination from the Estarreja Channel was brought into these channels by the currents, via the Laranjo Bay.

High concentrations of other toxic contaminants than Hg, such as Ni, Zn and As, also occurred in the Estarreja Channel sediments. For these metals and metalloid, significant differences were found between the contaminated and the reference channels. The persistence of high levels of metals and metalloid in the sediments several years after the source of contamination had ceased or the levels of contamination have been reduced to regulatory levels, has been documented elsewhere. The monitoring of the Seine River catchment, France, for about 55 years showed a decreased average level for Cd, Hg, Pb, Zn, Cu, Cr and Ni, due to the implementation of metal emission regulations and changes in the management of contaminated sediments. However, recent studies reported contamination levels in the sediments still high and above the natural background values (Meybeck et al., 2007). Bing et al. (2011) also showed that the Xijiu Lake sediments from the Taihu Lake catchment, China, were heavily contaminated by Cd, Cu, Pb, Cr, Zn and Hg, due to several decades of industrial discharges. The contamination state of heavy metals varied with the industrial development of the catchment area, and demonstrated that contamination started in the early 20th century, reached the maximal level between the mid-1970s and mid-1990s, and decreased after the implementation of constraints on highly-contaminating industries. However, concentrations of Cd, Zn and Hg were still high as reported by those authors. In Spain, ten years after the Aznalcóllar mines spill, Tornero et al. (2011) observed a decline in the sediment metal contamination. However, some elements were still found in high concentrations and bioavailable as was shown by the levels of zinc, lead, and arsenic in the bivalve *Scrobicularia plana*, which exceeded health advisory levels (Tornero et al., 2011).

This study also confirmed that sediment bound metals and metalloid in the Estarreja Channel were bioavailable. When exposed to the control, reference and contaminated sediments, the bioaccumulated concentrations in the bivalve *Scrobicularia plana* were positively correlated to the concentrations in the sediment. This was also observed by Coelho et al. (2006) for mercury and by Ereira (2011) for arsenic, in Ria de Aveiro, Portugal, and by Suñer et al. (1999) for arsenic in the Guadalquivir estuary, Spain. Non-essential metals, especially mercury, lead and cadmium can be toxic even at low concentrations. They may be accumulated in the tissues of shellfish at concentrations many times higher than those observed in the water and sediments, and transferred throughout the food chain to higher trophic levels, namely to human consumers (Elliott and Griffiths, 1986; Roberts et al., 1986). The low mortality experienced by *S. plana* after 28 days of exposure in the control sediments (8%), indicated that the conditions under which the assay was carried were satisfactory for this species, and that the higher mortalities observed in the other areas were due to the sediment contamination and the bioaccumulation of the toxic compounds. When absorbed, some metals (e.g. Zn, Cd, Pb, Cu and Hg) have been shown to form detoxification products by binding to special Heavy Metal Binding Proteins, metallothioneins (MTs). When associated with MTs, metals are thought to be removed from the toxic pool and would not cause adverse effects to the organism (Vašák, 2005). It is known that *Scrobicularia plana* synthesize MTs as a response to heavy metal contamination and that the salinity of the medium influences this physiological response (García-Luque et al., 2004; Coelho et al., 2007). High concentrations of metals induce stress that may result in biochemical and physiological alterations and elevated mortality in *S. plana* (Coelho et al., 2006), indicating that the disruption of this detoxifying mechanism may lead to the highest mortality found with the most contaminated sediments of the Estarreja Channel. The positive relationship found here between the sediment contamination and the mortality of *S. plana*, for all the contaminants suggests that as the organism is a deposit feeder and an important prey item to higher trophic levels, it can play an essential role in the mobilization of contaminants from the sediments to biota, increasing their bioavailability and progression through the food chain (see namely Elliott and Griffiths, 1986).

The effect of the sediment contamination and the bioavailability of the contaminants were reflected in the resident macrofauna of the Estarreja Channel. In this channel, the taxa richness increased from the most to the least contaminated areas, which in this case was from upstream to downstream. This also corresponded to a slight salinity increase along the channel, although the whole study area was within the mesohaline classification of the Venice System (1959). This trend is expected to occur naturally in the estuarine environment, as was shown in a comprehensive study of the macrofauna benthic communities in Ria de Aveiro (Rodrigues et al., 2011). This and other recent studies conducted in Ria de Aveiro in areas with similar salinity gradients, have shown that arthropods, namely amphipods, can be very abundant in the upper part of this estuarine system, in low salinity conditions (Quintino et al, 2011; Lopes et al., 2013). The absence of arthropods detected only in the upper, most contaminated part of the Estarreja channel, should result from their sensitivity to pollution, including metals (McLusky et al., 1986). This response of the resident macrofauna community was overall in good agreement with the highest levels of contamination and bioaccumulation in those areas as well as the highest mortality in *S. plana*. Several experimental studies have shown that annelids are more tolerant to metals contamination than arthropods, namely amphipods, which should explain their absence in the most contaminated areas of the Estarreja Channel (Wogram and

Liess, 2001; Amiard et al. 2006). In contrast, the annelids *Alkmaria romijni*, *Streblospio shrubsolii, Hediste diversicolor* and Oligochaeta were dominant in all areas of the contaminated and the reference channels. Amiard et al. (2006) showed that the annelid *Hediste diversicolor* had higher concentrations of compounds which share many characteristics of MT than those in other invertebrates. Nunes et al. (2008) in the Laranjo Bay also indicated that, contrarily to the bivalve *Scrobicularia plana,* the annelids *Hediste diversicolor* and *Alkmaria romijni* and the isopod *Cyathura carinata* were tolerant to contamination by metals, being present in areas with high levels of mercury. In our study, the isopod *Cyathura carinata* was not observed in the most contaminated area of the Estarreja Channel, while only few individuals were registered in the other areas. The bivalve *S. plana* was only observed in the less contaminated areas of the Estarreja Channel and in the reference channels. This pattern was also observed by several authors in other areas contaminated by metals. Rodrigues and Quintino (1993) described such characteristic in the macrobenthic impoverished areas in the outer Sado Estuary and Warwick (2001) reported the absence of crustacean species such as the amphipod *Corophium* spp. and the isopod *Cyathura carinata* in the Fal estuary (South England, Cornwall), which has a history of metal contamination. Mucha et al. (2003) in the Douro estuary, Portugal, also indicated that an increase in the concentrations of Zn, Cu, Pb, and Cr was associated with a decrease in species richness and diversity, namely the absence of the amphipod *Corophium volutator* and the isopod *Cyathura carinata* from the most contaminated areas. Stark (1998) studied the influence of the heavy metals Cu, Pb and Zn associated with urban runoff on the intertidal soft sediments macrofauna assemblages in two estuaries in Sydney, Australia, and verified that the contaminated areas were generally less diverse and, contrarily to the unpolluted areas, presented lower crustacean abundance.

In conclusion, the Sediment Quality Triad components applied in this study indicated and reinforced similar patterns, as summarized in Table 6.3. They indicated that, despite the emission of contaminants to the environment ceasing a decade ago, the sediments of the upstream part of the Estarreja channel remain contaminated with high levels of metals and metalloid, are bioavailable and induce severe consequences on the resident benthic macrofauna.

Table 6.3 Information provided by the various Sediment Quality Triad elements analyzed in this study. A statistical significant difference between the areas of the contaminated channel (Estarreja, E1 to E4) and the reference channels (Salreu and Canelas) is indicated by a (+) signal, whereas the (-) signal stands for non-significant difference.

Area	Environmental Chemistry	Bioaccumulation	Benthic macrofauna
E1	+	+	+
E2	-	+	+
E3	-	-	-
E4	-	-	-

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6.7 Supporting information

Table 6.A PERMANOVA main test pseudo-*F* values with associated significance between the contaminated (Estarreja) and the reference channels (Salreu and Canelas) areas in 2011, for the whole set of contaminants (column Total) and individually for each metal/metalloid (Cr, Ni, Cu, Zn, As, Cd, Pb and Hg). ns - non-significant ($p > 0.05$); * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Areas	Cr	Ni	Cu	Zn	As	Cd	Pb	Hg	Total
E1 - S1,C1	2.253 (ns)	225.330*	18.253 (ns)	9976.300**	14793.000**	8.333 (ns)	75.000 (ns)	1865.900*	31.310*
E2 - S2,C2	0.120 (ns)	12.813 (ns)	2.253 (ns)	2.848 (ns)	50.480 (ns)	0.333 (ns)	4.757 (ns)	163.930*	4.205 (ns)
E3 – S3,C3	13.887 (ns)	0.170 (ns)	1.552 (ns)	<0.001 (ns)	147.000 (ns)	59.853 (ns)	2.177 (ns)	290.080*	7.782 (ns)
E4 – S4,C4	0.116 (ns)	96.333 (ns)	0.100 (ns)	0.0006 (ns)	1.815 (ns)	0.006 (ns)	0.180 (ns)	12.731 (ns)	0.194 (ns)

Table 6.B Mean length of the specimens of *Scrobicularia plana* used in the bioaccumulation assay and mean individual lyophilized weight (± standard error) at the beginning (T_0) and at the end of the experiment (T_{28}). M = Mira; S = Salreu; C = Canelas; E = Estarreja Channel with areas 1 to 4 (E1 to E4).

Area/Time	Length (cm)	Weight (µg)
M T_0	3.792 ± 0.048	217.744 ± 18.865
M T_{28}	3.797 ± 0.058	177.086 ± 11.171
C T_{28}	3.734 ± 0.047	179.200 ± 11.024
S T_{28}	3.785 ± 0.055	172.200 ± 11.801
E1 T_{28}	3.784 ± 0.082	150.160 ± 15.463
E2 T_{28}	3.782 ± 0.074	157.033 ± 9.378
E3 T_{28}	3.787 ± 0.039	172.664 ± 10.759
E4 T_{28}	3.777 ± 0.050	163.367 ± 14.972

Table 6.C *Scrobicularia plana* bioaccumulation assay PERMANOVA main test pseudo-*F* values with associated significance. Control sediment = M (Mira); reference sediments = S (Salreu) and C (Canelas); contaminated sediments = E1, E2, E3 and E4 (Estarreja). ns - non-significant ($p > 0.05$); * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Areas	Cr	Ni	Cu	Zn	As	Cd	Pb	Hg	Total
M,(S,C)	5.353 (ns)	174.740 (ns)	0.156 (ns)	17.679 (ns)	151.930 (ns)	1.081 (ns)	50.388 (ns)	0.351 (ns)	1.480 (ns)
E1,(S,C)	7.945*	20.426***	0.002 (ns)	6.822*	236.600***	0.020 (ns)	20.328**	58.429*	10.113**
E2,(S,C)	0.315 (ns)	0.765 (ns)	0.198 (ns)	1.439 (ns)	5.407*	1.998 (ns)	36.787***	39.202*	4.712*
E3,(S,C)	1.226 (ns)	0.071 (ns)	0.024 (ns)	0.700 (ns)	1.088 (ns)	0.896 (ns)	0.146 (ns)	0.024 (ns)	0.491 (ns)
E4,(S,C)	1.362 (ns)	5.702*	0.468 (ns)	0.858 (ns)	16.381*	0.466 (ns)	0.487 (ns)	0.020 (ns)	0.563 (ns)

Table 6.D Abundance (*A*), Taxa richness (*S*), Shannon-Wiener diversity (*H'*, $\log_2$) and Margalef's richness (*d*) of the replicate samples and the composite sample per area (n=4) collected in 2013 in the Estarreja (E1 to E4), Salreu (S1) and Canelas (C1) Channels. The value in brackets represents the composite sample in the Estarreja Channel (n=16). The letters before the species/taxon name indicate the Phylum: An - Annelids; Ar - Arthropods; M – Molluscs. The taxa are ranked from highest to lowest total abundance and the four dominant taxa in each area are highlighted, in light gray.

		Abundance*					
	Taxa	E1	E2	E3	E4	S1	C1
An	*Hediste diversicolor*	57	122	75	74	170	138
An	*Alkmaria romijni*		35	1		29	433
An	*Streblospio shrubsolii*	3	211	1	4	36	201
An	Oligochaeta	2			22	150	274
Ar	*Gammarus chevreuxi*			11	4		9
Ar	*Cyathura carinata*			6	3	7	
Ar	Dolichopodidae			7	1	2	
M	*Scrobicularia plana*				2		1
Ar	*Corophium multisetosum*					1	1
Ar	*Heterotanais oerstedti*						1
A (0.01m²)	Individual sample (0.01m²)	15.5	92.0	25.2	27.5	264.5	98.7
	Composite sample		(40.1)				
S (0.01m²)	Individual sample (0.01m²)	1.5	3.0	3.0	3.0	5.2	4.7
	Composite sample	3	3	6	7	8	7
			(8)				
d	Individual sample (0.01m²)	0.17	0.44	0.60	0.58	0.77	0.87
	Composite sample	0.73	0.44	1.55	1.81	1.26	1.31
			(1.90)				
H' ($\log_2$)	Individual sample (0.01m²)	0.28	1.28	0.87	0.87	1.92	1.23
	Composite sample	0.48	1.31	1.31	1.51	1.96	1.81
			(1.75)				

*The species abundance values correspond to the sum of the 4 replicate samples per area.

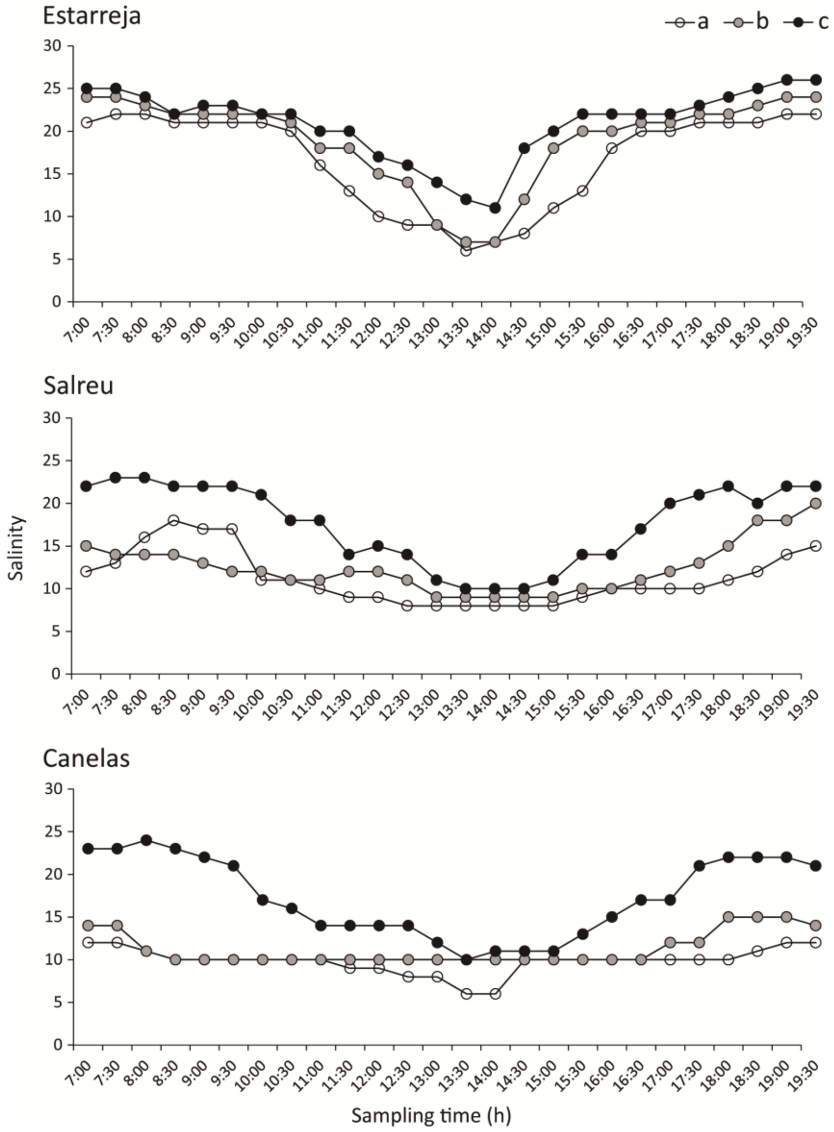

Figure 6.A Temporal variation of bottom water salinity during a tidal cycle in the sampling sites a to c in the Estarreja, Salreu and Canelas Channels, Ria de Aveiro, Portugal.

Chapter 7. Functional and structural benthic indicators in the assessment of estuarine contaminated sediments

7.1 Abstract

The Estarreja Channel, Ria de Aveiro, Portugal, received industrial effluents for more than 70 years. Despite the discharges stopped a decade ago, a recent study using the Sediment Quality Triad approach showed the negative ecological effects still associated with the contaminated sediments. The problem area being located within the mesohaline salinity gradient, posed additional diagnostic challenges, as the resident benthic community was naturally impoverished and included stress tolerant species, as expressed in the 'estuarine quality paradox'. The null hypothesis of no significant difference between the impacted and reference channels was tested using the composition and abundance of the benthic community conventionally sampled by a hand-held corer, but also captured in leaf-bags used in an experimental decomposition study. Synthesis indicators, including primary (*S*) and derived (*H'*) biological variables, as well as taxonomic (AMBI, M-AMBI) and non-taxonomic indices (ISS) and functional indicators (decomposition rates of several substrates), were also statistically tested.

Keywords: Heavy metals contamination; *Phragmites australis*; *Fucus vesiculosus*; Macroinvertebrate communities; Ecological quality assessment; Ria de Aveiro

7.2 Introduction

Estuaries have long attracted human settlement, sometimes with severe environmental consequences, justifying the importance of impact assessment of human activities in these systems (Ferreira et al., 2004; Chapman et al., 2013). The resident macroinvertebrate communities have also long received special attention on such impact assessment and water management studies, being one of the diagnostic biological elements included in the European Water Framework Directive (WDF, 2000/60/EC). Several quality indices have since been developed, mostly taxonomic based (AMBI and M-AMBI, Borja et al., 2000; 2003; BQI, Rosenberg et al., 2004), but also non-taxonomic, based namely in body size descriptors, such as biomass. Such indices can be based on the overall biomass distribution (Gray and Mirza, 1979; Clarke, 1990; De Eyto and Irvine, 2007), but also on size distribution metrics, namely skewness (ISD) (Reizopoulou and Nicolaidou, 2007), size diversity (Quintana et al., 2008), the proportion of large individuals or size-classes (Basset et al., 2008) or the sensitivity/tolerance to stress of macroinvertebrate body size classes (by weight) (ISS) (Basset et al., 2012). These approaches are nevertheless all structural, in the sense that they capture an image of the benthic community at a specified moment in time. Aquatic bio-monitoring studies using the resident macroinvertebrate communities and including structural as well as functional approaches are much less common. Most examples come from freshwater ecosystems, namely studying leaf-litter decomposition rates and the structural characteristics of the associated benthic invertebrate community (Pascoal et al., 2003; Bergfur et al., 2007; Castela et al., 2008).

This study focused on the use of structural and functional indicators of the benthic macrofauna community in a mesohaline area of Ria de Aveiro, Western Portugal, where the Estarreja Chemical Complex used to discharge industrial effluents. These discharges originated a strong contamination of the superficial sediments in the upper areas of the Estarreja Channel, (Hall et al., 1985; Lucas et al., 1986; Pereira et al., 1998a; Costa and Jesus-Rydin, 2001; Monterroso et al., 2003; Pereira et al., 2009). A recent study using multiple lines of evidence was conducted in this area by Lopes et al. (2014) and showed that although the emission of contaminants had stopped a decade ago, sediments remained contaminated with high metals and metalloid levels, mainly Hg and As, that these were bioavailable, bioaccumulated and had negative effects on the resident benthic community. In this work we extended the study of the benthic community sampled with corers to that collected with mesh-bags in a decomposition experiment using various substrates, namely *Phragmites australis*, *Fucus vesiculosus*, and an artificial control substrate. We wish to compare the benthic communities sampled by these various methods and their ability to perceive the problems associated with the contaminated sediments. Synthesis descriptors, including primary and derived biological variable, biotic structural indices, based in the taxonomic characteristics of the samples or in body size properties, as well as functional indicators related to the decomposition rates of the various experimental substrates, were all tested under the null hypothesis of no significant differences between the contaminated and the reference channels.

7.3 Material and methods

7.3.1 Study area

Ria de Aveiro is located on the Northwestern coast of Portugal, between 40º38`N and 40º57`N, with maximum width and length of 10 and 45 km, respectively. This system includes four main Channels, Mira, Ílhavo, Espinheiro and S. Jacinto, characterized by extensive intertidal mud and sand flats, salt marshes and islands. All the channels receive freshwater inputs and the Ria connects with the sea throughout an artificial inlet (Figure 7.1). This study was conducted in three smaller channels, one contaminated (Estarreja) and two reference (Salreu and Canelas), discharging to the Laranjo Bay, located in the central area of Ria de Aveiro (Figure 7.1). Estarreja Channel received continuous industrial discharges for decades which induced an environmental contamination gradient (Lopes et al., 2014).

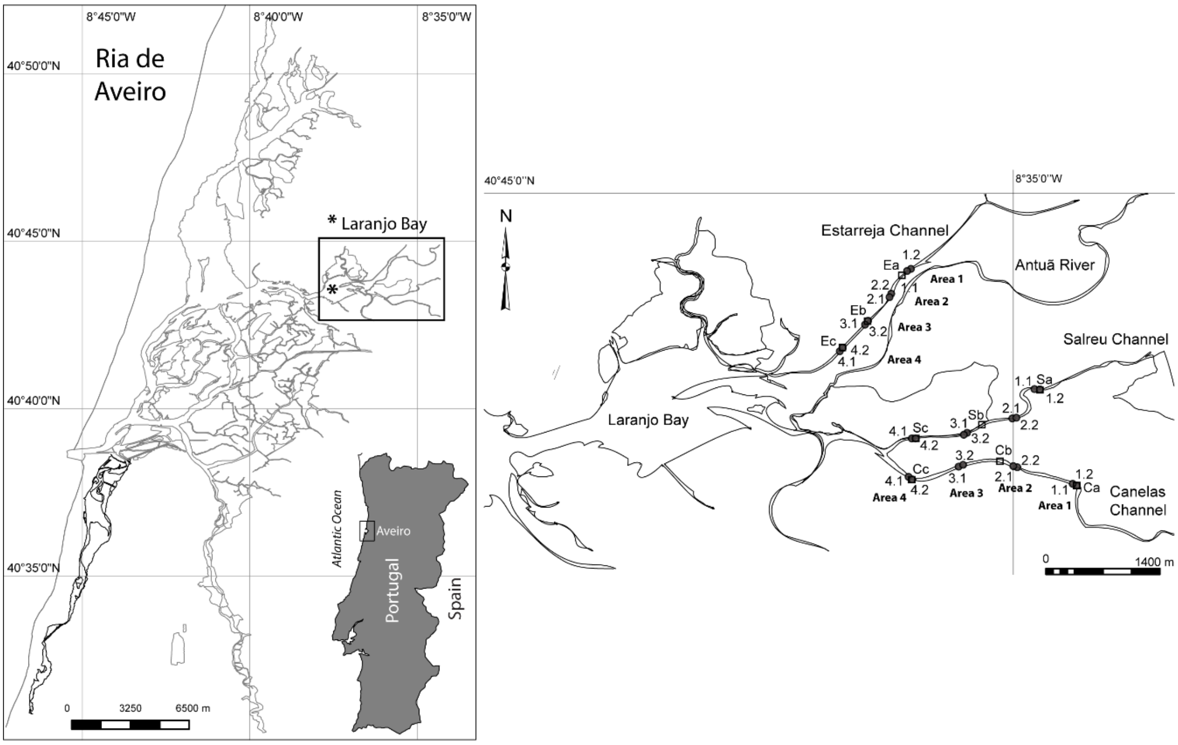

Figure 7.1 Sampling sites in Estarreja, Salreu and Canelas Channels in Ria de Aveiro, Western Portugal, including for the study of bottom water salinity (Ea to Ec, Sa to Sc and Ca to Cc, respectively).

7.3.2 Field and laboratory procedures

This study was performed during winter 2011 (February - April), in a total of four areas per channel and two sampling sites per area (Figure 7.1). Four corer sediment replicates were collected per site, totaling 96 replicates, 32 per channel. Each individual sample was collected with a 0.01 m^2 hand-held corer and placed in a plastic container, brought to the laboratory, washed through 0.5 mm mesh sieve and the residue preserved in 70% ethanol. In the same sites, an experimental field study of the decomposition of dry *Phragmites australis* and *Fucus vesiculosus* was undertaken, using the leaf-bag technique (Petersen and Cummins, 1974). Leaves of *P. australis* used in the experiment were collected simultaneously, from the same area, in the growing season and before the natural senescence. The algae were collected at the same time. Both species were air dried and later oven-dried to constant weight (60 °C for 72 h). Aliquots of 3.000 ± 0.005 g dry weight were placed in 5 mm mesh bags. Leaves were cut into 8 cm long fragments excluding the basal and apical parts. At the beginning of the experiment (day 0), the mesh-bags with dried *Phragmites australis* leaves, *Fucus vesiculosus* and with an artificial substrate used as control (plastic pieces), were placed in the field sites, at the bottom, in the subtidal. In each site, three replicates of each substrate were collected at days 3, 7, 15 and 30. Each replicate was placed in a separate plastic container, brought to the laboratory, washed through a 0.5 mm mesh sieve and the residue preserved in 70% ethanol for the macrofauna identification. The remaining biomass determinations and the decay of *Fucus vesiculosus* and *Phragmites australis* followed the methodology previously used by Lopes et al. (2011) in the decomposition study of these species. The decay rate (k) for each species was obtained by modeling the remaining biomass as a negative exponential decay function, from day 0 to 15 (k_{15}) and from 0 to 30 (k_{30}).

At the end of the experiment, a biological data matrix was prepared with the macroinvertebrate species/taxa and their abundance per replicate, per site for the corer samples and also per sampling time, for each substrate in the mesh-bag samples. This data was used to calculate synthesis descriptors, namely primary and derived biological variable and biotic indices. For the calculation of the non-taxonomic Index of Size Spectra Sensitivity (ISS, Basset et al., 2012), the benthic specimens were individually measured. Linear measurements of body size were obtained to the nearest 0.001 mm using a stereoscope and image analysis software. For the Insecta, Crustacea and Polychaeta the body length was measured as the distance from the anterior part of the head to the end of the last abdominal segment (excluding cerci, antennae and other appendages). The body length was measured as total shell length for Gastropoda and as valve length at the longest point for Bivalvia. The individual biomass was then estimated, whenever possible, from the length-mass relationship obtained per species in a smaller study previously conducted with samples from the Mira Channel (unpublished data) and from the length-mass relationships indicated by Rosati et al. (2012), assuming a relative invariance within and among populations and species pertaining to a same family or order.

7.3.3 Data analysis

The benthic macrofauna data were represented by the abundance of species per replicate and per site for the corer samples and per site and per sampling time for the mesh-bag samples. Primary and derived biological descriptors, namely the number of species/taxa (*S*) and the Shannon-Wiener diversity index (*H'*, $\log_2$), were calculated considering the individual replicates and the composite sample per site.

The AZTI Marine Biotic Index, AMBI (Borja et al., 2000), the multimetric AMBI, M-AMBI (Borja et al., 2003; Muxika et al., 2007) and the Index of Size Spectra Sensitivity, ISS (Basset et al., 2012) were calculated with the composite sample per site for the *Phragmites autralis*, the *Fucus vesiculosus* and the artificial substrate mesh-bags (3 replicates x 4 sampling times) and the corer samples (4 replicates). The AMBI index (Borja et al., 2000) is a frequent component of multimetric indices under scrutiny within the Ecological Quality Assessment of superficial waters in the European Union's Water Framework Directive (WDF, 2000/60/EC). The multimetric M-AMBI index combines the species richness, the Shannon-Wiener diversity and AMBI (Borja et al., 2003; Muxika et al., 2007). Both were calculated using the software AMBI v4.1 (www.azti.es). The threshold values used to translate the M-AMBI values into ecological quality statements (EQS) were based upon the European intercalibration exercise (Borja et al., 2007; 2009): 0 ≤ Bad ≤ 0.20 < Poor ≤ 0.38 < Moderate ≤ 0.53 < Good ≤ 0.77 < High > 1. The index ISS is based on the sensitivity/tolerance of body-size (by weight) macroinvertebrate classes as described by Basset et al. (2012). This index was calculated with and without a species richness correction factor. When using this correction, the values suggested by Basset el al. (2012) were considered: 1 for all samples with more than 5 taxa, 0.8 for 4 and 5 taxa, 0.4 for 2 and 3 taxa, and 0.2 for samples with 1 taxon. The EQS for this index was according to the boundaries established by Basset et al. (2013), considering Ria the Aveiro as a large ecosystem, with an average tidal amplitude > 0.5 m and a salinity < 30 in the study areas. All of our samples were obtained in a mesohaline area and so, the boundaries were Azoic = 0 < Bad < 0.8 ≤ Poor < 1.5 ≤ Moderate < 1.9 ≤ Good < 2.8 ≤ High ≤ 6.0.

All benthic data, the multispecies abundance, the synthesis indices and the decay rates were tested for the null hypothesis of no significant differences between the contaminated and the reference channels. A model with two factors was used: contamination, fixed with two levels, and channels, random and nested in the contamination (only the reference channels could be replicated). Also, knowing from Lopes et al. (2014) that the upstream areas of the impacted channel (areas 1 and 2, cf. Figure 7.1) were more contaminated than the downstream areas (3 and 4), all tests paired the data from areas [1 - 2] and [3 - 4], and the model was run separately for the two groups. The hypotheses testing were based on the composite sample of the replicates per site. This strategy allows to overcome low redundancy due to the low spcies richness of the individual replicates. For the multispecies abundance data, the resemblance matrix between samples was obtained with the Bray-Curtis similarity coefficient, following standardization and a dispersion weighting for the contamination factor of the original data, as in Lopes et al. (2014) (Clarke et al., 2006b). The benthic data was also represented in ordination analysis, using Non-metric Multidimensional Scaling (NMDS). For the biomass loss, decay rates, the biotic indices and the primary and derived biological variables, the resemblance matrix among samples was obtained with the Euclidean distance.

Hypothesis testing was performed by Permutation Multivariate Analysis of Variance (Anderson, 2001), using the software PRIMER v6 (Clarke and Gorley, 2006), with the add-on PERMANOVA+ (Anderson et al., 2008). This method allows partitioning the variability from a resemblance matrix and test individual terms, including interactions, using permutations (Anderson and ter Braak, 2003). The PERMANOVA tests were run with 9999 Monte Carlo permutations. The pseudo-F values in the main tests and the t-statistic in the pairwise comparisons were evaluated in terms of the significance among levels of the tested factor. Values of $p \leq 0.05$ revealed that the groups differed significantly.

7.4 Results

7.4.1 *Environmental characterization*

According to the Venice System (1959) classification of brackish waters, the study area was classified as mesohaline, although salinity variation reached polyhaline conditions in the downstream part of the Estarreja Channel (site Ec, see Figure 7.1). During a tidal cycle (Figure 7.2), salinity in the Estarreja, Salreu and Canelas Channels ranged respectively between 6 - 22, 8 - 18 and 6 - 12 in area A (upstream), 7 - 24, 9 - 20 and 10 -

15 in area B (mid channel) and 11 - 26, 10 - 23 and 10 - 24 in area C (downstream). Despite the three channels were classified as mesohaline, a salinity gradient was noticed from upstream to downstream in each channel, salinity being lower upstream. At low tide, significant salinity differences were found between areas A and C in the Estarreja Channel ($t = 4.548$, $p = 0.001$) and between areas A,B and A,C in Salreu (A,B: $t = 3.500$, $p = 0.009$; A,C: $t = 3.873$, $p = 0.005$) and Canelas (A,B: $t = 3.207$, $p = 0.013$; A,C: $t = 3.961$, $p = 0.004$). At high tide, significant differences were found between areas A,B and A,C in the contaminated channel (A,B: $t = 4.707$, $p = 0.0002$; A,C: $t = 6.396$, $p = 0.0001$), between areas A,C and B,C in Salreu (A,C: $t = 9.152$, $p = 0.0001$; B,C: $t = 8.008$, $p = 0.0001$) and between all the areas in Canelas (A,B: $t = 4.707$, $p = 0.0004$; A,C: $t = 6.396$, $p = 0.0001$; B,C: $t = 2.512$, $p = 0.022$). Despite these differences along each channel, no significant differences were found between the contaminated and the reference channels at low tide and high tide (low tide: Area A - pseudo-$F = <0.0001$, $p = 1.000$; Area B - pseudo-$F = 0.037$, $p = 0.877$; Area C - pseudo-$F = 31.148$, $p = 0.111$; high tide: Area A - pseudo-$F = 13.776$, $p = 0.164$; Area B - pseudo-$F = 17.926$, $p = 0.148$; Area C - pseudo-$F = 40.333$, $p = 0.100$).

7.4.2 Biomass loss and decay rates

The remaining biomass of each substrate along time showed a similar trend in all channels, with *Fucus vesiculosus* loosing biomass faster than *Phragmites australis* as indicated in Figure 7.3. No significant differences were found between the contaminated and the reference channels for the biomass loss of the macrophyte and the alga in the upstream and downstream areas (upstream: *P. australis* - pseudo-$F = 3.442$, $p = 0.310$; *F. vesiculosus* - pseudo-$F = 0.002$, $p = 0.973$; downstream: *P. australis* - pseudo-$F = 0.334$, $p = 0.668$; *F. vesiculosus* - pseudo-$F = 0.090$, $p = 0.815$) The highest loss of biomass by the alga was reflected in the fastest decomposition rates as shown in Table 7.1. The decay rate (k) was always higher for *Fucus vesiculosus*, considering the two time intervals (k_{15} and k_{30}) and always increased from the upstream to the downstream areas (cf. Table 7.1). No significant differences were found in the decay rates between the contaminated and the reference channels, for the macrophyte or the alga, for both time intervals (k_{15} and k_{30}) and for both the upstream and the downstream areas (upstream: *P. australis* - k_{15} - pseudo-$F = 0.167$, $p = 0.757$; k_{30} - pseudo-$F = 3.853$, $p = 0.291$; *F. vesiculosus* - k_{15} - pseudo-$F = 0.003$, p = 0.969; k_{30} - pseudo-$F = 0.0123$, $p = 0.784$; downstream: *P. australis* - k_{15} - pseudo-F =

6.918, *p* = 0.232; k_{30} - pseudo-*F* = 3.255, *p* = 0.325; *F. vesiculosus* - k_{15} - pseudo-*F* = 2.393, *p* = 0.371; k_{30} - pseudo-*F* = 74.202, *p* = 0.074).

Within each channel, for day 30 no significant differences were found in the decay rates among areas for all channels (Estarreja - pseudo-*F* = 1.534, *p* = 0.333; Salreu - pseudo-*F* = 4.546, *p* = 0.093; Canelas - pseudo-*F* = 3.415, *p* = 0.131) but, for day 15, significant differences were found in the Estarreja and Canelas Channels (Estarreja - pseudo-*F* = 34.876, p = 0.003; Salreu - pseudo-*F* = 3.962, *p* = 0.112; Canelas - pseudo-*F* = 13.184, *p* = 0.016). In the contaminated channel significant differences were found between all the areas, excepting between areas 3 and 4 (1,2 - *t* = 4.502 , *p* = 0.045; 1,3 - *t* = 44.100, *p* = 0.0004; 1,4 - *t* = 6.816 , *p* = 0.020; 2,3 - *t* = 10.983, *p* = 0.008; 2,4 - *t* = 5.559, *p* = 0.031) and in the Canelas Cannel, significant differences were found between areas [1 and 3], [1 and 4] and [2 and 3] (1,3 - *t* = 4.552, *p* = 0.048; 1,4 - *t* = 4.332, *p* = 0.049; 2,3 - *t* = 4.552, *p* = 0.047). The differences found within each channel could reflect the salinity gradient previously reported for each channel. Comparing the decay rates of the contaminated and the reference channels with those obtained by Quintino et al. (2009) and Lopes et al. (2011) for the same substrates in the mesohaline area of the Mira Channel, one of the most pristine channels in Ria de Aveiro (Castro et al., 2006), the decay rates of the macrophyte obtained in this study were lower than those observed by Quintino et al. (2009) but higher than those obtained by Lopes et al. (2011). On the other hand, the decay rates of the alga were much lower in this study, in Estarreja, Salreu and Canelas Channels, than those reported previously for the Mira Channel (Lopes et al., 2011) (cf. Table 7.1).

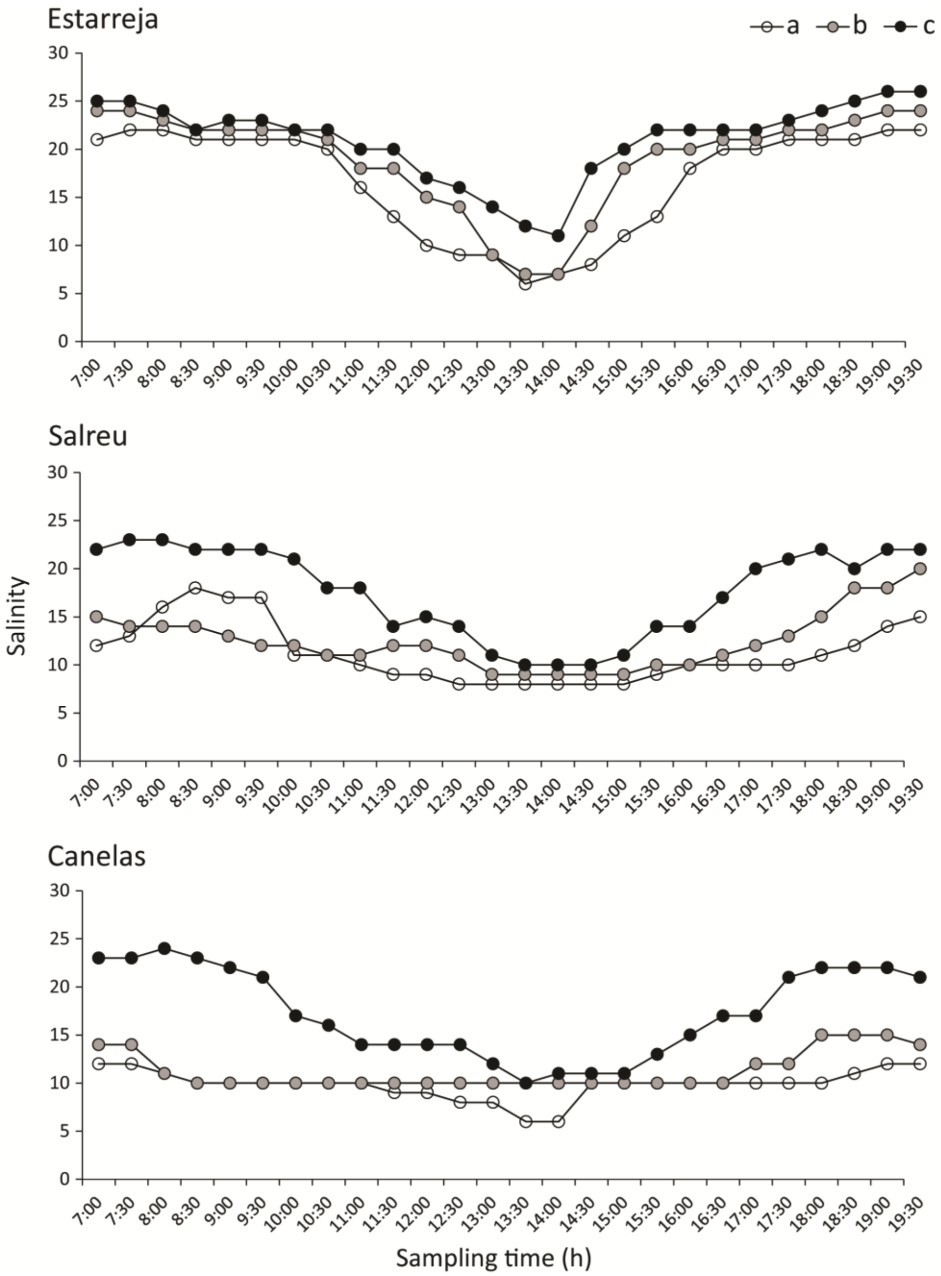

Figure 7.2 Temporal variation of bottom water salinity during a tidal cycle in the sampling sites A to C in the Estarreja, Salreu and Canelas Channels, Ria de Aveiro, Portugal.

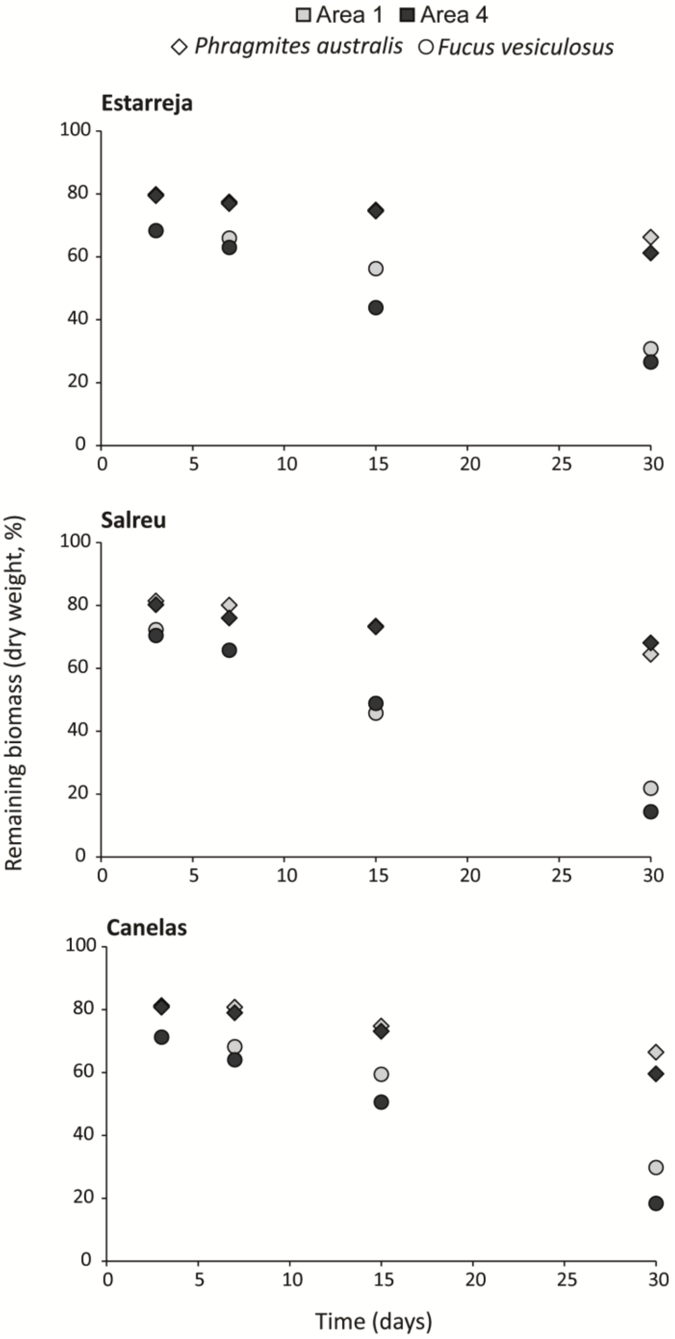

Figure 7.3 Evolution of the remaining biomass of *Phragmites australis* and *Fucus vesiculosus* during the 30-day decay period in areas 1 and 4 in Estarreja, Salreu and Canelas Channels, Ria de Aveiro. Each value corresponds to the mean for all replicates and all sampling sites per area. For clarity the graph only shows the data from the most upstream and downstream areas, 1 and 4, respectively.

Table 7.1 *Phragmites australis* (Pa) and *Fucus vesiculosus* (Fv) decay rates calculated per area (1 to 4) in Estarreja, Salreu and Canelas Channels from day 0 to day 15 (k_{15}) and from day 0 to day 30 (k_{30}). The decay rates for the mesohaline area in Mira Channel obtained by Quintino et al. (2009) (A) and by Lopes et al. (2011) (B) are also shown. n.a. - not available.

		Estarreja		Salreu		Canelas		Mira (A)		Mira (B)	
Substrate	Area	k15	k30	k15	k30	k15	k30	k15	k30	k15	k30
Pa	1	0.024	0.016	0.024	0.017	0.023	0.016	0.026	0.020	0.020	0.015
	2	0.023	0.018	0.023	0.017	0.022	0.017				
	3	0.024	0.017	0.024	0.021	0.025	0.018				
	4	0.024	0.018	0.026	0.016	0.025	0.019				
Fv	1	0.045	0.041	0.051	0.051	0.041	0.040	n.a.	n.a.	0.085	0.073
	2	0.047	0.044	0.050	0.049	0.041	0.040				
	3	0.053	0.045	0.055	0.051	0.051	0.055				
	4	0.059	0.048	0.052	0.061	0.051	0.055				

7.4.3 Benthic macrofauna

Tables 7.A to 7.D, given as supporting information, show the distribution of total abundance per species collected with each sampler in each area per channel. The relative proportion of Annelids, Arthropod, Molluscs and other groups is given in Figure 7.4. Contrarily to the reference channels (Salreu and Canelas), the corer samples obtained in the contaminated channel (Estarreja) were devoid of Arthropods upstream, in the most contaminated areas (Figure 7.4). This pattern was not observed in the mesh-bag samples for any of the substrates (cf. Figure 7.4). In the corer samples, the Annelids *Alkmaria romijni*, *Streblospio shrubsolii*, *Hediste diversicolor* and Oligochaeta were dominant in all areas of the contaminated and the reference channels, except in areas 3 and 4 of the Estarreja Channel, where the Oligochaeta was replaced by the Arthropod *Melita palmata* (area 3) or by the Annelid *Capitella* spp. (area 4) (cf. Tables 7.A to 7.D, supporting information). A different scenario could be observed in the mesh-bag samples, for which the Arthropod *Gammarus chevreuxi* was the dominant species in all channels and all substrates (cf. Tables 7.A to 7.D and Figure 7.4).

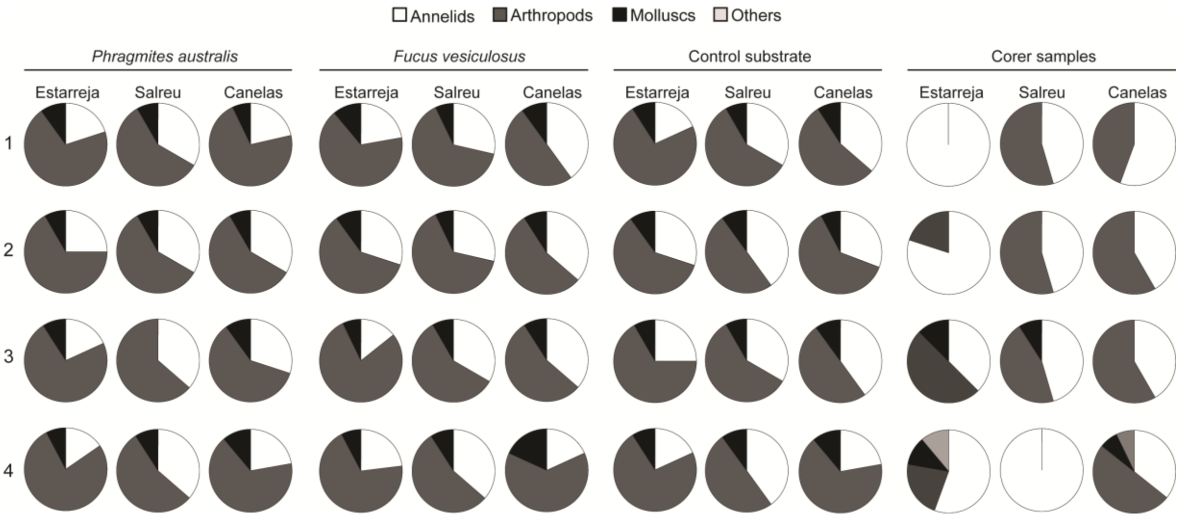

Figure 7.4 Pie charts representing the proportion of Annelids, Molluscs, Arthropods and other groups in the mesh-bags of *Phragmites australis*, *Fucus vesiculosus* and control substrate and in the corer samples collected in the study areas 1 to 4, in the Estarreja, Salreu and Canelas Channels.

Ordination analysis of the corer and mesh-bag macrofauna samples are shown in Figure 7.5. Figure 7.5A corresponds to the whole fauna and it was noticed that the mesh-bags and the corer samples were represented in opposite sides of the horizontal axis. This was the result of the differences in the most characteristic species in the two types of samplers, mesh-bags with epifauna dominated by Arthropods and corers with endofauna dominated by Annelids. This is well shown by the species vectors in Figure 7.5A. In the diagram representing the upstream areas, the vertical axis opposed the contaminated and the reference channels. However, the corer and the mesh-bag samples obtained in the Estarreja Channel were represented in opposite sides of the vertical axis (cf. Figure 7.5A). This could be explained by opposite abundance proportion of the Oligochaeta in the mesh-bag and the corers: in the mesh-bags Oligochaeta were more abundant in Estarreja than in the reference channels while the opposite was seen in the corers (cf. Tables 7.A to 7.D, supporting information). This opposite trend was no longer observed in the downstream part of the study area, (cf. Figure 7.5A). The distribution of the Oligochaeta abundance between the samplers in the upstream part of the study area seemed to be a casual event, unrelated to the sediment contamination. When Oligochaeta were excluded from the analysis, the separation of the corer and mesh-bag samples remained evident along the horizontal axis (Figure 7.5B), but now the corer and the mesh-bag samples from the contaminated channel were no longer represented in opposite sides of the vertical axis. Also the mesh-bag samples from the contaminated and the reference channels were no longer separated (cf. Figure 7.5A and 7.5B). In the downstream areas, the distribution pattern of the samples in the ordination diagram was not affected by the removal of the Oligochaeta. The mesh-bags and the corer samples remained in opposite sides of the horizontal axis as a result of the fundamental differences in the most characteristic species of the two types of samplers (Figure 7.5B).

Statistically, when the Oligochaeta were kept in the analysis, the null hypothesis of no significant differences between the contaminated and the reference channels was rejected for all the samplers in the upstream part of the channels, areas 1 and 2, except with the data from *Fucus vesiculosus* (*P. australis*: pseudo-*F* = 32.520, *p* = 0.023; control substrate: pseudo-*F* = 25.516, *p* = 0.016; corer samples: pseudo-*F* = 14.388, *p* = 0.047; *F. vesiculosus*: pseudo-*F* = 8.000, *p* = 0.055, very close to the significance threshold). For the downstream part, areas 3 and 4, the null hypothesis was never rejected (*P. australis*: pseudo-*F* = 1.806, *p* = 0.311; *F. vesiculosus*: pseudo-*F* = 1.940, *p* = 0.255; control substrate: pseudo-*F* = 2.363, *p* = 0.231; corer samples: pseudo-*F* = 2.959, *p* = 0.197). When the Oligochaeta were excluded from the mesh-bag samples, the null hypothesis in the upstream part was no longer rejected, *P. australis*: pseudo-*F* = 2.013, *p* = 0.272; control substrate: pseudo-*F* = 4.501, *p* = 0.086; *Fucus vesiculosus*: pseudo-*F* = 0.603, *p* = 0.665). In the downstream areas, the removal of the Oligochaeta had no statistical consequences, and the difference between the contaminated and the reference channels remained non-significant for all the mesh-bag samples (*P. australis*: pseudo-*F* = 1.912, *p* = 0.295; *F. vesiculosus*: pseudo-*F* = 1.150, *p* = 0.440; control subatrate: pseudo-*F* = 1.9740, *p* = 0.279).

When the analysis focused on the sub-set of specimens that could be measured, for the purpose of calculating the ISS index, the ordination analysis showed a pattern similar to that obtained after removing the Oligochaeta (Figure 7.A, given as supporting information). This could be explained by the fact that Oligochaeta could not be measured, given that the specimens were not complete. With this sub-set, the null hypothesis of no significant differences between the contaminated and the reference channels was never rejected, in the upstream or downstream areas, in the corer and in the mesh-bag samples (upstream, areas 1 and 2 - *P. australis*: pseudo-*F* = 1.823 , *p* = 0.321; *F. vesiculosus*: pseudo-*F* = 3.927 , *p* = 0.124; control substrate: pseudo-*F* = 3.437, *p* = 0.135; corer samples pseudo-*F* = 6.343, *p* = 0.107; downstream, areas 3 and 4 - *P. australis*: pseudo-*F* = 2.003, *p* = 0.283; *F. vesiculosus*: pseudo-*F* = 0.642, *p* = 0.657; control substrate: pseudo-*F* = 1.854, *p* = 0.311; corer samples pseudo-*F* = 2.741, *p* = 0.198).

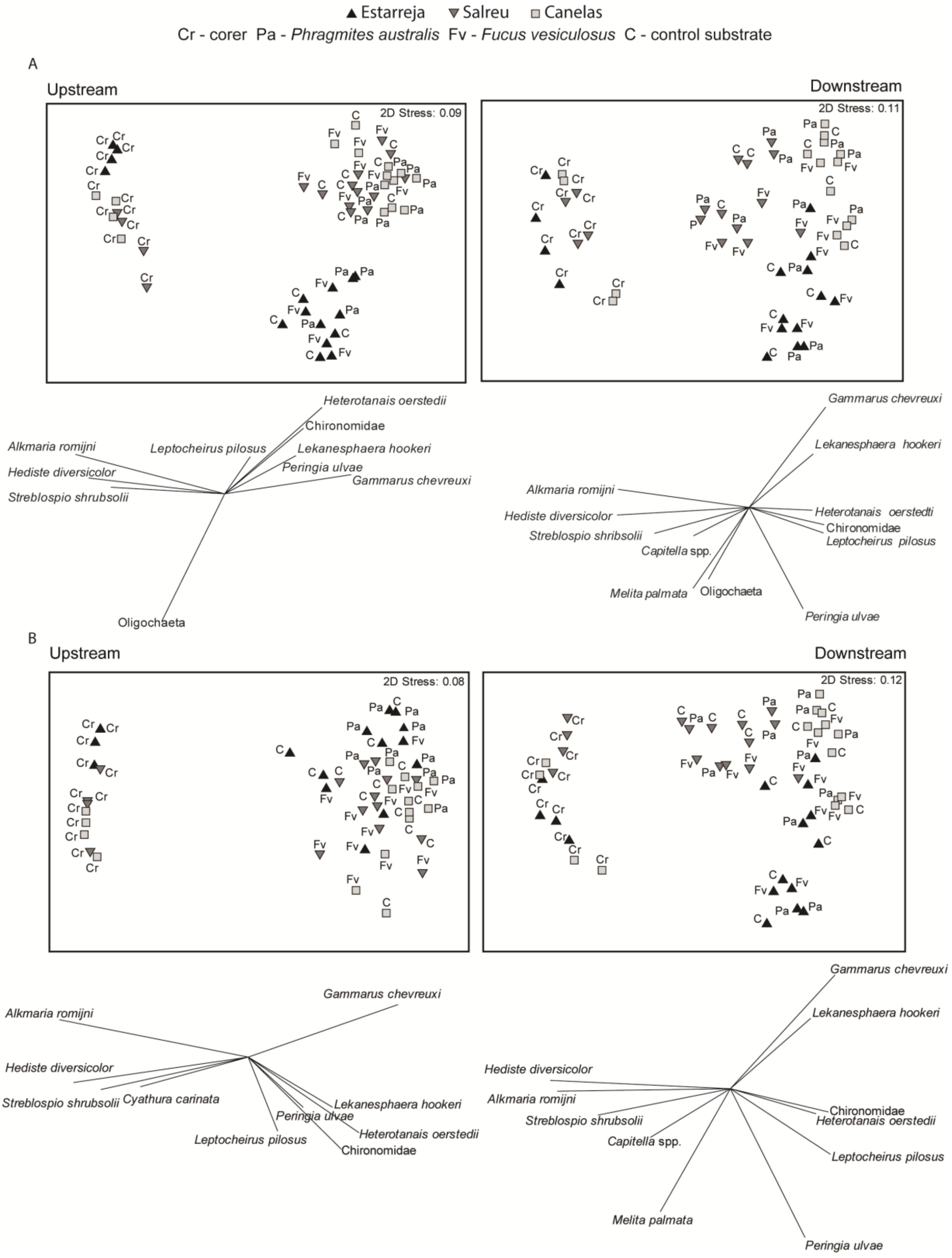

Figure 7.5 Ordination diagram (NMDS) of the macrobenthic fauna collected by the several samplers in the upstream and downstream areas of the study area, with (A) and without (B) Oligoqueta. The vectors represent the species which contribute at least with 5% to the overall sample abundance.

7.4.4 Primary and derived biological variables and biotic indices

The benthic macrofauna abundance in the Estarreja, Salreu and Canelas Channels in the mesh-bag samples (*Phragmites australis*, *Fucus vesiculosus* and the control substrate) and in the corer samples are shown in Tables 7.A to 7.D (supporting information). The taxa richness (*S*) and Shannon-Wiener diversity (*H'*, $\log_2$) were calculated considering the replicate samples and the composite sample per area, which, for the mesh-bags, also combined the individual replicates per sampling time. In the Estarreja Channel, excepting the control mesh-bags, the mean taxa richness increased from the more contaminated to the less contaminated areas, in the corer and the mesh-bags. In the reference channels the mean taxa richness did not show any particular pattern for the mesh-bags nor for the corer samples. The patterns observed with the composite sample were more evident. *H'* was higher in the reference (Salreu and Canelas) than in the contaminated channel (Estarreja), for both the mesh-bags and the corer samples. An increase of *H'* from the more to the less contaminated areas of the Estarreja Channel was also observed in all the samples (corer, *P. australis*, *F. vesiculosus* and control mesh-bags). A similar increase from upstream to downstream was also observed in the Salreu Channel for *P. australis* and *F. vesiculosus* mesh-bag samples, while the opposite was seen in the control substrate. In the corer samples, *H'* was also lower downstream. In the Canelas Channel, *H'* did not show any particular spatial pattern in the mesh-bags and in the corer samples.

The full macrobenthic data and the sub-set representing the specimens that could be measured for the calculation of the ISS index were also used for the calculation of AMBI and M-AMBI indices. The mean values of the primary and derived biological variables and of the indices for the full data set are shown in Table 7.2. The data were individually submitted to hypothesis testing, under the null hypothesis of no significant differences between the contaminated and the reference channels, separately for the upstream and downstream areas. The *S* and *H'* values obtained with the corer samples were able to reflect the sediment contamination, as in both cases, significant differences were found between the contaminated and the reference channels, in the upstream areas (*S*: pseudo-F = 456.330, p = 0.030; *H'*: pseudo-F = 718.510, p = 0.027). *S* and *H'* values did not reject the null hypothesis for the corer samples downstream nor for any of the mesh-bags data. With the AMBI, the null hypothesis was never rejected for any of the samplers: upstream, areas 1 and 2 - *P. australis*: pseudo-F = 3.760, p = 0.310; *F. vesiculosus*: pseudo-F = 0.748, p = 0.552; control substrate: pseudo-F = 0.182, p = 0.751; corer samples pseudo-F = 1.575, p = 0.422; downstream, areas 3 and 4 - *P. australis*: pseudo-F = 1.012, p = 0.506; *F.*

vesiculosus: pseudo-*F* = 0.445, *p* = 0.635; control substrate: pseudo-*F* = 1.961, *p* =0.391; corer samples: pseudo-*F* = 2.063, *p* = 0.228. For M-AMBI, significant differences were only found for the corer samples in the upstream areas. The lower values were obtained in the contaminated channel, the only one with (Poor) EQS (cf. Table 7.2) (*P. australis*: pseudo-*F* = 21.259, *p* = 0.1321; *F. vesiculosus*: pseudo-*F* = 11.547, *p* = 0.184; control substrate: pseudo-*F* = 46.032, *p* = 0.094; corer samples: pseudo-*F* = 5539.100, *p* = 0.010). In the downstream areas, no significant differences were found for any of the samplers.

The relationships between the AMBI and the M-AMBI values and the taxa richness (*S*) and Shannon-Wiener diversity (*H'*), as well as the relationship between *S* and *H'* for the corer samples are shown in Figure 7.6. It was clear the direct relationship between the M-AMBI and the *S* and *H'* values, whit a Spearman rank correlation of 0.95 and 0.80, respectively, both significant at $p < 0.001$ (cf. Figure 7.6). These results indicate that the M-AMBI response to sediment contamination was due to the lower taxa richness and diversity and not to the tolerance/sensitivity of the resident benthic fauna (the AMBI component). The direct relationship between *S* and *H'* was very clear in the upstream areas, with a Spearman rank correlation of 0.80, significant at $p \leq 0.01$ (cf. Figure 7.6). The same null hypothesis was never rejected when AMBI and M-AMBI values were obtained with the sub-set of individuals that could be measured, for the calculation of the ISS index. The ecological quality statement of the corer samples remained the same as before.

The ISS values for the various samplers and areas are also given in Table 7.2. Likewise the M-AMBI values, the null hypothesis with the ISS was only rejected for the corer samples and in the upstream areas (pseudo-*F* = 340.070, *p* = 0.034), where values were lower in the contaminated channel. These areas were the single to present ISS EQS as ‘Moderate’. In all other areas and samplers, ISS was classified as ‘High’, denoting a generalized better ecological quality status than that obtained with M-AMBI (cf. Table 7.2).

Table 7.2 Mean values for the taxa richness (*S*), Shannon-Wiener diversity (*H'*), AMBI, M-AMBI and ISS indices, for the corer (Cr) and the mesh-bags with *Phragmites australis* (Pa), *Fucus vesiculosus* (Fv) and the control substrate (C) in the upstream (areas 1 and 2) and downstream (areas 3 and 4) parts of the Estarreja, Salreu and Canelas Channels. EQS - Ecological Quality Statement. All the values correspond to the mean of the composite sample per site (n = 4 per channel for upstream and for downstream).

		Pa			Fv			C			Cr		
		Estarreja	Salreu	Canelas	Estarreja	Salreu	Canelas	Estarreja	Salreu	Canelas	Estarreja	Salreu	Canelas
Upstream	S	8.75	10.25	10.00	9.00	9.75	8.75	7.75	8.75	9.00	4.00	8.75	8.50
	H'	0.86	1.16	1.28	1.74	1.80	1.87	1.37	1.58	1.71	0.62	1.72	1.67
	AMBI	0.44	0.60	0.73	1.26	1.44	1.30	1.04	1.01	1.22	3.02	3.34	3.14
	M-AMBI	0.64	0.71	0.71	0.64	0.72	0.70	0.63	0.68	0.69	0.33	0.59	0.59
	EQS	Good	Good	Good	Good	Good	Good	Good	Good	Good	Poor	Good	Good
	ISS	3.694	3.662	3.260	3.200	3.104	3.215	3.500	3.396	3.033	1.700	3.289	3.393
	EQS	High	High	High	High	High	High	High	High	High	Moderate	High	High
Downstream	S	9.25	8.50	8.25	10.50	9.75	8.75	8.25	8.75	7.25	6.75	6.50	10.25
	H'	1.79	1.73	1.10	2.36	2.31	1.75	2.08	1.91	1.39	1.05	1.48	1.54
	AMBI	1.67	1.40	0.65	2.03	1.96	1.16	2.26	1.68	0.86	3.02	3.08	3.18
	M-AMBI	0.69	0.67	0.64	0.77	0.74	0.77	0.65	0.68	0.62	0.46	0.50	0.63
	EQS	Good	Good	Good	Good	Good	Good	Good	Good	Good	Moderate	Moderate	Good
	ISS	2.962	3.706	3.582	2.900	3.388	3.302	2.900	3.593	3.418	3.500	2.750	3.467
	EQS	High	High	High	High	High	High	High	High	High	High	Good	High

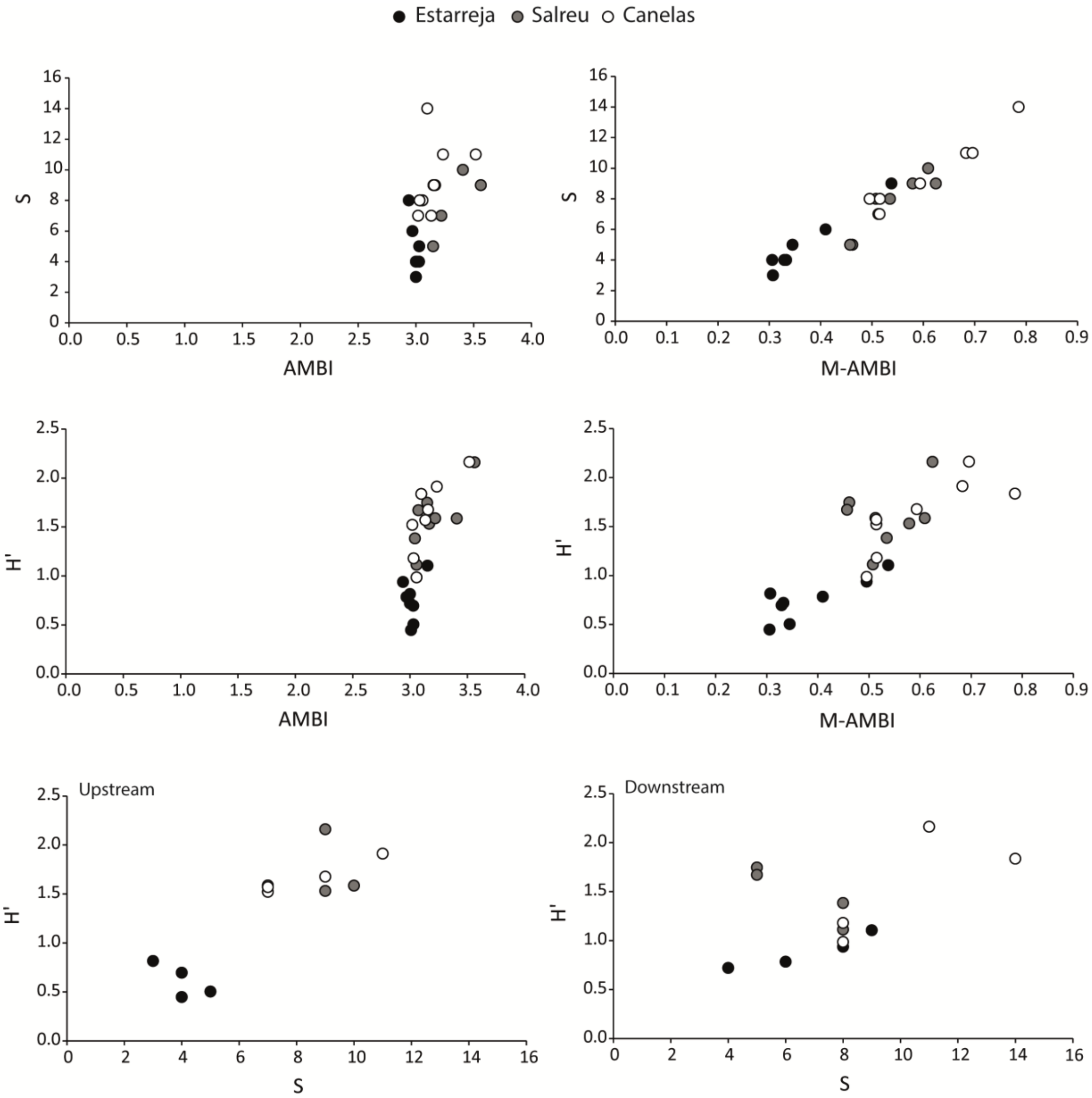

Figure 7.6 Relationship between the AMBI and M-AMBI indices and the taxa richness (*S*) and Shannon-Wiener diversity (*H'*) for the corer samples from the contaminated (Estarreja) and the reference (Salreu and Canelas) channels. The relationship between *S* and *H'* was presented separately for the upstream and downstream areas.

Considering that the ISS calculation involves the abundance distribution of the benthic fauna by the body size classes, the sensitivity/tolerance of macroinvertebrate body size classes to stress and a correction factor associated with the taxa richness, the calculations were also made without the influence of the taxa correction in order to understand its effect on the index response. The relationship between the taxa richness and the ISS values, calculated with and without the correction factor is shown in Figure 7.7, for the corer samples only, where the index rejected the null hypothesis. When the correction factor was applied, the samples with lower taxa richness (less than 5 species) were penalized and classified with a lower ISS value. These samples were mainly located upstream and in the contaminated channel (cf. Figure 7.7). When the taxa richness correction factor was not included, the ISS values increased (cf. Figure 7.7), and the differences between the contaminated and the reference channels in the upstream areas were no longer significant (pseudo-*F* = 0.158, *p* = 0.708). This indicates the overall dependence of the ISS index from species richness, as was the case of the M-AMBI.

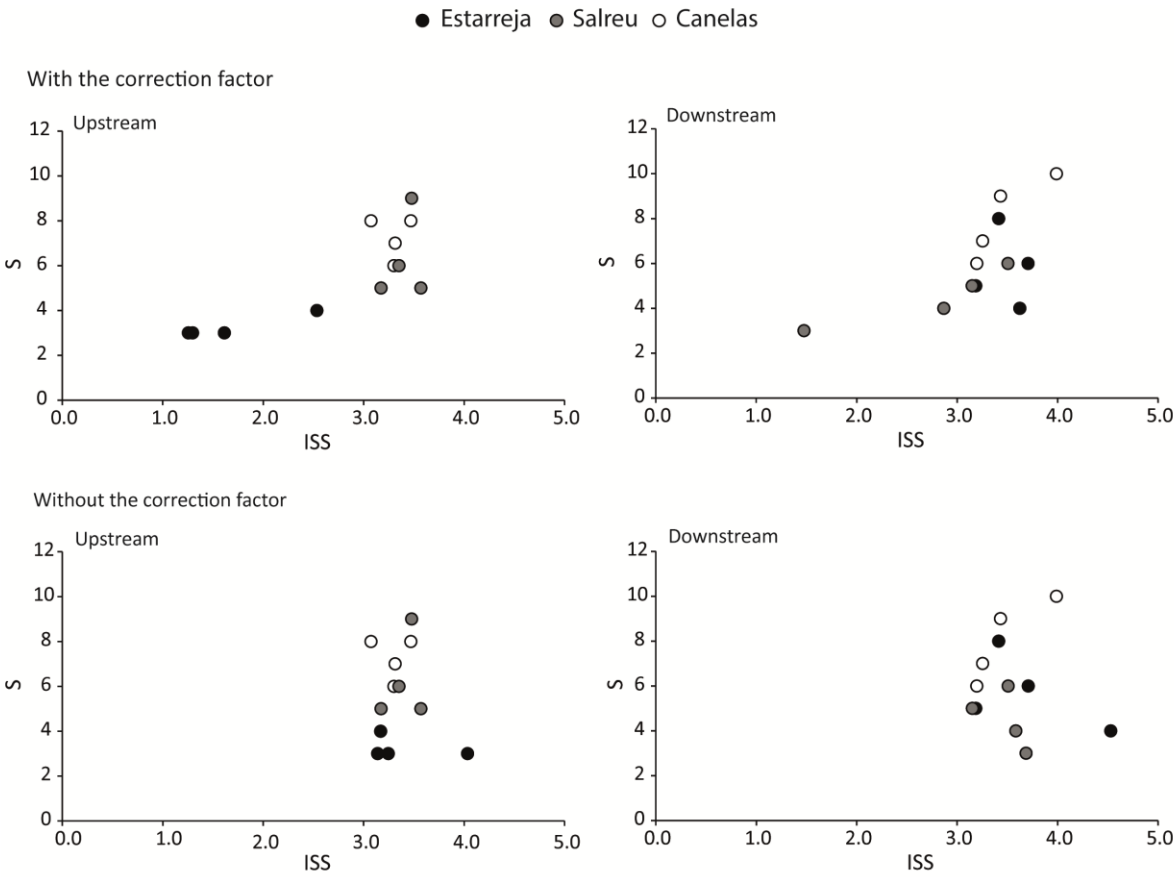

Figure 7.7 Relationship between the ISS index and the taxa richness (S) in the upstream and downstream areas of the contaminated (Estarreja) and the reference (Salreu and Canelas) channels, calculated with (A) and without (B) the taxa richness correction factor.

7.5 Discussion

The bio-assessment of human impact on littoral ecosystems has been intensified since the implementation of the European Water Framework Directive (WDF, 2000/60/EC) in 2000. Functional (e.g. decomposition rate of organic matter) and structural (AMBI, Borja et al., 2000; M-AMBI, Borja et al., 2003; BENTIX, Simboura and Zenetos, 2002; BQI, Rosenberg et al., 2004; BOPA, Dauvin and Ruellet, 2007) indices were since developed but the complexity of the transitional systems advices precautions in their implementation (Elliott and Quintino, 2007). The ecological negative impact of sediment contamination by metals and metalloid has been shown in many studies (Luoma and Rainbow, 2011). The strong contamination of the superficial sediments in the upper areas of the Estarreja Channel, namely by mercury, has been studied over the years by several authors (Hall et al., 1985; Costa and Jesus-Rydin, 2001; Lucas et al., 1986; Monterroso et al., 2003; Pereira et al., 1998a, 2009). In a recent study, Lopes et al. (2014) showed that the metals and metalloid sediment contamination in this channel was bio-available and affected negatively the resident endofauna community, by comparison to reference channels. In this study, we tested if such impact was also detected by the macrofauna sampled in mesh-bags used in a decomposition field experimental study. The decomposition rates, used as a functional indicator, and various synthesis descriptors, including taxonomic and non-taxonomic biotic indices, were also tested. The null hypothesis of no significant differences between the contaminated and the reference channels was not rejected in the downstream part of the study area, the least contaminated (Lopes et al., 2014), by any of these descriptors. Some were able to reject the null hypothesis in the upstream part, as summarized in Table 7.3.

The evaluation of the macrophyte and the alga remaining biomass and decay rates showed the same pattern in the contaminated and the reference channels, with the alga loosing biomass faster than the macrophyte and with higher decay rates downstream, where salinity was also higher. Such overall pattern was similar to that previously observed by Quintino el al. (2009) and Lopes et al. (2011) in Mira Channel, one of the most pristine in Ria de Aveiro (Castro et al., 2006). The decay rates of the alga in this study were about half of those obtained in the Mira Channel. This difference could be related to the strongest currents in the Mira Channel, promoting a faster decay of the alga due to its structural fragility. Near the mouth of the Ria de Aveiro, the tidal currents velocities can be higher than 2 m/s but they are weak in the many innermost small channels (Dias et al., 2000; Vaz and Dias, 2008). No significant differences were found between the contaminated and the reference channels for the biomass loss and decay rates of *P. australis* and of *F. vesiculosus*. Although decomposition has been successfully used as a functional tool to assess organic and inorganic water contamination in small woodland streams (e.g. Pascoal et al., 2003; Castela, et al., 2008), this study indicated that it was not a suitable functional indicator for sediment contamination by metals and metalloid in estuarine systems.

Table 7.3 Overall test results for the upstream part of the contaminated (Estarreja) and the reference (Salreu and Canelas) channels with the various studied descriptors. Pa - *Phragmites australis*, Fv - *Fucus vesiculosus*, C - control substrate and Cr - corer samples. A statistical significant difference between the contaminated and reference channels is indicated by a (+) signal, whereas the (-) signal stands for non-significant difference. n.a. – not applicable.

Descriptors	Pa	Fv	C	Cr
Decomposition rates	**-**	**-**	**-**	**n.a.**
Macrobenthic Community	**-**	**-**	**-**	**+**
Species richness (*S*)	**-**	**-**	**-**	**+**
Species diversity (*H'*)	**-**	**-**	**-**	**+**
AMBI	**-**	**-**	**-**	**-**
M-AMBI	**-**	**-**	**-**	**+**
ISS without correction factor	**-**	**-**	**-**	**-**
ISS with correction factor	**-**	**-**	**-**	**+**

The selection of the sampling technique is one of the most important decisions to make prior to any study focusing on the description of benthic macroinvertebrate communities as they differ in the sampling efficiency on both specific aquatic taxa and benthic habitat types (Rosenberg and Resh, 1993; Somerfield and Clarke, 1997). Grabs and box corers are the most commonly used in transitional waters, particularly in estuaries (Baker and Wolff, 1987), but in lagoons and other shallow transitional ecosystems as well as in streams, hand-nets (Lucena-Moya et al., 2009) and mesh-bags (Cortes et al., 1997) are also common. The macrobenthic fauna sampled by mesh-bags in decomposition studies has been successfully used as a biological indicator of organic and inorganic enrichment in freshwater ecosystems (Pascoal et al., 2003; Bergfur et al., 2007; Castela et al., 2008). Although the use of this sampling procedure is much less common in estuaries, it has been shown to conveniently describe the full salinity gradient (Quintino et al., 2011) and not to differ significantly when the leaf-bags are set-up with different natural substrates or even artificial substrates (Lopes et al., 2013). In this study we used the leaf-bag technique to understand if the fauna sampled by mesh-bags with *P. australis, F. vesiculosus* and a control substrate were able to identify the sediment metals and metallloid contamination as described by Lopes et al. (2014). The benthic macroinvertebrates sampled with the hand-held corer, mostly dominated by endofauna, namely annelids, responded to the sediment contamination. Significant differences were found between the contaminated and the reference channels for the upstream part of the study area when using the whole community data, but also when testing species richness or species diversity alone (cf. Table 7.3). The absence of arthropods in the more contaminated areas of the Estarreja Channel was also indicative of the existing problem (McLusky et al., 1986; Wogram and Liess, 2001; Amiard et al. 2006). The macrofauna associated with the *Phragmites australis*, *Fucus vesiculosus* and the control substrate mesh-bags on the other hand, did not show significant differences between the contaminated and the reference channels, and was mostly dominated by epifauna, namely arthropods. These results suggest that the sediment contamination by metals and metalloid is mainly affecting the resident endofauna but not the epifauna captured by the mesh-bags, so that the leaf-bag technique is not a good approach for this kind of contamination problem.

The biotic indices AMBI and M-AMBI have been successfully used to detect different anthropogenic impacts worldwide in coastal and estuarine systems, including anoxia and hypoxia, eutrophication, nutrient loads, sediment toxicity (metals, PAH) and aquaculture organic enrichment (Borja et al., 2000; 2003; 2007; 2008; 2009b; 2011; Salas et al., 2004; Muxika et al., 2005; 2007; Dauvin et al., 2007; Teixeira et al., 2007; Bigot et al., 2008; Josefson et al., 2008; Pinto et al., 2009; Cai et al., 2013; Li et al., 2013). In this study, the implementation of the taxonomic index AMBI to the corer and the mesh-bag samples did not show significant differences between the contaminated and the reference channels, indicating that the tolerance/sensitivity of species to organic enrichment in which this index is based was not able to identify the sediment contamination by metals and metalloid. Other studies have also indicated the failure of this biotic index, namely concerning alterations induced by dredging activities (Ware et al., 2009) but also in situations of mild subllitoral organic enrichment from sewage outfall (Sampaio et al., 2011), or estuarine intertidal organic enrichment from oyster farming (Quintino et al., 2012). However, when the AMBI was combined with the species richness and the Shannon-Wiener diversity, the M-AMBI values showed significant differences between the upstream areas of the contaminated and the reference channels for the corer samples (cf. Table 7.3). The upstream areas of transitional systems are naturally characterized by a lower diversity and taxa richness (Quintino and Rodrigues, 1989; Rodrigues and Quintino, 1993; Uwadiae, 2009; Rodrigues et al., 2006; 2011; Whitfield et al., 2012). Transitional systems can be exposed to anthropogenic stress but are also naturally stressed and highly variable ecosystems, being difficult to separate these causes of change, as discussed by Elliott and Quintino (2007) under the theme of the ‘Estuarine Quality Paradox’. This study is thus conducted in an inherently problematic area for ecological quality diagnostic, the mesohaline part of the salinity gradient. However, the contaminated and the reference channels were located close to each other and presented similar baseline environmental characteristics, such as salinity, grain-size and organic matter (see also Lopes et al., 2014). The significant lower taxa richness and diversity of the upstream areas of the Estarreja Channel when compared to the reference channels (cf. Table 7.3), should not be seen as a false positive. On the contrary, it is the result of the high levels of sediment contamination by metals and metalloid, which has been shown in another study to cause aggravated mortality on a bioaccumulation assay test species (Lopes et al., 2014). Such diminished taxa richness and diversity was at the base of the rejection of the null hypothesis by the M-AMBI index as well as the ISS index when calculated using the taxa richness correction factor (cf. Table 7.3). Although such statistical significant should not be attributed to a false positive, the interest of using these complex biotic indices could be questioned, when much simpler ones, like the primary and the derived biological variables (*S* and *H'*) allow to reach the same conclusions. The strong direct relationship between M-AMBI and *S* and *H'* was previously exposed namely by Rodrigues et al. (2011) in a comprehensive survey of the benthic population in Ria de Aveiro and by Puente and Diaz, (2008) in highly stress estuaries in Cantabria, along the northern coast of Spain and this study now also shows that ISS can be unfavorably affected by the introduction of a correction factor due to taxa richness. In this case, however, because this factor may not be used in the calculation of the index, it is possible to understand how it affects the ecological statement, even without alteration of the size-class stress sensitivity component of the index, as in this case.

Pollice et al. (2014) studied the response of several indices to a number of anthropogenic pressures (agricultural diffuse inputs, domestic discharges, industrial discharges, fin fisheries) in transitional water ecosystems in Apulia and, despite some disagreement among the indices and contrasting assessment of the ecological status of the same ecosystem, M-AMBI and ISS showed a stronger linear correlation (r = 0.84), confirming that they convey similar information. Authors, however, did not verify if this was due to the influence of a taxa richness (S) upon the two indices. In our study, the ecological quality assessment obtained with the ISS was always higher than that obtained with the M-AMBI. This could be related to the fact that M-AMBI was primarily developed to be used in coastal environments, not in estuaries. Estuaries are naturally stressed environments due to the high variability in their physico-chemical and hydro-morphological characteristics. However, their biota is well-adapted to cope with it without adverse effects (Environmental Homeostasis) (Elliott and Quintino, 2007). Thus, transitional systems may only be regarded as stressful for marine or freshwater-adapted organisms and not for the organisms that naturally live in these stressed ecosystems. Elliott and Quintino (2007) suggested that the characteristics of natural stress in estuaries are similar to those for anthropogenic stress, making the detection of the anthropogenic stress more difficult. It could also be related to the ISS being relatively recent and needing a better intercalibration with other indices. Basset et al. (2012) indicated that ISS was able to respond to organic load and enrichment gradients and also discriminate between anthropogenic stresses and other stress sources characterizing transitional waters. Being a non-taxonomic index, it may be better adapted to respond to stress than other taxonomic based indices. Nevertheless, this work indicate that it was insensitive to the stressor of concern, whereas showing a dependency from species richness that needs careful thinking as the correction factors will not be universally applicable.

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7.6 Supporting information

Table 7.A Taxa richness (*S*) and Shannon-Wiener diversity (*H’*, log$_2$) of the *Phragmites australis* replicate samples and the composite sample per area (n=24 mesh-bags) collected in 2011 in areas 1 to 4 in the Estarreja (E), Salreu (S) and Canelas (C) Channels. The value in brackets represents the composite sample per channel (n=96). The letters before the species/taxon name indicate the Phylum: An - Annelids; Ar - Arthropods; M – Molluscs. The taxa are ranked from highest to lowest total abundance and the four dominant taxa in each area are highlighted, in light gray.

		*Abundance											
	Taxa	E1	E2	E3	E4	S1	S2	S3	S4	C1	C2	C3	C4
Ar	*Gammarus chevreuxi*	587	504	208	110	954	877	813	150	789	706	751	318
Ar	Oligochaeta	378	366	55	53	11	7	15	53	5	14	1	8
M	*Peringia ulvae*	4	10	44	356	5	4		6	10	223	22	57
Ar	*Alkmaria romijni*	30	69	27	16	31	106	198	88	10	24	13	
Ar	Chironomidae	11	7	16	22	94	56	17	23	36	45	9	33
An	*Hediste diversicolor*	3	5	2	6	9	32	34	143	4	12	4	2
Ar	*Heterotanais oerstedii*	1		1	6	47	11	15	6	61	14	5	8
Ar	*Leptocheirus pilosus*	6	3	16	19	10	14	5	1	16	4	8	46
Ar	*Lekanesphaera hookeri*	6	6	3	2	9	6	5	7	22	33	12	7
Ar	*Melita palmata*			3	34			1		1			
An	*Streblospio shrubsoli*		1			8	5	4	29		2		
Ar	*Paragnathia formica*	2	3	1	1		3	1		1		5	
Ar	Copepoda									1	1		
Ar	*Corophium insidiosum*										2		
Ar	*Corophium multisetosum*						2						
Ar	Caenidae				1								
Ar	Dysticidae												1
Ar	Elmidae									1			
Ar	Ephydridae				1								
Ar	*Cyathura carinata*		1										
Ar	Sminthuridae		1										
Ar	Dolichopodidae									1			
Ar	*Corophium* sp.					1							
S	Individual sample	6.50	7.00	6.33	8,33	8,00	8,83	7,67	7,67	8,33	8,67	6,33	6,50
	Composite sample	10	12	11	13	11	12	11	10	14	12	10	9
		(16)				(14)				(17)			
H' (log$_2$)	Individual sample	1.29	1.44	1.91	1,91	1,12	1,26	1,26	2,39	1,10	1,48	0,73	1,55
	Composite sample	1.38	1.58	2.10	2,09	1,18	1,30	1,30	2,51	1,15	1,65	0,72	1,69
		(2.05)				(1.60)				(1.41)			

*The species abundance values correspond to the sum of the 24 replicate samples per area.

Table 7.B Taxa richness (*S*) and Shannon-Wiener diversity (*H'*, $\log_2$) of the *Fucus vesiculosus* replicate samples and the composite sample per area (n=24 mesh-bags) collected in 2011 in areas 1 to 4 in the Estarreja (E), Salreu (S) and Canelas (C) Channels. The value in brackets represents the composite sample per channel (n=96). The letters before the species/taxon name indicate the Phylum: An - Annelids; Ar - Arthropods; M – Molluscs. The taxa are ranked from highest to lowest total abundance and the four dominant taxa in each area are highlighted, in light gray.

		*Abundance													
	Taxa	E1	E2	E3	E4	S1	S2	S3	S4	C1	C2	C3	C4
Ar	*Gammarus chevreuxi*	150	136	97	45	399	436	291	67	556	217	418	175
An	Oligochaeta	435	247	163	193	36	59	70	56	6	12	3	33
Ar	Chironomidae	19	45	54	45	255	184	147	29	74	67	25	88
Ar	*Alkmaria romijni*	5	90	18	60	25	149	211	47	7	34	7	
M	*Peringia ulvae*	3	16	19	139	6	1	4	3	10	71	32	62
Ar	*Leptocheirus pilosus*	2	5	5	14	7	4	15	3	7	80	13	34
Ar	*Heterotanais oerstedii*	4	3	2	10	48	15	18	3	30	16	8	30
Ar	*Lekanesphaera hookeri*	12	6	7		5	6	13	9	41	45	25	10
An	*Hediste diversicolor*	2	2	1	4	5	30	23	63	8	13	7	6
Ar	*Streblospio shrubsolii*		8		2	9	4	3	24	1	3	2	
Ar	*Melita palmata*			2	19								
Ar	*Paragnathia formica*			1	1	1	3	1	4		2		2
Ar	Diptera			1		1							1
Ar	*Idotea chelipes*				2								
Ar	Copepoda						1	1					
Ar	Caenidae			1									
Ar	Corixidae						1						
Ar	Dysticidae			1									
Ar	Hemiptera											1	
Ar	Colembola				1								
M	*Lymnea peregra*												1
Ar	*Corophium volutator*						1						
Ar	*Procambarus clarkii*					1							
	Individual sample	5.67	7.00	7.83	9.33	7.83	8.33	8.83	8.00	6.83	9.00	7,83	7,67
S	Composite sample	9	10	14	13	13	14	12	11	10	11	11	11
		(17)				(16)				(14)			
	Individual sample	1.27	1.87	2.04	2.39	1.84	1.97	2.25	2.41	1.36	2.37	1,34	2,27
H' ($\log_2$)	Composite sample	1.31	2.17	2.25	2.55	1.94	2.12	2.37	2.84	1.41	2.67	1,41	2,50
		(2.25)				(2.38)				(2.11)			

*The species abundance values correspond to the sum of the 24 replicate samples per area.

Table 7.C Taxa richness (*S*) and Shannon-Wiener diversity (*H’*, $\log_2$) of the control replicate samples and the composite sample per area (n=24 mesh-bags) collected in 2011 in areas 1 to 4 in the Estarreja (E), Salreu (S) and Canelas (C) Channels. The value in brackets represents the composite sample per channel (n=96). The letters before the species/taxon name indicate the Phylum: An - Annelids; Ar - Arthropods; M – Molluscs. The taxa are ranked from highest to lowest total abundance and the four dominant taxa in each area are highlighted, in light gray.

		*Abundance											
	Taxa	E1	E2	E3	E4	S1	S2	S3	S4	C1	C2	C3	C4
Ar	*Gammarus chevreuxi*	306	173	69	44	452	471	287	83	267	144	307	112
A	Oligochaeta	469	244	102	108	28	10	14	27	8	13	1	25
A	*Alkmaria romijni*	25	139	35	72	28	63	195	33	3	12	8	4
Ar	Chironomidae	18	13	22	12	128	85	22	6	55	25	12	44
M	*Peringia ulvae*	2	19	50	195		1	3	2	7	91	16	28
A	*Hediste diversicolor*	4	10	3	4	12	37	23	84	3	2	6	
Ar	*Heterotanais oerstedti*	3	2	4	4	55	13	12	3	29	12	5	22
Ar	*Leptocheirus pilosus*	2	2	3	19	8	12	7		6	1	5	10
A	*Streblospio shrubsoli*		4	2		10	3	3	22	1	6	2	
Ar	*Lekanesphaera hookeri*		2	1		3	4		2	9	12	2	2
Ar	*Melita palmata*				12								
Ar	*Paragnathia formica*	3		1					2				
Ar	Diptera				1	1					1		
Ar	Corixidae	1						1					
Ar	Acarina			1							1		
Ar	*Corophium insidiosum*									1	1		
Ar	Elmidae							1					
Ar	Hemiptera	1											
Ar	*Idotea chelipes*												1
Ar	Copepoda				1								
Ar	*Corophium multisetosum*					1							
Ar	Collembola							1					
	Individual sample	6.17	6.50	6.67	7,33	7,17	7,50	8,00	6,50	6,50	7,17	5,17	5,67
S	Composite sample	11	10	12	11	11	10	12	10	11	13	10	9
		(17)				(16)				(14)			
	Individual sample	1.35	1.79	2.09	2,14	1,65	1,60	1,69	2,18	1,60	1,89	0,91	1,82
H' ($\log_2$)	Composite sample	1.43	2.03	2.46	2,36	1,82	1,68	1,85	2,42	1,64	2,30	1,06	2,33
		(2.23)				(2.08)				(1.96)			

*The species abundance values correspond to the sum of the 24 replicate samples per area.

Table 7.D Taxa richness (S) and Shannon-Wiener diversity (H', $\log_2$) of the corer replicate samples and the composite sample per area (n=8) collected in 2011 in areas 1 to 4 in the Estarreja (E), Salreu (S) and Canelas (C) Channels. The value in brackets represents the composite sample per channel (n=32). The letters before the species/taxon name indicate the Phylum: An - Annelids; Ar - Arthropods; M – Molluscs. The taxa are ranked from highest to lowest total abundance and the four dominant taxa in each area are highlighted, in light gray.

		*Abundance											
	Taxa	1E	2E	3E	4E	1S	2S	3S	4S	1C	2C	3C	4C
An	*Alkmaria romijni*	1393	1799	1287	1121	697	1301	1387	692	524	1441	1925	346
An	*Streblospio shrubsolii*	159	50	77	110	157	372	243	570	113	773	232	927
An	*Hediste diversicolor*	69	75	138	69	63	279	197	324	130	229	193	226
An	Oligochaeta	9	13		3	186	131	32	54	31	150	47	146
An	*Capitella* spp.				36		5	1	9	4	10		89
Ar	*Corophium multisetosum*					3	1			20	59	4	
Ar	*Melita palmata*			23	1	10	1	1					18
Ar	*Gammarus chevreuxi*					7	1	1		6	6	8	19
Ar	*Cyathura carinata*		3	2	2	13	3			1	2		3
Ar	*Leptocheirus pilosus*			1		1					4	1	7
Ar	Chironomidae					8	1	1			1		2
M	*Scrobicularia plana*				3			1				7	1
Ar	*Heterotanais oerstedtii*										3		6
N	Nemertini ni				5								3
Ar	Dolichopodidae							5		2	1		
Ar	*Lekanesphaera hookeri*						1	1					1
M	*Peringia ulvae*			2									
Ar	*Carcinus maenas*			2									
Ar	*Neomysis integer*											2	
An	*Heteromastus* sp.								1				
An	*Autolytus brachichephalus*					1							
S	Individual sample (0.01m²)	3.25	3.50	4.38	4.13	6.50	5.00	4.63	4.25	5.75	6.88	5.38	7.75
	Composite sample	4	5	8	9	11	11	11	6	9	12	9	14
		(12)				(16)				(17)			
H' ($\log_2$)	Individual sample (0.01m²)	0.75	0.45	0.84	0.86	1.81	1.49	1.26	1.66	1.49	1.74	0.99	1.93
	Composite sample	0.76	0.48	0.88	0.97	1.75	1.57	1.20	1.73	1.66	1.75	1.07	2.08
		(0.79)				(1.63)				(1.80)			

*The species abundance values correspond to the sum of the 8 replicate samples per area.

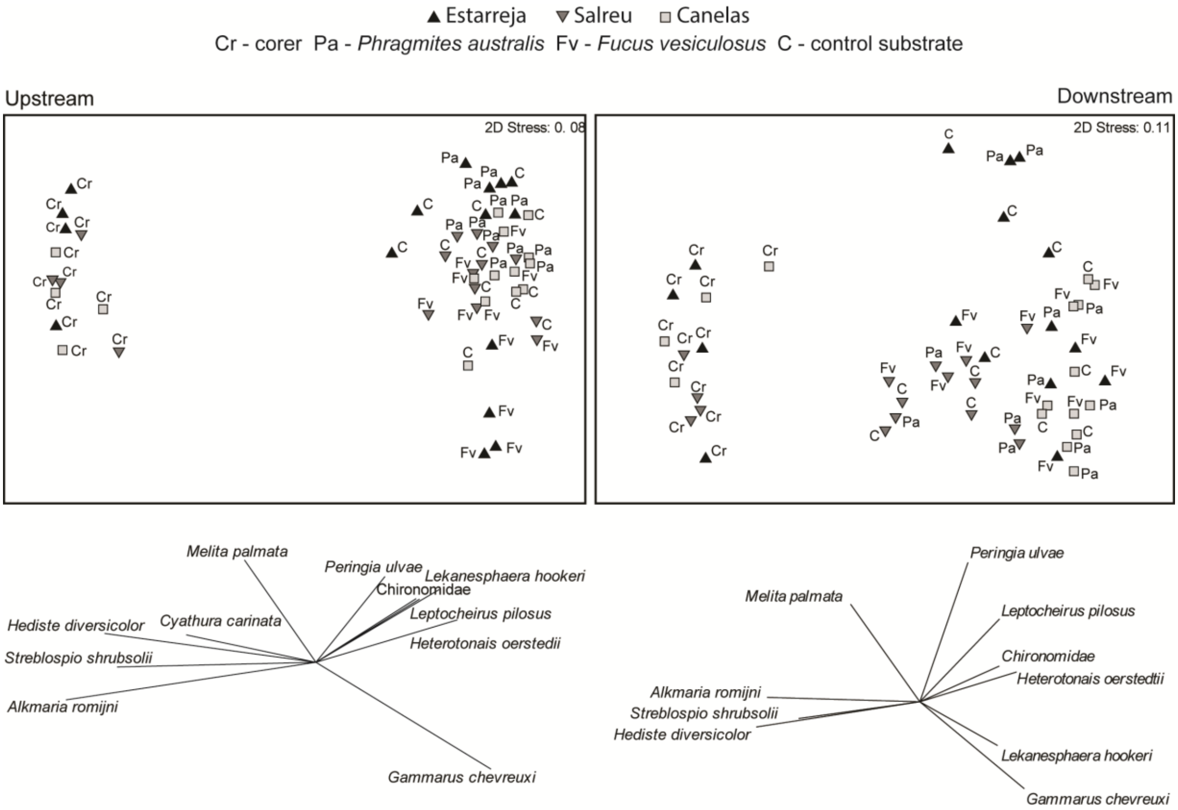

Figure 7.A Ordination diagram (NMDS) of the sub-set of macrobenthic fauna that could be measured for the calculation of ISS. The vectors correspond to the species which contribute at least with 5% to the overall sample abundance.

Chapter 8. Conclusions

8.1 Conclusions

The implementation of the European Water Framework Directive (WDF, 2000/60/EC) has increased the European investigation in order to develop methodologies to assess the Ecological Quality Status of aquatic ecosystems. Several functional, taxonomic and non-taxonomic indices were developed but, due to the high complexity of the transitional systems, the implementation of these indices should be done with some precautions. The present work showed important information about the ability of several taxonomic, non-taxonomic and functional indices to evaluate the environmental quality in transitional systems, being a step forwards in the development of suitable environmental quality indicators.

The main conclusions of this study were synthesized below.

Chapter 2 - *In situ* experimental decomposition studies in estuaries: A comparison of *Phragmites australis* and *Fucus vesiculosus*

The experimental study of decomposition using the leaf-bag technique with the macrophyte *Phragmites australis* and the alga *Fucus vesiculosus* along a full salinity gradient in Mira Channel at days 3, 7, 15, 30 and 60 showed that the alga and the macrophyte presented an opposite trend in the decomposition rate along the salinity gradient, with the decomposition rates of the algae always higher than those of the macrophyte. The decomposition rate of *P. australis* was higher in the mid estuarine area, corresponding to the natural environment of the species. In the case of *Fucus vesiculosus*, the higher decomposition rate was observed in areas of higher salinity, where the alga occurs naturally, and much lower towards the freshwater environment. Thus, the alga, distributed in the euhaline and polyhaline areas of the estuary, is unsuitable to evaluate decomposition in the oligohaline and limnetic reaches of the estuary, and conversely the macrophyte, mainly distributed in the oligohaline and mesohaline part of the estuary, is unsuitable for assessing decomposition in the outer, euhaline areas.

The decomposition rates of *Fucus vesiculosus* and *Phragmites australis* decreased with time ($k_{15} > k_{30} > k_{60}$), with the decomposition rates of the alga always higher than those of the macrophyte.

The use of decomposition rates as a measure of ecosystem integrity or quality status in transitional waters will not be straight forward and must take into account, among others, the test species, the study area positioning along the estuarine gradient, and the time interval for the calculation of the decomposition rate.

Chapter 3 - Macroinvertebrates communities associated with the decomposition of *Phragmites australis* and *Fucus vesiculosus* in transitional systems

The macrofauna communities associated with the decomposition of a macrophyte (*Phragmites australis*) and an alga (*Fucus vesiculosus*) were studied along a full salinity gradient, using the leaf-bag technique and four sampling times (days 3, 7, 15 and 30). A control was set up using an artificial substrate. A subsequent study was also conducted in two other channels representing the mesohaline region, Canelas and Salreu and included empty bags as procedure control.

This study showed that the macrobenthic fauna associated with the decaying (*Phragmites autralis* and *Fucus vesiculosus*) and the artificial substrate showed equally well the benthic succession from the marine to the freshwater areas, in all sampling times.

Arthropods were dominant in all substrates along the estuarine gradient and replaced by annelids in freshwater. No significant differences were found between the benthic communities associated with *P. australis* and *F. vesiculosus*, despite the strong differences in the decay rates, suggesting that these did not seem to be primarily related to the benthic colonizers.

Although the organic substrates sustained a more abundant fauna, the benthic communities did not show significant differences between the organic and the artificial substrates, especially at the level of the species composition, suggesting that the macroinvertebrates may colonize both substrates to feed on the biofilm and/or to seek shelter. The strongly impoverished benthic community sampled by the empty bags reinforced this idea.

Chapter 4 - Variability of body size descriptors in benthic invertebrates along a full salinity gradient sampled by leaf-bags and corers

The abundance distribution of macrobenthic invertebrates body size classes and the variability of length, weight and length-weight relationships of Arthropods, Molluscs and Annelids was studied in a system without anthropogenic stresses, the Mira Channel, using the macrobenthic fauna collected with different sample techniques, the leaf-bag technique with *Phragmites australis* and two mesh-sizes (1 mm and 5 mm) and hand-held corer. The same study was carried out considering individual species with at least three distribution areas along the full salinity gradient.

Significant differences were found between all the salinity areas excepting between the euhaline and polyhaline areas for all the macrobenthic fauna sampled and the sub-set of measured specimens for all the samplers. Despite the exclusion of some dominant taxa in the 1mm leaf-bag and corer samples, mainly Annelids, the sub-set of measured specimens was able to show the benthic succession from the marine to the freshwater areas showed by the original macrobenthic fauna sampled.

The body size abundance distribution of the benthic macroinvertebrates according to the ISS size classes did not show any particular trend from the marine to the freshwater environment for the leaf-bag and corer samples, indicating that a salinity range from 0 to 35 will not act like a masking factor when this index is applied in areas affected by anthropogenic stresses.

Along the salinity gradient significant differences were found in the length, weigh and length-weight relationships of Annelids, Arthropods and Molluscs. These differences occur between the upstream (oligohaline and limnetic) and the downstream (euhaline and polyhaline) areas of the channel, suggesting that these differences could be related with the species succession along the estuarine gradient. At the species level, significant differences were found in the length and weight of the Arthropod *Microdeutopus gryllotalpa* in the 1 mm and the Arthropods *Gammarus insensibilis* and *Melita palmata* 5 mm leaf-bags, which could be related with their preferential distribution areas. The length-weight relationships of individual species, showed significant differences for the Arthropods *Gammarus insensibilis* and *Melita palmata* between their distribution areas in the 5 mm leaf-bags but not in the 1 mm leaf-bags suggesting that this mesh size could condition the free circulation of the individuals, interfering with the corporeal condition of macroinvertebrates inside the bags. In the corer samples no significant differences were found in the length-weight relationship of the Annelid *Streblospio shrubsolii* along its distribution areas.

Chapter 5 - Variability of taxonomic and non-taxonomic biotic indices applied to macroinvertebrates sampled leaf-bags and corers

The variability of taxonomic and non-taxonomic biotic indices was studied in macroinvertebrates sampled along a full salinity gradient in Mira Channel, Ria the Aveiro, using leaf-bags with *Phragmites australis* and two mesh-sizes (1 mm and 5 mm) and a hand-held corer.

No significant differences were found in the AMBI, M-AMBI and ISS values along the salinity gradient for all the samplers, considering all full species data set and the sub-set of measured specimens.

When comparing AMBI and M-AMBI calculated from the full species data and the sub-set of measured specimens, no significant differences were found for none of the samplers, indicating that the exclusion of some specimens and even some taxa from the original data set did not affect the results.

When all the macrofauna sampled was considered, the ecological quality statement (EQS) obtained with the 1 mm and 5 mm leaf-bags along the salinity gradient was the same. Considering only the sub-set of the measured specimens, the EQS in the 5 mm leaf-bags decreased. The EQS of the corer samples considering only the sub-set of measured specimens increased in the oligohaline area which could be explained by the exclusion of annelids. The EQS of the corer samples was lower than that of the leaf-bags due to the difference between the dominant species in both types of samples, Annelids in the corers and Arthropods in the leaf-bags.

The EQS obtained with the ISS index was higher than that obtained with the M-AMBI index in the leaf-bags. An opposite trend was observed in the corer samples, which could be related to the low taxa richness and abundance of the corer samples which compromise the ISS index calculation. The inability of the M-AMBI to classify the Mira

Channel in such a good ecological quality as the ISS could be related to the fact that M-AMBI has been developed to coastal environments, in which the presence of species that naturally occur in transitional systems are indicative of environmental problems or to the necessity of the ISS to be better intercalibrated with other indices.

Chapter 6 - Ecological effects of contaminated sediments following a decade of no industrial effluents emissions: the Sediment Quality Triad approach

The Estarreja Channel received industrial effluents from the Estarreja Chemical Complex (CQE) for more than 70 years. A decade after the emissions were stopped, sediments from that channel were statistically compared to sediments from reference channels using the Sediment Quality Triad approach.

Several decades of industrial discharges created a strong superficial sediments contamination by Cr, Ni, Cu, Cd, Zn, Pb and, mainly, Hg and As, in the upper areas of the Estarreja Channel where the industrial discharge was located.

In a bioaccumulation assay with the bivalve *Scrobicularia plana*, the highest mortality of the bivalve was observed in the most contaminated sediments and the bioaccumulation within tissues strongly related with the sediments metals and metalloid concentrations.

The resident macroinvertebrates community also showed significant differences between the impacted and the reference channels in the upper areas, where the community was most affected, resulting mainly in the absence of Arthropods in these areas.

All three elements of the Sediment Quality Triad rejected the null hypothesis of no significant differences between the contaminated and the reference channels, indicating that, despite the industrial emissions cessed a decade ago, sediments remain polluted with high levels of metals and metalloid, available to bioaccumulation and with severe community level consequences (Table 8.1).

Table 8.1 Information provided by the various Sediment Quality Triad elements analyzed in this study. A statistical significant difference between the areas of the contaminated channel (Estarreja, E1 to E4) and the reference channels (Salreu and Canelas) is indicated by a (+) signal, whereas the (-) signal stands for non-significant difference.

Area	Environmental Chemistry	Bioaccumulation	Benthic macrofauna
E1	+	+	+
E2	-	+	+
E3	-	-	-
E4	-	-	-

Chapter 7 - Functional and structural benthic indicators in the assessment of estuarine contaminated sediments

In this study the benthic communities sampled by a hand-held corer were compared to those captured in leaf-bags used in an experimental decomposition study with *Phragmites australis* and *Fucus vesiculosus*. An artificial substrate was also used as control. Synthesis indicators, including primary (*S*) and derived (*H'*) biological variables, as well as taxonomic (AMBI, M-AMBI) and non-taxonomic indices (ISS) and functional indicators (decomposition rates) were statistically tested under the null hypothesis of no significant difference between an impacted (Estarreja) and reference channels (Canelas and Salreu).

The null hypothesis of no significant differences between a contaminated and the reference channels was not rejected in the downstream part of the study area, the least contaminated, by any of the descriptors used in the corer and leaf-bag samples.

No significant differences were found between the contaminated and the reference channels for the biomass loss and decay rates of *P. australis* and *F. vesiculosus*, indicating that the decay rate is not a suitable functional indicator for sediment contamination by metals and metalloid in estuarine systems.

Significant differences were found between the contaminated and the reference channels for the corer samples when using the whole community data, suggesting that the sediment contamination by metals and metalloid is mainly affecting the resident endofauna but not the epifauna captured by the leaf-bags, indicating that the leaf-bag technique is not a good approach for this kind of contamination problem.

The implementation of the taxonomic index AMBI to the corer and the mesh-bag samples did not show significant differences between the contaminated and the reference channels, indicating that the tolerance/sensitivity of species to organic enrichment in which this index is based was not able to identify the sediment contamination by metals and metalloid.

The significant lower taxa richness and diversity of the upstream areas of the Estarreja Channel due to the sediment contamination by metals and metalloid was at the base of the rejection of the null hypothesis by the M-AMBI index as well as the ISS index when calculated using the taxa richness correction factor. Although such statistical significance should not be attributed to a false positive, the interest of using these complex biotic indices could be questioned, when much simple ones, like the primary and the derived biological variables (*S* and *H'*) allow to reach the same conclusions.

The ecological quality assessment obtained with the ISS was always higher than that obtained with the M-AMBI. This could be related to the fact that M-AMBI was primarily developed to be used in coastal environments, not in estuaries. It could also be related to the ISS being relatively recent and needing a better intercalibration with other indices.

The results obtained with the study of synthesis indicators (*S* and *H'*) biological variables, taxonomic (AMBI, M-AMBI) and non-taxonomic indices (ISS) and functional indicators (decomposition rates) using the leaf-bag technique and corer samples under the null hypothesis of no significant differences between the contaminated and the reference channels are synthetized in Table 8.2.

Table 8.2 Overall test results for the upstream part of the contaminated (Estarreja) and the reference (Salreu and Canelas) channels with the various studied descriptors. Pa - *Phragmites australis*, Fv - *Fucus vesiculosus*, C - control substrate and Cr - corer samples. A statistical significant difference between the contaminated and reference channels is indicated by a (+) signal, whereas the (-) signal stands for non-significant difference. n.a. – not applicable.

Descriptors	Pa	Fv	C	Cr
Decomposition rates	**-**	**-**	**-**	**n.a.**
Macrobenthic Community	**-**	**-**	**-**	**+**
Species richness (*S*)	**-**	**-**	**-**	**+**
Species diversity (*H'*)	**-**	**-**	**-**	**+**
AMBI	**-**	**-**	**-**	**-**
M-AMBI	**-**	**-**	**-**	**+**
ISS without correction factor	**-**	**-**	**-**	**-**
ISS with correction factor	**-**	**-**	**-**	**+**

Chapter 9. Literature cited in this thesis

9.1 Literature cited in this thesis

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