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DECLARAÇÃO

Para efeitos do disposto nº2 do Art. 8º do Dec-Lei 388/70, o autor desta tese declara que interveio na conceção do trabalho experimental, na interpretação dos resultados e na redação dos manuscritos publicados e submetidos para publicação.

Sílvia Susana Ferreira Pedro

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ABSTRACT

Estuaries face different anthropogenic pressures as a consequence of their privileged location and high productivity, and thus a diverse array of pollutants enter the ecosystem. Metals are of particular concern, due to their persistent and non-degradable character and pernicious effects exerted on the biota. Metals are found in several compartments of the estuarine ecosystem. They may be in dissolved or particulate forms in the water column, sorbeb on the sediments or accumulated in the biota. This thesis aimed to determine the effect of the sediment-organism interactions in metals' cycling in salt marsh and intertidal sediments. Special attention was given to metal speciation, to assess the mobility and bioavailability of such elements. Total metal concentration was determined in tissues of two fish species occupying different levels in the estuarine trophic web, as well as in bottom sediments, to assess metal exposure and accumulation in fish tissues. Sequential extractions were made in rhizosediments of three halophytes and adjacent bare mud flat sediments from two different salt marshes. Operationally defined fractions were obtained using solution of increasing strength and acidity, to evaluate the effect of halophytes on metal availability. Two laboratory trials were conducted in which metal fractionation was assessed in on sediments before and after passing through the gut of the two species. The results of these works indicate that metal accumulation in estuarine sediments can affect the accumulation in fish tissues. Sediment-organism interactions alter metal mobility in the sediments. Salt marsh plants tend to immobilize metals in their rhizosediments, while the ingestion of sediment by and detritivorous species, and subsequent excretion of fecal pellets, makes some metals more bioavailable to the estuarine trophic web.

Keywords: Metals; Speciation; Influence of organisms; Sediments; Estuary.

RESUMO

INFLUÊNCIA DAS PLANTAS, DOS INVERTEBRADOS E DOS PEIXES NA MOBILIZAÇÃO DE METAIS EM SEDIMENTOS DE SAPAL E ZONA ESTUARINA ADJACENTE

Os estuários enfrentam diferentes pressões antropogénicas inerentes à sua localização privilegiada e elevada produtividade, e têm como consequência a presença mais ou menos acentuada de diversos tipos de poluentes. A persistência e o carácter não degradável dos metais no ambiente é particularmente preocupante, tendo em conta os efeitos nocivos que podem exercer no biota. Os metais podem ocupar vários compartimentos num estuário, e.g., na coluna de água (dissolvidos ou particulados), adsorvidos ao sedimento ou acumulados nos organismos. A presente tese teve como objetivo avaliar o efeito das interações organismo-sedimento na dinâmica de metais em sedimentos de sapal e áreas intertidais adjacentes, incidindo em particular na especiação dos metais e na sua disponibilização para a teia trófica estuarina. Para avaliar a exposição de duas espécies de peixes de diferentes níveis da teia trófica estuarina à contaminação por metais no sedimento, determinaram-se as concentrações totais em tecidos e em sedimentos superficiais. Analisou-se também a especiação dos metais nos sedimentos entre raízes de três halófitas e nos sedimentos sem coberto vegetal, em dois sapais. Extraíram-se sequencialmente frações operacionais com soluções de força e/ou acidez crescente, para avaliar o impacto dos organismos na mobilidade dos metais. Realizaram-se ainda duas experiências em que se determinou a especiação dos metais no sedimento antes e depois da ingestão por duas espécies de diferentes grupos taxonómicos. Concluiu-se que a acumulação de metais no sedimento estuarino pode afetar a acumulação nos tecidos das espécies selecionadas. A interação sedimento-organismo conduz a alterações da dinâmica dos metais; a ação das plantas de sapal potencia a imobilização de alguns elementos, sendo o efeito da ingestão de sedimento por organismos detritívoros aparentemente contrário, disponibilizando os metais sob formas mais acessíveis à teia trófica estuarina.

Palavras-Chave: Metais; Especiação; Influência dos organismos; Sedimentos; Estuário

Resumo Alargado

Os estuários têm uma localização privilegiada, situando-se em zonas de interface entre a terra e o mar, em ambientes de elevada produtividade de elevada produtividade, tanto a nível terrestre, como aquático. Estão, reconhecidamente, entre os ecossistemas de maior valor ecológico e económico, conduzindo a uma elevada atratividade para a ocupação por populações humanas e atividades associadas. A multitude de usos levou a que intensas pressões se fizessem sentir nos estuários a nível mundial. A contaminação por metais é uma entre a miríade de consequências dessas pressões antropogénicas, e uma das mais preocupantes pelos efeitos perniciosos para o biota e, em última análise, para as populações humanas.

Os sedimentos, pela sua facilidade de obtenção e análise, são tradicionalmente recomendados em programas de monitorização de contaminação por metais como a primeira abordagem a ser tomada. Têm contudo a desvantagem da concentração metálica total não corresponder na sua generalidade ao teor disponível para o biota, e, consequentemente, a avaliação da toxicidade inerente ao material analisado ser bastante limitada. Não obstante, a comparação da concentração total dos metais presentes no sedimento com, por exemplo, os respetivos níveis pré-industriais permite obter um valor designado por “fator de enriquecimento”. A comparação entre fatores de enriquecimento calculados para diferentes áreas e ao longo de intervalos de tempo definidos pode revelar assim o grau de impacto sofrido pelo ecossistema. Apesar de nem sempre ser observada uma relação entre o teor de metais no sedimento e a acumulação nos organismos, há todavia estudos em que tal relação foi verificada.

A presente tese teve como objetivo avaliar o efeito das interações organismo-sedimento na dinâmica de metais em sedimentos de sapal e áreas intertidais adjacentes, incidindo em particular na especiação dos metais nos sedimentos. Nos trabalhos que compõem esta tese, o termo ‘especiação’ refere-se à partição ou fracionamento geoquímico dos metais, ou seja, à sua distribuição por diferentes fases sólidas sedimentares, como sejam por exemplo os carbonatos ou óxidos de ferro. Na Introdução Geral, capítulo 1, foi realizado um enquadramento do tema da presente tese, com foco sobre a dinâmica de metais nos sedimentos estuarinos, e nas implicações da mobilidade e disponibilização desses elementos para os organismos aquáticos. Destacou-se igualmente a importância da avaliação da especiação dos metais, e como diferentes formas químicas representam diferentes consequências na disponibilidade e toxicidade dos metais para o biota.

No capítulo 2 avaliou-se a exposição potencial da tainha *Liza ramada* à contaminação do sedimento por metais. Esta espécie ocupa um nível trófico baixo na teia trófica estuarina podendo ser considerada como predominantemente detritívora. Analisou-se a acumulação de um conjunto de elementos essenciais (Co, Cr, Cu, Ni e Zn) e não-essenciais (Cd, Pb e Hg) no sedimento superficial proveniente de áreas utilizadas por esta espécie para a sua alimentação. A preferência alimentar de *L. ramada* foi estudada com base na dimensão das partículas ingeridas, tendo sido constatada a maior preferência dos exemplares de menor dimensão por partículas de sedimento mais finas. Considerando a correlação significativa normalmente encontrada entre os sedimentos mais finos e uma maior concentração de metais associados aos mesmos, concluiu-se que os juvenis de *L. ramada*, estando potencialmente mais expostos a teores mais elevados de metais no sedimento, por via alimentar, teriam a tendência para acumularem os mesmos elementos numa maior extensão que os adultos da mesma espécie. Esta hipótese foi testada na segunda parte do capítulo 2, em que se determinou a acumulação de metais nos tecidos de exemplares de diferentes classes etárias/dimensionais. Os exemplares de menor dimensão apresentaram de facto maior concentração de metais nos tecidos, excetuando a concentração de Hg, para o qual é reconhecida a bioacumulação crescente com a idade nos teleósteos marinhos/estuarinos. A maior exposição dos juvenis ao sedimento mais contaminado, aliada ao seu metabolismo mais elevado, contribuirá certamente para esse resultado. A análise da concentração de metais nos conteúdos estomacais de *L. ramada* revelou não ser essa uma abordagem eficaz para determinar a exposição diferenciada a que juvenis e adultos estarão sujeitos. A este respeito, os conteúdos estomacais parecem mostrar apenas um “instantâneo”, tendo sido considerados como maus indicadores da exposição destes organismos à contaminação por metais do ecossistema estuarino. Dada a heterogeneidade dos sedimentos superficiais, e considerando os amplos movimentos que as tainhas efetuam no estuário para se alimentarem, a concentração de metais determinada nos conteúdos estomacais não será refletida na bioacumulação nos tecidos, nomeadamente no músculo.

A forma como os organismos podem influenciar a dinâmica de metais no sedimento foi estudada no capítulo 3. As plantas de sapal aprisionam os metais nos sedimentos entre raízes, e através da modificação de características físico-químicas destes últimos afetam a mobilidade dos metais que aí ocorrem. Por outro lado, é também reconhecida a interferência que os organismos bentónicos exercem sobre a dinâmica sedimentar, nomeadamente através da bioturbação que o seu comportamento gera, e que pode atingir profundidades de até 20 cm. Desta forma, no capítulo 3 descreveu-se a influência de três espécies de plantas de sapal (*Halimione portulacoides*, *Sarcocornia fruticosa* e *Spartina maritima*), provenientes de dois sapais do estuário do Tejo com morfologia distinta (Hortas e Rosário), na especiação de metais nos sedimentos. Adicionalmente selecionaram-se duas espécies animais cuja ecologia e nível trófico teriam potencialmente a capacidade de afetar igualmente a especiação de metais nos sedimentos estuarinos: um bivalve, *Scrobicularia plana*, e um peixe, *L. ramada*. Os resultados obtidos evidenciaram a capacidade das plantas imobilizarem os metais em formas menos biodisponíveis, tendo contudo sido constatado que o comportamento químico dos metais se sobrepõe a condicionantes relativas à espécie colonizadora ou ao sapal selecionado. Dois dos elementos, Cd e Zn, apresentaram maior mobilidade no sedimento, enquanto o Cu e o Zn se revelaram elementos bastante mais estáveis (em particular o Cu), predominando a associação a frações mais refartarias do sedimento. Não obstante o peso do comportamento químico dos metais na sua partição geoquímica, observaram-se, ainda assim, diferenças na influência da morfologia do sapal sobre o ciclo de metais. No sapal do Rosário, mais desenvolvido/maduro, com maior teor de matéria orgânica e sedimentos finos, foi evidente uma maior capacidade de retenção e imobilização dos metais no sedimento. Ao comparar os sedimentos, de entre raízes com os das áreas intertidais adjacentes (sem coberto vegetal), verificou-se que a presença de *S. fruticosa* promoveu maiores diferenças no fracionamento dos metais que as outras duas espécies. Por outro lado, no sapal das Hortas, um sapal menos desenvolvido/mais jovem, a presença das três halófitas promoveu diferenças significativas em relação à partição dos metais. Em qualquer dos casos, a disponibilidade dos metais era inferior nos sedimentos entre raízes do que nos que provinham das zonas sem vegetação. Ao contrário do efeito exercido pelas plantas, a ação das duas espécies de animais parece ter promovido maior biodisponibilidade de alguns metais no sedimento estuarino, nomeadamente do Cd, Cu e Zn. A partição destes três elementos nas frações mais lábeis evidenciou um aumento após a passagem pelo trato digestivo das duas espécies, o que não parece acontecer no caso do Ni.

No capítulo 4 estudou-se o potencial de um predador de topo da teia trófica estuarina como indicador da contaminação de metais no sedimento. À semelhança do estudo realizado na primeira parte desta tese, determinaram-se as concentrações de vários elementos essenciais e não-essenciais no músculo e fígado de uma espécie piscívora, neste caso o xarroco, *Halobatrachus didactylus*. Observaram-se variações na acumulação de metais no fígado facilmente atribuíveis às alterações metabólicas durante a época de reprodução desta espécie, tendo também sido verificadas diferenças entre machos e fêmeas. Por conseguinte, decidiu-se não ser o fígado um órgão aconselhável para estudos de monitorização de metais, apesar de poder refletir potencialmente o aporte recente de metais no ambiente. Para obviar a influência do género e da fase reprodutora no metabolismo dos metais, comparou-se a acumulação no músculo de machos adultos com idade estimada superior a 5 anos. Por serem mais sedentários que as fêmeas, nomeadamente porque são os machos que guardam os ninhos durante o desenvolvimento dos ovos, foi admitida a hipótese de que estes indivíduos poderiam refletir de uma forma mais precisa a contaminação de metais no sedimento. Foram comparados exemplares e amostras de sedimento superficial de duas áreas com níveis muito distintos de contaminação por metais. A frente estuarina do Portinho da Costa (Almada), perto da embocadura do Tejo, é uma área com hidrodinamismo e profundidade elevados e onde a contaminação por metais é relativamente baixa. Na Baía do Seixal, localizada numa área mais interior do estuário, a pressão urbana e industrial sente-se de uma forma particularmente acentuada, o que aliado a um baixo hidrodinamismo e profundidade reduzida conduz a que neste local seja possível encontrar níveis consideravelmente elevados de metais. Os resultados obtidos mostraram que os elementos essenciais, como o Zn e o Cu, por serem regulados metabolicamente, não exibem diferenças que possam refletir a concentração desses elementos patente no sedimento. Contudo, para elementos não-essenciais ao metabolismo destes animais, como o Cd, o Pb, e o Ni[1] concluiu-se que o xarroco tem potencial como indicador da sua biodisponibilidade no ecossistema.

Por último, no capítulo 5 teceram-se algumas considerações finais sobre os trabalhos suprarreferidos, integrando-se os principais resultados e conclusões dos capítulos anteriores. Concluiu-se que a acumulação de metais no sedimento estuarino afeta potencialmente a acumulação em determinados organismos, havendo uma interação sedimento-organismo que resulta na alteração da dinâmica dos metais neste ecossistema. A ação das plantas de sapal potencia a diminuição da biodisponibilidade de alguns elementos, ao passo que a ação de espécies predominantemente detritívoras parece ter o efeito contrário, disponibilizando os metais sob formas mais móveis e acessíveis à teia trófica estuarina. Cenários como o da subida do nível médio da água do mar podem potenciar a exportação e disponibilização de metais nos estuários.

[1] Apesar de ser essencial para o metabolismo de diversas espécies, nomeadamente de microrganismos, plantas, aves e mamíferos, o Ni não parece desempenhar nenhuma função metabólica no metabolismo dos peixes.

List of Papers

This thesis is based on the following published or submitted manuscripts:

S. Pedro, V. Canastreiro, I. Caçador, E. Pereira, A. C. Duarte, P. R. Almeida (2008). Granulometric selectivity in *Liza ramado* and potential contamination resulting from heavy metal load in feeding areas. Estuarine, Coastal and Shelf Science 80: 281-288. *DOI: 10.1016/j.ecss.2008.08.011.*

S. Pedro, I. Caçador, E. Pereira, A. C. Duarte, P. R. Almeida. Bioaccumulation of trace metals in thin-lipped grey mullet (*Liza ramada*): relationship with size and ecological repercussions. Submitted to Estuarine, Coastal and Shelf Science.

S. Pedro, B. Duarte, I. Caçador, P. R. Almeida. Metal speciation in salt marsh sediments: influence of halophyte vegetation. Submitted to Estuarine, Coastal and Shelf Science.

S. Pedro, B. Duarte, G. Reis, J. L. Costa, I. Caçador, E. Pereira, A.C. Duarte, P. R. Almeida. Metal partitioning and availability in estuarine surface sediments: changes promoted by feeding activity of *Scrobicularia plana* and *Liza ramada.* Submitted to Estuarine, Coastal and Shelf Science.

S. Pedro, B. Duarte, N. Castro, P. R. Almeida., I. Caçador, J. L. Costa (2014) The Lusitanian toadfish as bioindicator of estuarine sediment metal burden: the influence of gender and reproductive metabolism. Ecological Indicators 48: 370-379. *DOI: 10.1016/j.ecolind.2014.08.041.*

CHAPTER 1

General Introduction

Aims and Structure of the Thesis

Estuaries and saltmarshes: systems under human pressure

Estuaries are among the most productive and valuable, not only regarding their ecological importance, but also when considering the average (per hectare) of the estimated values of the services they provide (Costanza *et al.*, 1997; McLusky and Elliott, 2004).

The definition of estuaries given by Pritchard (1967) is probably one of the most cited: "*an estuary is a semi-enclosed coastal body of water which has a free connection with the open sea and within which sea water is measurably diluted with fresh water derived from land drainage*". This definition was modified throughout the time, adding more features than the connection with the sea and the salinity gradient. A more comprehensive definition, where geomorphological, physical, chemical and biological criteria are comprised, is the one given by Perillo (1995), in which "*an estuary is a semi-enclosed coastal body of water that extends to the effective limit of tidal influence, within which sea water entering from one or more free connections with the open sea, or any other saline coastal body of water, is significantly diluted with fresh water derived from land drainage, and can sustain euryhaline biological species from either part or the whole of their life cycle*". Defining what is an estuary raises debate (Day, 1980; Reddering, 1980) because of the divergent proprieties found within and among estuaries from different regions of the world (Bianchi, 2013), but in a broader perspective, estuaries may be defined as a "*portion of the earth's coastal zone where there is interaction of ocean water, fresh water, land, and atmosphere*" (Day *et al.*, 2013), including areas of land and sea affected by coastal activities (French, 1997). What stands common to all the definitions is that an estuary has an interface dimension, which challenges species' physiology to adapt to severe environmental gradients, such as temperature, turbidity, dissolved oxygen or salinity fluctuations (Chapman and Wang, 2001).

The presence of human populations around coastal areas in general has been occurring during the course of history, with the first human civilizations settling around estuaries about 5000 years ago (Day *et al.*, 2013). While the first reasons for population settlement around the estuarine areas were related with their high productivity, given the richness of the soils in these areas and the abundant natural biota, rivers provided important routes for navigation and the logistic advantages made these regions appealing for establishing ports and industries. This development, of course, did not arise without a price: the increasing growth of human populations settlements, and the increase of uses made on estuaries, led to intense pressures and demands over the natural resources (French, 1997), rendering coastal waters, and especially estuarine waters, widely polluted and subjected to severe environmental degradation (McLusky and Elliott, 2004). Chemical pollution, in particular, occurs when a chemical substance promotes changes in the natural system as a result of man's activities, and as a consequence the fitness of individuals, populations, species or communities to survive is reduced (McLusky and Elliott, 2004). Within chemical pollutants, metals (commonly referred to as heavy metals[2]) have received particular interest in the last decades. In a review study published in 2012, the term "heavy metals" was among the five most used keywords in estuarine pollution research, "metals" was in the top three words in titles and abstract, and "sediments" was the most important issue in estuarine pollution related papers (Sun *et al.*, 2012). Environmental awareness on estuarine pollution by metals and its impending pernicious effects on the biota has consequently become an issue of increasing concern, especially in the last two to three decades. This concern comes from the fact that metals may become adsorbed onto sediment particles, that way becoming stored with deposited sediment, transforming mudflats and salt marshes into metal sinks (Caçador *et al.*, 1996a,b).

Salt marshes are complex ecosystems distributed in mid and high latitudes, and are among the most productive environments in the world. They are natural or semi-natural ecosystems that develop on alluvial sediments and border saline water bodies, depending on favorable conditions of wave energy, tidal regime, and substrate to grow (Beeftink, 1977; Dijkema *et al.*, 1990; Kennish, 2001).

[2] Although commonly used in biological and environmental studies, given its connotation with toxicity and deleterious effects on biota, it is currently agreed that the term "heavy metal(s)" should be avoided, as there is no chemical basis in the choice of metals included in this classification (Duffus, 2002), not to mention the cases of metalloids and nonmetals commonly addressed as "heavy metals".

Halophytic vegetation (mainly grasses and small shrubs), along with algae, are responsible for the extraordinary primary production associated with these ecosystems (Ibáñez *et al.*, 2013; Teixeira *et al.*, 2014), and the zonation and distribution of vegetation are affected by factors like frequency and duration of flood, sulfide concentration, and substrate composition (Ibáñez *et al.*, 2013). Salt marshes provide many other services beyond the aforementioned high productivity. For example, they absorb wave energy, mitigating shoreline erosion and attenuating flood events, functioning like a buffer (Dijkema *et al.*, 1990; Kennish, 2001). Other vital functions are undertaken by salt marshes, like providing nursery areas in tidal creeks for fish species, and resting, breeding and feeding areas to many resident and migratory bird species. The importance of these wetlands was officially recognized by their inclusion in the European Habitats Directive and in the Water Framework Directive (WFD). Notwithstanding the ecological importance of salt marshes, human pressures and impacts over them have long been observed. Physical alterations have led to the direct and indirect destruction of wetlands, with drainage, filling and land reclamation being common processes. Another significant impact is the one caused by the construction of dams, weirs or other water retention structures upstream, compromising the supply of sediment to salt marshes and estuaries in general. Sediment starvation, together with sea level rise may condemn wetlands subsistence (Ibáñez *et al.*, 2013). Salt marsh vegetation plays an important role in sediment retention (whose inputs come not only from rivers, but also from tidal flooding), acting as a trap for sediment ant thus increasing accretion rates (Pethick, 1981). As pointed out earlier, these sediments will act as sinks of pollutants, namely metals. As a consequence of salt marsh locations - usually surrounded by urban and industrial areas, they consequently receive important discharges of these contaminants.

Metal cycling in estuaries

Salt marsh sediments are not only a *sink* for metals: they may also become a *source*, given the appropriate conditions. For example, metals stored in sediment may be remobilized during erosion events, or when the sediment is somehow disturbed. This may be a significant path for metals to re-enter the aquatic ecosystem (French, 1997). Depending on the form of the metals, and if these are in more or less bioavailable states, sediments become a source of metals to the biota, including to salt marsh plants. Vegetation has a direct involvement in the retention and transformation of metals (Caçador *et al.*, 1996b; Caçador *et al.*, 2009), in addition to the role in the entrapment of sediments. Salt marsh plants accumulate metals to a great extent in their roots (Caçador *et al.*, 2000), and metal concentrations in the sediments around the root system (rhizosediments) are greatly influenced by the presence of halophytic vegetation (Caçador *et al.*, 1996a; Doyle and Otte, 1997; Reboreda and Caçador, 2007b). When comparing metal levels in bare mudflats with adjacent colonized sediment, higher concentrations are usually found in the latter, surrounding the root systems of halophytes (Reboreda *et al.*, 2008). The complexity of the interaction between plants and sediments goes further, as the vegetation presence modifies metal partition in the sediment where it stands (Caçador *et al.*, 1996b; Reboreda and Caçador, 2007a; Reboreda *et al.*, 2008).

Metal contamination of estuarine bed sediments can have a significant impact on concentrations along the estuary (Wu *et al.*, 2005). In the middle reaches of an estuary, tidal sediment disturbance and surface sediment resuspension may be the main generators of dissolved estuarine trace metals (Morris *et al.*, 1986). The salt marsh surface microlayer (a very thin layer, typically less than 100 µm that exists on top of most natural water bodies, and is usually enriched in organic and metal bearing materials) was proven to be responsible for the concentration of a significant proportion of the trace metal burden for the salt marsh (Pellenbarg and Church, 1979). Additionally, vegetation litter can sorb the surface microlayer trace metals, and the organic acids released by the decaying vegetation can chelate dissolved trace metals, making them available for scavenging by the litter of the marsh. The uptake, distribution and removal of metals from the sediment by marsh plants are part of the determining processes that may turn salt marshes into sources or sinks of metals to the estuarine ecosystem (Weis and Weis, 2004). The uptake by the plants' roots usually increases during growth season, and part of the metals is translocated to the aboveground tissues (Caçador *et al.*, 2009); by the end of the growing season, the senescent plants tissues decay into organic detritus. Therefore, plant tissues may be sources of metals, through leaching and mineralization of plant litter, or sinks, through litter adsorption or microbial immobilization (Weis and Weis, 2004). Metals establish strong bounds with organic compounds, and organic matter is known to be a stable sink for metals. Hence, when metals are bound to living organisms, detritus, etc., they become stabilized and less available to the ecosystem (Duarte *et al.*, 2008). The hydrolysis and breaking down of the organic matter under oxidizing conditions can lead to the release of soluble metals into the environment (Tessier *et al.*, 1979), unless they form a stable complex with other sediment components.

Lacking the vegetation cover, mudflats are more than meet the eye. These areas are also responsible for intense primary productivity, due to the activity of benthic communities (Underwood and Kromkamp, 1999). Important inputs of organic particles and detritus (and the inherently associated metals) are exported from nearby salt marshes and disseminated by tidal action into intertidal mudflats, creating an exceptionally rich habitat for benthic communities. Benthic invertebrates play an important role in cycling nutrients and inorganic compounds between sediments and the water column. Taking the example of suspension- and deposit-feeders, exposure to metals occurs via dietary intake, from pore water derived fluxes and from burrow and overlying waters. These organisms play a particularly significant part in the transformation of the physical and chemical properties of suspended particles and their subsequent transport to the sediment surface (Turner and Millward, 2002). Particle ingestion is a primary pathway of exposure to trace metals whereby metals can enter estuarine trophic webs. In the case of some bivalves, for example, a strategy to reduce the exposure to bioavailable contaminants involves a flexible digestion, balancing the ingestion rate and the intra- and extracellular digestive (digestive gland and intestine, respectively) processing of particles (Decho and Luoma, 1996). Suspended matter is ingested, and after sorting and rejection, part of those particles (coated with nutrients and, e.g., trace metals) enter the digestion/absorption phase, after which the resulting wastes are eliminated. Chemical speciation, bioavailability, gut passage time and assimilation efficiency are some of the physiological and chemical characteristics that will determine what is assimilated and what is egested (Turner and Millward, 2002). In the end of the suspended particle process, faeces and pseudofaeces form modified biodeposits in the surface sediment, change cohesiveness and distribution of particles, and attract and hold material that would otherwise remain in suspension (Graf and Rosenberg, 1997). Large populations, such as those of the deposit-feeder *Scrobicularia plana* (da Costa, 1778) (Hughes, 1970), may thus significantly modify the chemical and ecological characteristics of the local substratum and suspended particle load (Turner and Millward, 2002), and subsequently modify the distribution and availability of metals therein. But bivalves like the peppery furrow shell (*S. plana*) are not the only deposit feeders that build up large populations with the ability to modify the estuarine sediment surface and suspended matter: such properties are also verified among nekton species. Grey mullets, like *Liza ramada* (Risso, 1827), access tidal creeks during flood periods, where they can feed on the extensive biofilm of diatoms that surfaced during the ebb tide. These fishes have an extraordinary feeding plasticity. Their feeding ecology and the travelling distances within the estuary in each tidal cycle (Almeida, 1996) lead to a massive resuspension of the bottom sediments. Bioturbation is an important process in the control of the interactions between the dissolved metal ions and the particulate matter in estuaries (Bianchi, 2007). As part of the estuarine trophic web, this fish species plays an important role in the trophic transfer of metals, either by predation or death and decay in the ecosystem. The numerous levels and organisms taking part in the estuarine trophic web make it rather complex to analyse. Dietary uptake of metals, and inherent trophic transfer, is an important pathway for the entry of metals into estuarine and marine animals (Wang, 2002). Another process important for the estuarine trophic web is biomagnification of metals, i.e., the progressive increase of those elements as we go up in the trophic level. Even though the extent to which it occurs remains uncertain, there is a great proximity between the estuarine trophic web and humans, raises interest on this subject (Mathews and Fisher, 2008).

Metal mobility, availability and toxicity to aquatic organisms

When comparing metal levels in the sediments with those of the overlying water, differences can be remarkably high, reaching values between three and five orders of magnitude (Bryan and Langston, 1992). Decision makers and general public have been traditionally more familiarized with total concentrations and with the necessity of remediation (or “clean-up”) that consequently arises when concentrations of metals exceed certain levels, due to the usual connotation with pernicious effects on the biota (Long *et al.*, 1995). Nevertheless, total concentrations of metals and other contaminants in general, are not necessarily correlated with the eventually observed biological toxicity - such relationship is actually connected to the bioavailable fraction (Harmsen, 2007). In this context, bioavailability refers to the amount of metal available to be assimilated by the organisms (Griscom and Fisher, 2004). Many different processes influence both the concentration and the bioavailability of metals in estuarine sediments, among which are the mobilization of metals to pore waters and chemical speciation, the influence of bioturbation, salinity, redox potential or pH, the transformation of metals (e.g. by methylation), or sediment phases to which metals are preferentially bound, such as Fe oxides and organic matter (Bryan and Langston, 1992). The various binding phases and processes that influence metal exposure in sediments are, in fact, one of the factors that make predicting the bioavailability of metals in sediments more problematic.

In terms of the biota, one of the various problems that metals pose is that they may act as, or mimic, nutrients. The latter poses a problem due to competition with the uptake of the actual nutrient by the organism, e.g. Cd^{2+} competing with Ca^{2+} site in Photosystem II during photoactivation (Faller *et al.*, 2005); competition between sediment metals for uptake sites in organisms (like Cu and Ag; Zn and Cd) (Bryan and Langston, 1992). When a metal acts as a nutrient, it becomes a matter of concern because an essential metal can quickly become toxic above certain levels (Strom *et al.*, 2011). This toxicity is variable among organisms, and several factors control the accumulation of metals in tissues, e.g. temperature, trophic behaviour or metabolism (Bianchi, 2013).

As a response to metal exposure, organisms have developed mechanisms to avoid metal toxicity in order to prevent the impairment of vital functions. Among plants, it is possible to find different strategies to deal with excess metals: most plant species are basically metal-excluders, and avoid the transport of metals to the shoot photosynthetic tissues by sequestering them in the vacuoles and cell walls in the roots; others have adapted to live in metal enriched environments, and have the capacity to accumulate large amounts of metals in the aboveground tissues (Weis and Weis, 2004; Hanikenne and Nouet, 2011). Adaptations like increased rates of root-to-shoot transfer and metal detoxification and sequestration in the leaves, comprising high vacuolar storage capacity, are involved in the latter strategy (Krämer, 2010). Animals also display adaptation strategies to address environmental exposure to metals, and elevated concentrations may induce resistance mechanisms. These mechanisms may encompass enhanced ability to detoxify the metal internally, release compounds that chelate metals, reducing their bioavailability, or increasing the excretion rates of metals (Brown and Depledge, 1998). The presence of metal binding proteins is a common metal tolerance and detoxification strategy, and it is found in both plants and animals (Amiard *et al.*, 2006).

Metal speciation

As referred above, total concentration of metals is not as important in determining their toxicity for the environment as their available forms (Ankley *et al.*, 1994). The notion of availability has started for some time to be a part of risk assessment approaches, even though it is challenging to integrate the methods implied therein to regulation, particularly because of the difficulty to reach a consensus capable to be integrated in decision making (Harmsen, 2007). Scientific community has been working towards the understanding of biological availability of metals, and a large array of chemical and biological methods to assess bioavailability have been developed (Harmsen, 2007). Chemical speciation is determinant in the toxicity of a metal to organisms. For example, organic forms of metals are generally more toxic than inorganic forms, as it can be observed for elements like Hg (Kamps *et al.*, 1972; Canário *et al.*, 2005; Mergler *et al.*, 2007; Mason *et al.*, 2012); but the contrary may also be witnessed – e.g. inorganic arsenic forms present more toxicity to the biota than organic forms (De Bettencourt, 1988; Jain and Ali, 2000; Hughes, 2002; Sharma and Sohn, 2009). The oxidation state is another factor influencing the toxicity of metals – a good example is the case of the two stable oxidation states of Cr: while the Cr (III) has low solubility, reactivity, mobility, and low toxicity to organisms, the hexavalent oxidation state of Cr is considerably more soluble and toxic to the biota, and presents high risks to humans (Rai *et al.*, 1989; Barnhart, 1997; Barceloux, 1999; Becker *et al.*, 2006; Duarte *et al.*, 2012).

Sediment geochemistry is of the utmost importance for the differential speciation and availability of metals, inducing considerable differences in the bioaccumulation by plants and animals, irrespective of the environmental total concentration of an element (Luoma, 1989). Sorption/desorption, dissolution/precipitation, complexation, acidification and redox reactions are determinant for the capacity of sediments to retain a certain element, which will in turn influence its bioaccumulation (Kersten, 2007). Chemical extraction sequences have been developed in order to estimate the potential remobilization of metals under changing environmental conditions (Förstner and Kersten, 1988), but these sequential extraction schemes do not allow the determination of the 'true' species of the metal at the molecular level (Kersten, 2007). For that reason, the term 'form' is usually more adequate when referring to the results of those procedures. Of the multitude of methods developed in the past decades to assess metal speciation and fractionation in sediments and soils (e.g. Tessier *et al.*, 1979; Rauret *et al.*, 1989; Rauret *et al.*, 1999; Maiz *et al.*, 2000; van Hullebusch *et al.*, 2005), one of the most established and adapted is the one described by Tessier *et al.* (1979). In common, all these methods have that sequential reagents of increasing strength are to be used to accomplish the partition of the trace element into different forms. The successive fractions should correspond to metal association forms of progressively less mobility. In the case of the Tessier method, specifically, five fractions are obtained in the end of the sequential extraction: 1) the exchangeable fraction, where changes in the water ionic composition are likely to affect sorption-desorption processes; 2) the carbonates fraction, a fraction susceptible to changes in the pH; 3) the reducible fraction (Fe/Mn oxides), which is unstable under anoxic conditions (low Eh); 4) the fraction bound to organic matter (e.g. living organisms, detritus, coatings on mineral particles, etc.), that by the complexation processes can affect the mobility of released metals; and 5) the residual fraction, which is expected to contain strongly bound metals, and the release of such metals is not expected to occur under normal environmental conditions (Tessier *et al.*, 1979). The carbonates and exchangeable fractions together, the labile phase (Griscom *et al.*, 2000), can be considered a proxy of alterations that are susceptible to be observed in the environment The results of sequential extraction schemes will ultimately contribute to better understand the mobility, transport and partitioning of trace metals and assess the potential metal toxicity of sediments to the biota.

The study area

The Tagus is the longest Portuguese river, draining an area of 86 000 km^2. It has its origin in Albarracin (Spain) and outflows into the Atlantic Ocean, near Lisbon. The Tagus estuary (38º44'N; 9º08'W) is a partially stratified estuary in the Atlantic coast of Europe. It has a deep, straight inlet channel (the deepest area of the estuary, reaching a depth of 40 m), and a broad, shallow, inner bay (with 25 km long and 15 km wide) (Vale and Sundby, 1987). Its topography presents a complex system of channels, intertidal mudflats and small islands (Vale, 1990). The estuary occupies an area of about 320 km^2, which extends landward to about 50 km north of Lisbon (De Bettencourt, 1988), and includes approximately 97 km^2 of tidal flats (Catarino *et al.*, 1985) and 17 km^2 of salt marshes (Caçador *et al.*, 2013). The predominant halophyte species of the Tagus estuary salt marshes are *Spartina maritima* Fernald (Poales, Poaceae), *Halimione portulacoides* (L.) Aellen (Caryophyllalles, Chenopodiaceae) and *Sarcocornia fruticosa* (L.) A.J. Scott (Caryophyllalles, Chenopodiaceae) (Caçador *et al.*, 1996a; Caçador *et al.*, 2013). The tidal regime is semi-diurnal, ranging from 0.4 m from the lowest neap tide to 4.1 m at the highest spring tide, and the tidal influence reaches 80 km upstream from Lisbon (Vale and Sundby, 1987).

The main sources of pollution in the Tagus estuary come from agricultural runoff, domestic effluents from the metropolitan area of Lisbon and two main industrial areas located in both margins of the estuary: in the right margin, between Vila Franca de Xira and Alverca, and in the left margin the Seixal-Barreiro industrial axis. The Barreiro Quimigal complex (which included a pyrite roasting plant) and Siderurgia Nacional (a smelter) were identified as being among the most likely sources of trace metals contamination in the estuary (Cotté-Krief *et al.*, 2000).

AIMS AND STRUCTURE OF THE THESIS

Metals entering the estuarine system end up dissolved in the water column or sorbed to particulate matter and sediments. The form of those metals, i.e., their chemical (or geochemical) species, is determinant in characterizing the bioavailability and/or toxicity of those elements to the biota.

Previous works established that salt marsh plants modify metal speciation in the sediments by promoting changes in their characteristics, such as oxygenation of otherwise anoxic layers, changes in pH, Eh or organic matter content. Additionally, bioturbation promoted by benthic organisms alters the sediments' dynamics, causing their resuspension and redistribution. Based on this information, the present study was focused on assessing the effect of sediment-organism interaction on metals' dynamic in salt marsh and intertidal mudflats sediments, aiming particularly to evaluate changes promoted by the organisms on metal speciation, and also how metal contamination in estuarine sediments could be reflected on important populations of estuarine fishes from different trophic levels.

This thesis is organized in five chapters. Chapter 1 comprises the current general introduction, where a framework of the topic of the thesis is made. The importance of metal speciation and metal cycling in the estuarine ecosystem is highlighted. Chapter 2 is entitled "*Sediment metal availability to the estuarine biota*" and includes two papers, one of them already published in an international journal and the other submitted for publication. This chapter describes the potential effect of sediment metal contamination on the teleost *Liza ramada*, assessed indirectly based on the feeding preferences of this mugilid and on metal accumulation on its tissues and organs. Chapter 3 is entitled "*Metal speciation in salt marsh sediments and intertidal mudflats*", and comprises two papers submitted for publication in an international journal. The first one focuses on the effect of different halophytes on the sediments of two salt marshes with different morphology. Three halophytes species (*Halimione portulacoides*, *Sarcocornia fruticosa* and *Spartina maritima*) were chosen, based on their abundance in the Tagus salt marshes. In the second paper, an assessment on the effect that deposit feeders have on metal speciation in estuarine sediments was made with two separate laboratory trials, by determining the geochemical partition of metals in the sediment before and after passing through the gut of *Scrobicularia plana* and *L. ramada*. Chapter 4 is entitled "*Estuarine biota as sentinel organisms for sediment metal contamination: A case study*", and contains one paper already published in an international journal. In this work, a top predator from the estuarine trophic web, *Halobatrachus didactylus*, was chosen to assess the species potential as an indicator of metal availability from the sediment. For that, metal concentrations were determined in the liver of male and female specimens captured during reproductive and non-reproductive periods, and metal accumulation in the muscle of adult males captured in areas with distinct sediment metal loads was also studied. Finally, Chapter 5 concludes with some final considerations and integration of the results obtained in the previous chapters.

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CHAPTER 2

SEDIMENT METAL AVAILABILITY TO THE ESTUARINE BIOTA

GRANULOMETRIC SELECTIVITY IN *LIZA RAMADO*[3] AND POTENTIAL CONTAMINATION RESULTING FROM HEAVY METAL LOAD IN FEEDING AREAS

ABSTRACT

The stomach contents of thin-lipped grey mullets *Liza ramado* were analyzed in terms of granulometric composition and compared to the sediment of potential feeding areas in the Tagus estuary. Total organic matter (TOM) content and heavy metal content were determined in the surface sediment of three areas and eight trace elements were quantified: Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn. The three sampled areas did not differ in TOM; and the heavy metal content was below Effects Range-Low level for most elements. The mean observed concentrations were present in the following sequence: Zn > Pb > Cr > Cu > Ni> Co > Cd > Hg. Stomach contents granulometric composition provided information about the feeding selectivity of the mullets. Sediment fractions with particle size between 20 and 50 mm are preferred, independently of the fishes' length. Smaller standard length (SL) fishes have a higher positive selection of fine grained sediments than those with a larger SL. Finer fractions usually have higher concentration of heavy metals, which makes younger specimens of the thin-lipped grey mullet potentially more exposed to heavy metal load in the estuary. Metal concentration was not independent from the sampling point, presenting higher values near the margins and the estuary tidal drainage system. This means that during the first period of each tidal cycle, the mullets will feed first on the most contaminated areas, as a consequence of their movement following the rising tide to feed on previously exposed areas.

Keywords: Heavy metals; Mugilidae; Feeding behavior; Grain size; Sediment pollution; Tagus estuary

[3] At the time this paper was published, databases presented the species name as *Liza ramado*, which was afterwards considered a misspelling (www.fishbase.org). Although presently, this it is not a valid synonym, and the valid species name is *Liza ramada,* a choice was made to maintain the name used in the published paper.

INTRODUCTION

The effect of contaminants depends on their biogeochemical transformations and the mobility of soluble forms induced by chemical gradients, bioturbation, and resuspension by the tide's activity (Caetano *et al.*, 2003). In muddy cohesive sediments biotic activity is a very important factor in sediment transport, deposition, resuspension and mixing of previously redox-stratified layers (Tolhurst *et al.*, 2003; Atkinson *et al.*, 2007). Biological activity in contaminated sediments thus becomes an important factor in the release of contaminants into the water column.

The Tagus estuary is one of the largest of Western Europe and one of the most important brackish water ecosystems of the Portuguese coast. For decades this estuary has been widely used for industrial development, agriculture and urbanization (Cabral *et al.*, 2001). Urban and industrial effluents are regularly discharged into the estuary (Caçador *et al.*, 1996a; Costa 1999) along with agricultural runoff, yielding substantial quantities of anthropogenic pollutants, with heavy metals playing an important role in the contamination status of the estuary (Caçador *et al.*, 1996a, 2000).

The thin-lipped grey mullet (*Liza ramado*) feeds on the extensive intertidal mudflats of the estuary, filtering the superficial layer of the sediment and particles in the water column (Almeida, 1996). The biological activity favors the availability of smaller particles into the water column (Atkinson *et al.*, 2007), along with metals and other contaminants bond to these particles (Buol *et al.*, 1997). These animals move in the estuary following the tidal currents (Almeida *et al.*, 1993) and with these movements are responsible for the re-distribution of particles from one point of the estuary to another, acting as a transportation vehicle for sediment. Mullets play an important part in the estuarine trophic web. They are essentially primary consumers (Almeida, 2003), presenting a great feeding plasticity (Bruslé, 1981), which allows them to exploit energy resources easily accessible (Almeida *et al.*, 1993). This species is one of the most abundant mugilids in the Tagus estuary, being commercially fished mainly by local fishermen. An increase in the abundance of the thin-lipped grey mullet has been reported for several decades (Oliveira and Ferreira, 1997).In spite of its abundance, it is not an important economic resource in the Tagus, but it is widely exploited in many Mediterranean countries, where it represents an important halieutic resource for local populations (Oliveira and Ferreira, 1997). They are also used in intensive and semi-intensive policultures with other species all over the world (Drake *et al.*, 1984).

The evaluation of sediment contamination and possible transference of contaminants to biologic communities is a major concern on the assessment of anthropogenic impact in aquatic ecosystems and is essential to an integrate management of estuaries. Mugilids are known to be selective in what concerns the particle size of the sediment that they ingest. This means that they prefer some parts of the estuary as preferential feeding areas and will be expose to the contaminants that are present in the sediment fraction collected during their feeding activity. This work's objective was to assess the contamination level to which these mugilids are exposed by feeding in potentially contaminated areas.

MATERIALS AND METHODS

Site description

The Tagus estuary is located in the West coast of Portugal (38°44'N, 9°08'W) and covers an area of about 320 km^2, which makes it one of the largest estuaries on the Atlantic coast of Europe. Within the estuary, salt marshes occupy approximately 20 km^2 (ca. 6%) and intertidal mudflats extend over 80 km^2 (ca. 20%), mostly located on the left bank of the upper part of the estuary. The study was carried out in the southern part of the middle zone of the estuary (Fig. 1), characterized by a complex branched system and high tidal range (max. 4 m). Due to these characteristics, sampling was performed from a boat to minimize sediment disturbance and reduce sampling time.

Sediment sampling

Sediment samples were collected in three different sites (A, B, C, Fig. 1) of ca. 4 km^2 each, located on a zone known to be used as a feeding area by the thin-lipped grey mullet, *Liza ramado* (P.R. Almeida, *personal communication*). Samples were collected from 25 points in each site (Fig. 1). Sediment cores were collected directly with PVC containers placed inside the corers; the containers were kept in an upright position inside a cooler box until arrival at laboratory and then preserved at -20°C until further analysis. Only the top 5 mm of the sediment surface layer were analyzed in order to allow the comparison with the stomach contents, considering the mullets grazing behavior (Romer and Mclachlan, 1986; Almeida *et al.*, 1993).

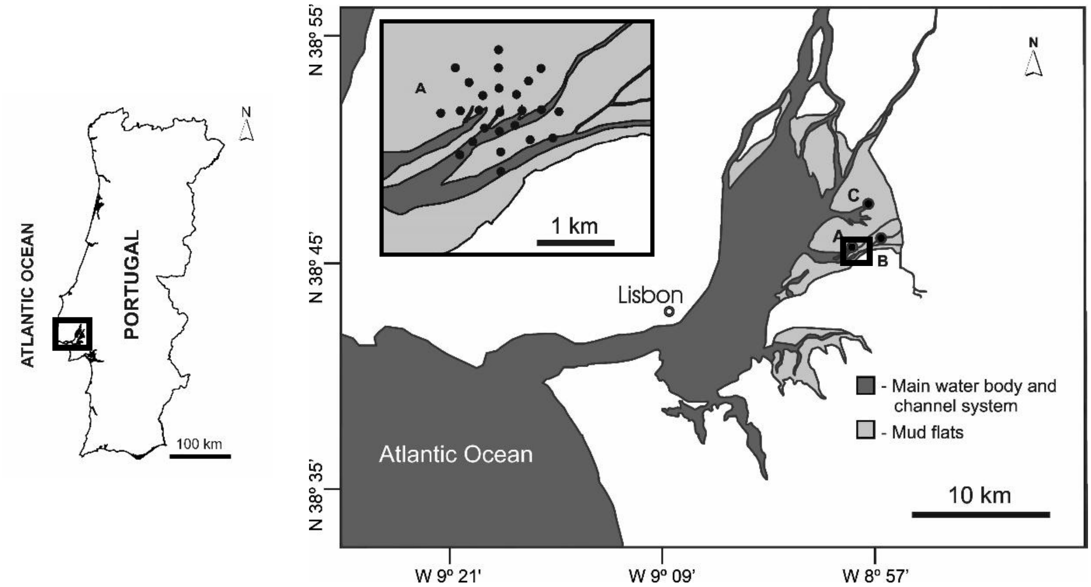

Figure 1 – Tagus estuary. Sampling sites identified (A, B, C), with detail of site A.

Particle size and organic matter quantification

For particle size evaluation, samples were dried to constant weight at 60°C for about five days and then homogenized. Particle size was determined using two distinct methods: for fractions larger than 50 mm a column of five sieves with calibrated mesh size (AFNOR type) was used, while for particle size fractions less than 50 µm the pipette method was applied (Gee and Bauder, 1986). All samples were primarily sieved through a 500 µm mesh size and no particles were retained. A total of six grain size classes were considered: 100–500 µm (medium sand), 50–100 µm (fine sand), 20–50 µm, 5–20 µm, 2–5 µm (silt) and 0–2 µm (clay) (USDA Soil Texture Classification System, Buol *et al*., 1997).

The total organic matter (TOM) content was determined as loss on ignition (LOI) by ashing 1.5 -5.5 g of sediment (dry weight) for 2 h at 600 °C.

Stomach contents analysis

The contents of the cardiac portion of the stomach of 225 thin-lipped grey mullets were used to determine particle size composition. Stomachs were frozen after the specimens' dissection for later removal of contents. For particle size determination, the same methodology that was applied to sediment samples was used.

Trace metals analysis

Heavy metal analyses were performed on freeze-dried sediment. Total Cd, Co, Cr, Cu, Ni, Pb and Zn concentrations were determined by flame atomic absorption spectrometry (AAS), using a Perkin Elmer A Analyst 100. Sediment samples were digested using 2 mL of an HNO_3/HCl mixture (3:1) in Teflon® reactors, heated at 110°C for 3 h. Extracts were filtered through Whatman 42 filters after cooling (room temperature) and diluted to 10 mL with deionized water. The accuracy of this analytical method was assessed by the analysis of international certificate standards. Standard additions and sludge reference materials were used for sediment (EC standards CRM 145 and 146). Blanks and the concurrent analysis of the standard reference material were used to detect possible contamination/losses during analysis.
Sediment samples were also analyzed for total mercury by AAS with thermal decomposition and gold amalgamation, using an Advanced Mercury Analyzer (AMA) LECO 254 (Costley *et al.*, 2000). The accuracy and precision of the analytical methodology for total mercury determinations were assessed by replicate analysis of certified reference materials (CRMs), namely MESS-2 and IAEA-356 for sediments.

Statistical treatment of the data

The Kruskal–Wallis test (*KW*) (Zar, 1999) was performed to evaluate the null hypotheses that the samples from the three sites did not have differences (1) between any of the granulometric classes and (2) in heavy metal content. We also assessed the relationship between sediment samples granulometry and metal contamination with Spearman's correlation coefficient (Zar, 1999). Simultaneous test procedure (STP) (Siegel and Castellan, 1988) was used when significant differences were found ($p < 0.05$).

A G-test of independence with Williams' correction (GW) was used to test the null hypothesis that the proportion of the most contaminated points (i.e. points that were in the 90th percentile – P90 – of the observed contamination level) was independent of the area (i.e. A, B or C) from where they were sampled. A spatial analysis using ArcGis 8.3 was performed to evaluate the distribution pattern of the points in the P90. A G-test of independence with Williams' correction was used to test the null hypothesis that the proportion of the points in the P90 of the observed contamination level was independent of some physic characteristic of the study area (e.g. the tidal drainage system). To perform the independence tests mentioned above, contingency tables were built with ***a*** number of columns and ***b*** number of rows. In these tables, ***a*** represented the spatial variable (i.e. the sampling area or the estuary channel drainage system), and ***b*** the sum of counts belonging or not to the P90 group (see example used for the channel system in Table 1). All points that were no more than 50 m apart from the channels were considered to be under the direct influence of the channel system, for classification purposes.

Table 1 – Contingency table used to test the independency of the proportion of P90 counts regarding the intertidal channel system

	Channel (C)	Not Channel (NC)
P90	$\Sigma\ C_{P90}$	$\Sigma\ NC_{P90}$
Not P90	$\Sigma\ C_{NotP90}$	$\Sigma\ NC_{NotP90}$

To investigate possible particle size selection by *Liza ramado*, feeding selectivity was assessed for each granulometric fraction using Strauss' Linear Index of Selectivity, $L_i=r_i-p_i$, where r_i and p_i are the relative frequency of the fraction *i* (in this case, the granulometric classes) in the stomach content and in the environment, respectively. The linearity of this index makes it less sensitive to sampling error associated with rare dietary items (Strauss, 1979). The index varies from -1.0 (strong negative selection) to 0 (random selection) and to +1.0 (strong positive selection). L_i was compared to the specimens' standard length (SL) in order to evaluate the possible variation of particle size selection with the fishes' length. Regression analysis (Sokal and Rohlf, 1995) used in this evaluation in each granulometric class and the three areas were tested for differences in the regression coefficients by means of an analysis of covariance (ANCOVA) (Sokal and Rohlf, 1995). All statistical analyses were performed using SPSS 15.0 (SPSS, 2006), STATISTICA 6.0 (StatSoft, 2001) and BIOMstat 3.01 (BIOMstat, 1996).

RESULTS

Sediment

Comparison of the granulometric composition of the three selected areas revealed significant ($p < 0.05$) and very significant ($p < 0.01$) differences in four of the six granulometric classes: 100–500 µm, 50–100 µm, 20–50 µm and 5–20 µm (Table 2). Areas B and C showed a higher percentage of smaller particles in their composition, mainly silts and clays (<50 µm), possibly due to favorable hydraulic conditions for fine grain sediments to settle in those areas.

Table 2 – Results of the Kruskal-Wallis test (*KW*) and *a posteriori* comparisons between the three areas (A, B, C) for the six granulometric classes under study (N=75, d.f.=2)

	]100-500 µm]	]50-100 µm]	]20-50 µm]	]5-20 µm]	]2-5 µm]	[0-2 µm]
Areas	*KW*=13,91**	*KW*=7,91*	*KW*=10,73**	*KW*=11,85**	*KW*=6,12^{ns}	*KW*=1,57^{ns}
A *vs* B	ns	**	**	**	-	-
A *vs* C	**	ns	**	ns	-	-
B *vs* C	**	ns	ns	**	-	-

ns – non significant; * - $p < 0.05$; ** - $p < 0.01$

The three sampling sites did not show significant differences regarding the total organic matter (TOM) content. This probably resulted from the fact that the layer of sediment analyzed (top 5 mm) is mainly constituted by organic matter. Mean TOM content for areas A, B and C was, respectively, 10.3±1.3%, 10.9±1.4% and 10.8±1.2% (mean±sd).

Mean concentration of metals in the sediment samples varied substantially and presented the following sequence: Zn> Pb >Cr> C ≈ Ni> Co> Cd >Hg (Fig. 2). Significant and very significant differences were found for Pb and Cd (KW=6.86, d.f.=2, p < 0.05 and KW=18.00, d.f.=2, $p < 0.01$, respectively). Cd had higher accumulation on sampling site C, further from the margin, while Pb had higher values in sampling site B. The other metals did not differ statistically among areas.

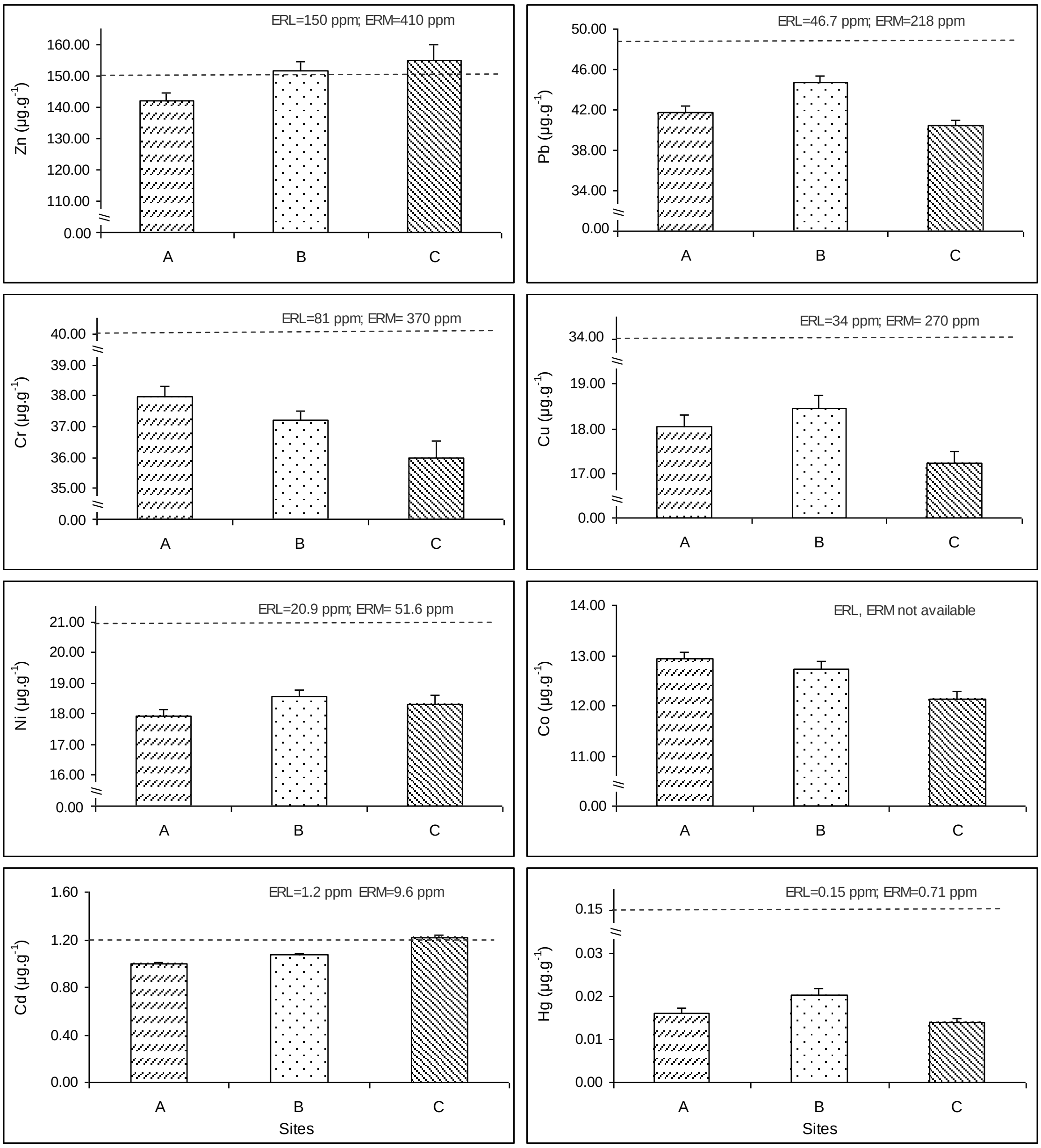

Figure 2 – Concentrations of Zn, Pb, Cr, Cu, Ni, Co, Cd and Hg (mean±se) (µg.g^{-1} dry weight). Effects Range-Low (ERL) and Effects Range-Median (ERM) (Long *et al.,* 1995) are given for each metal, except Co. Dashed lines indicate the value of ERL.

The comparison of granulometric content with heavy metal accumulation showed significant ($p < 0.05$) and very significant ($p < 0.01$) positive correlation for sediment particles between 100 and 500 µm and with less than 20 µm for the following elements: Co, Cr, Cu, Ni and Pb. Zinc had significant positive correlation only with sediment particles with less than 20 µm. The most representative granulometric fraction in all samples (20–50 µm) did not show significant correlation with any of the analyzed metals (Table 3).

Table 3 – Spearman's correlation coefficient between granulometric composition and heavy metal accumulation in sediment samples

	Spearman's R							
Particle size (µm)	Cd (df=68)	Co (df=64)	Cr (df=70)	Cu (df=70)	Hg (df=73)	Ni (df=69)	Pb (df=70)	Zn (df=70)
]100 - 500]	-0.074	0.415**	0.275*	0.360**	0.144	0.249*	0.326**	0.210
]50 - 100]	-0.206	-0.184	-0.161	-0.250*	0.077	-0.275*	-0.219*	-0.244*
]20 - 50]	0.204	-0.051	-0.004	0.018	-0.158	0.097	0.015	0.105
]5 - 20]	0.127	0.416**	0.275*	0.439**	-0.133	0.343**	0.412**	0.392**
]2 - 5]	0.228	0.504**	0.451**	0.613**	0.094	0.511**	0.577**	0.505**
[0 - 2]	0.221	0.437**	0.335**	0.567**	-0.083	0.417**	0.536**	0.484**

* $p \leq 0.05$; ** $p \leq 0.01$

The *G*-test of independence for the three areas (A, B and C) was not statistically significant (G_W=4.93, p=0.08, d.f.=2), denoting that the proportion of points belonging to the P90 of the observed contamination level was independent of the areas from where they were sampled. The spatial analysis of the distribution of the P90 (Fig. 3) revealed a preferential path of accumulation next to the branched channels of the estuary. The *G*-test of independence results showed that the distribution of the P90 of the observed contamination level was influenced by the channels localisation (G_W=13.24, p=0.003, d.f.=1).

Stomach contents

Stomach content dry weight (dw) varied between 0.872 and 15.857 g and their contents consisted mostly on particles between 50 and 20 µm. The same fraction was found to be the most abundant in the sediments, although its proportion was higher in the stomach contents (Table 4).

Feeding selectivity (L_i) was calculated only for particle size larger than 5 µm due to the low percentage of smaller particles in the stomach contents (less than 0.1%) (Table 4). Grain size particles with 100–500 µm were ingested approximately in the same proportion to their abundance in the environment in the three areas ($L_{100–500} \approx 0$).

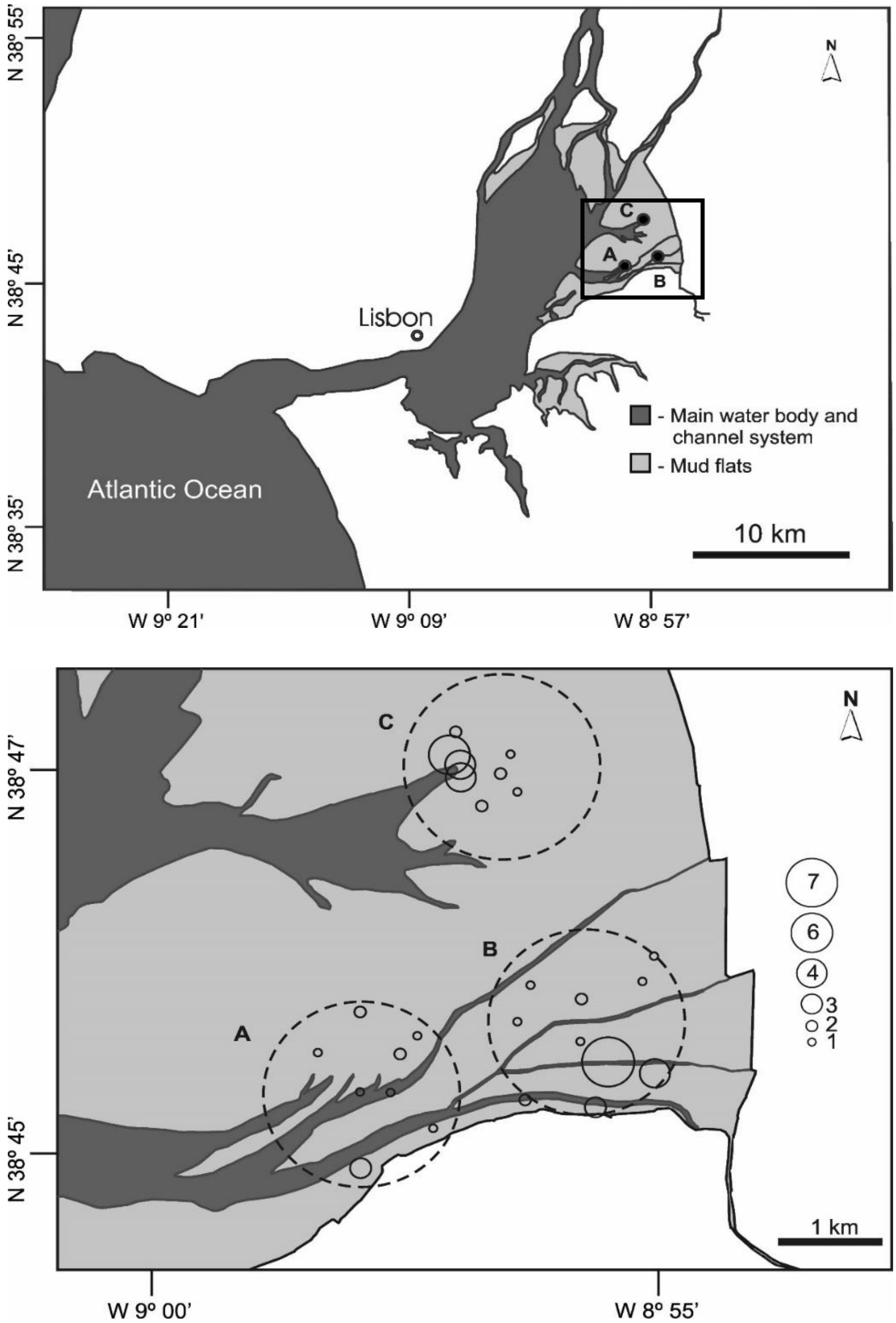

Figure 3 – Distribution pattern of the P90 of the observed metal contamination level. Different areas of the black circles represent the number of counts within the P90; dashed circles indicate areas A, B and C limits.

Regression between SL and L_i was not significant and no specific pattern was found between these two variables (Fig. 4). Medium sand particles are therefore ingested in similar proportions by *Liza ramado* specimens regardless of their size. Results for particles between 50–100 µm and 5–20 µm also pointed to near random selection in the environment by the fishes

($L_{50–100}$ and $L_{5–20}$≈0), but in this case, a significant relationship between the L_i and the fish's SL was found (Fig. 4). For fine sand particles (50–100 µm), a negative selection was observed, with rejection diminishing as SL increased. Silt particles (5–20 µm) were positively selected by fishes with sizes between 275-305 mm and rejected by larger specimens. Finally, the most common fraction in both sediment samples and stomach contents (20–50 µm) was positively selected by the entire SL range, with $L_{20–50}$ decreasing with the fish's size.

Table 4 – Comparison of the stomach contents' granulometric composition with the sediment samples from the three study areas (mean ± se; percentages)

Particle Size (µm)	*Liza ramado*	Sediment		
	stomach contents	A	B	C
]100-500]	2.73 ± 0.14	7.03 ± 0.45	6.37 ± 0.50	4.37 ± 0.47
]50-100]	9.28 ± 0.34	15.14 ± 1.12	11.81 ± 0.44	13.60 ± 0.70
]20-50]	87.62 ± 0.38	77.23 ± 1.22	81.17 ± 0.67	81.44 ± 0.84
]5-20]	0.27 ± 0.02	0.30 ± 0.01	0.33 ± 0.01	0.30 ± 0.01
]2-5]	0.06 ± 0.02	0.12 ± 0.01	0.15 ± 0.01	0.13 ± 0.01
[0-2]	0.05 ± 0.002	0.17 ± 0.01	0.18 ± 0.01	0.17 ± 0.01

This analysis showed that smaller specimens of *L. ramado* have the tendency to reject more particles of larger size or have a higher positive selection of smaller particles. On the other hand, larger animals may have a negative selection of larger particles but reject them less than smaller fishes. As the grain size decreases, larger fishes will show either a weaker positive selection or a negative selection towards smaller granulometric classes. It was possible to identify a preference of the fishes analyzed towards sediments with characteristics of areas B and C, i.e. higher quantity of particles belonging to the class 20–50 mm.

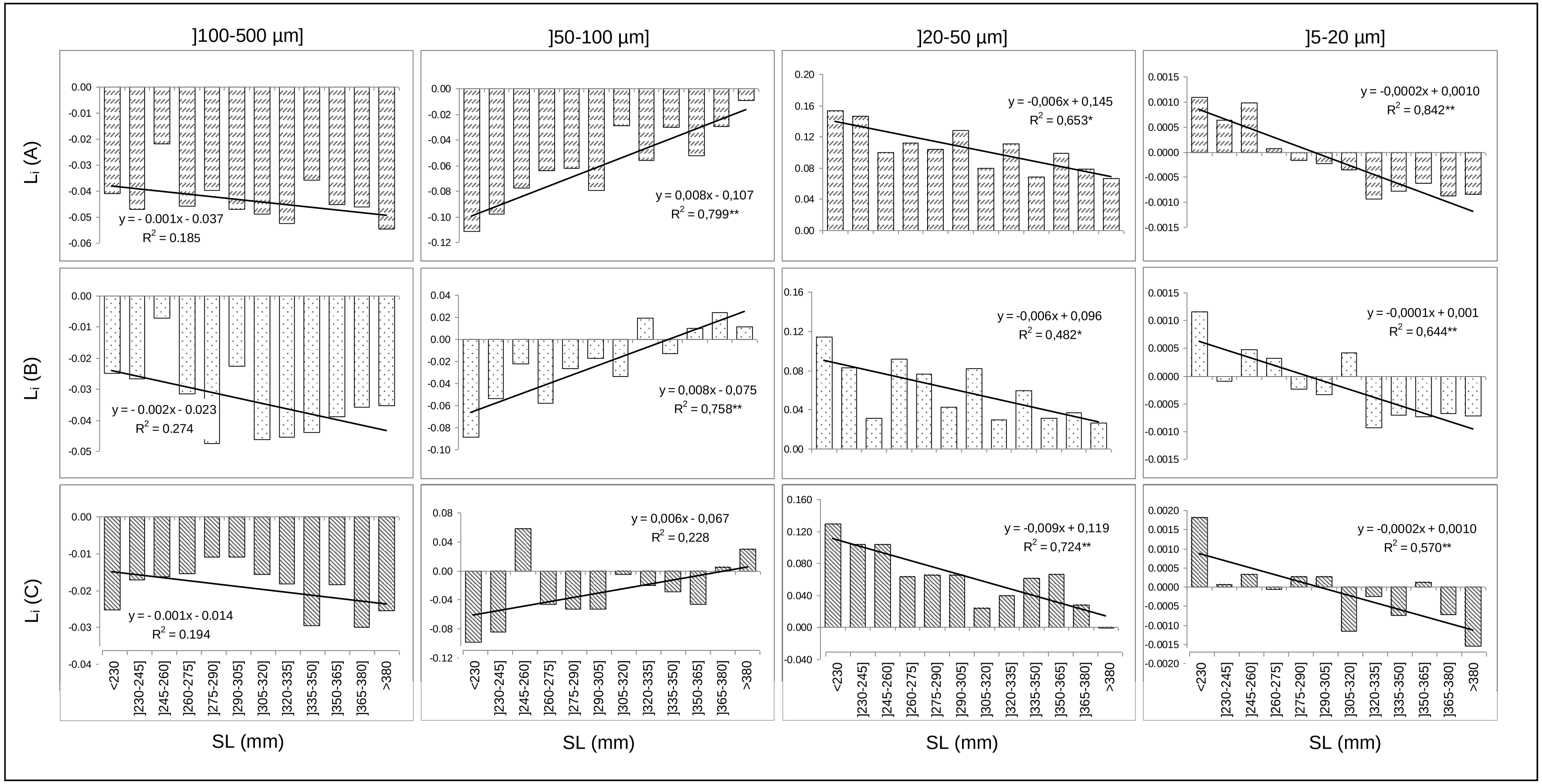

Figure 4 – Feeding selectivity of *Liza ramado* on different granulometric classes of sediment. Linear regression of Strauss' Selectivity Index (L_i) on fishes' standard length (SL, mm). *p<0.05; **p<0.01.

Higher value of $L_{20–50}$ in area A (mean $L_{20–50}$ for each area: A=0.104, B=0.059, C=0.062, Fig. 4) indicates that animals would have to invest more energy on finding the preferred sediment grain size, since this granulometric class was less abundant there than in the other two areas (Table 5).

Table 5 – ANCOVA results for the linear regression of the feeding selectivity index according to the fishes' standard length

	]100-500 µm]		]50-100 µm]		]20-50 µm]		]5-20 µm]	
A *vs* B *vs* C	Slope	Y-inter.	Slope	Y-inter.	Slope	Y-inter.	Slope	Y-inter.
F-statistics	0,414	26,281	0,211	6,659	0,933	18,915	0,343	0,029
p-value	0,665	0,000**	0,811	0,004**	0,405	0,000**	0,712	0,971

** - $p \leq 0.01$

DISCUSSION

The top layer sediment in the study area displayed a low heavy metal load. Metals concentrations, in general, were below the Effects Range-Low (ERL) values defined by Long *et al.* (1995). Exceptions to this were the accumulation of Cd (sites B and C) and Zn (site C), where the ERL values were slightly passed. ERL represents a minimal effects range on biological communities and it is calculated using the 10th percentile of the effects data for each chemical. It is a range intended to estimate conditions below which effects would rarely be observed.

The study area includes a part of the Nature Reserve of the Tagus estuary, near Hortas salt marsh, one of the least polluted salt marshes of the Tagus estuary (França *et al.*, 2005). Metal input of anthropogenic source has been reduced on the last two decades after several industries ceased their activity but urban and some industrial pressure are still present throughout the estuary. França *et al.* (2005) reported values of metal accumulation on Hortas salt marsh sediments (20 cm depth cores) slightly higher than those found in this work (0.5 cm top layer). Increasing metal loads with depth are usually an indicator that present concentrations are a consequence of background contamination levels and not recent anthropogenic sources. In recently polluted areas, surface sediments usually present higher concentration of contaminants than deeper layers (Ujevic *et al.*, 2000). Mercury levels were up to 10 times lower than the ones observed in a recent study for the same area in surface sediments (0-5 cm) (Canário *et al.*, 2005). The present work has shown that sampling points' location along the estuary influence metal concentration. Sampling near the estuary margins or in the branched channel system will yield higher values of metals accumulation than in the intertidal mudflats, so the differences found may not indicate a significant decrease of mercury in this particular area of the Tagus estuary, but only a different sampling approach. Our comparison of metal concentration in tidal channels and in the intertidal mudflats showed that, in general, heavy metals have a tendency to accumulate more in the deeper areas. Channels are less exposed to sediment resuspension processes, specifically to surface wave action, than the shallower mudflats (van Leussen, 1991), and this creates good conditions for contaminants deposition (van Leussen, 1991; Ujevic *et al.*, 2000). Almeida (1996) showed that the thin-lipped grey mullet follows the tidal movement when feeding demonstrating an increase in feeding intensity during the flood; other mullets display the same behavior, as described by Odum (1970), where a marked increase in the amount of food ingested as the tide rises for the striped mullet *Mugil cephalus* was reported. The main reason for this should be the fact that optimal feeding areas become accessible to the mullets with the flooding tide. Considering what was mentioned above, the first areas available for the mullets to feed upon are those where contaminants display a preferred accumulation path, i.e. the tidal channels system.

Several studies have described a direct correlation of fine grained sediments (<63 µm) with metal content, where the total amount of metals increases with decreasing grain size (e.g. Biksham *et al.*, 1991; Baptista Neto *et al.*, 2000; Ujevic *et al.*, 2000; Ikem *et al.*, 2003). The association of heavy metals with fine particles is generally attributed to the characteristics of finer grain sediments, namely: 1) the increasing surface area/volume ratio with decreasing size; 2) the negatively charged clay particles, which attract the positively charged metal ions; and 3) the organic matter content (Buol *et al.*, 1997). We found a positive correlation of most metals with silts and clays but also with the medium sand fraction. Ducaroir and Lamy (1995) have related the accumulation of metals in the coarser fractions as an indicator of natural accumulation processes, since it could not be attributed to the reasons that explain the association of metals with fine grain fractions. Zou *et al.* (2007) have recently described Cu as being mainly associated with coarser grain size particles (163-280 µm) in the contaminated surface sediment on a lake, but no causal explanation was advanced. Hg levels and Cd were not correlated with granulometric properties of the sediment. A study on Blanca Bay, Argentina, reported the same lack of correlation between Cd and sediment texture (Sericano and Pucci, 1982). The reduced variability of the TOM in the three locations may explain the lack of a positive correlation of this parameter with the smaller fractions of the sediment and heavy metals content, unlike what was described in other works (e.g. Ujevic *et al.*, 2000). When analyzing the feeding selectivity of *Liza ramado* for different grain size fractions available, we found a general trend of random selection or even rejection of sand and most silt and clay fractions, except for coarse silt (50-20 mm), where a distinct positive selection was observed. In addition, smaller animals seemed to reject larger particles in a greater extent than larger animals did, and the opposite selectivity was verified for smaller particles. Growth differences may be on the basis for the trends found in our work. Guinea and Fernandez (1992) found significant differences when comparing gill rakers of juveniles and adults of *L. ramado*; according to the same authors, gaps between structures on the gill rakers correspond to the size limit of particles which might be retained by them. Hence, differences between juveniles and adults could be translated into a possibility of selecting different size particles, which would corroborate the different selectivity found in the present study. Selectivity differences found for particles with 5–20 µm between different lengths of *L. ramado* specimens, where smaller animals showed a positive selection while larger ones rejected it, may be reflected in a higher exposure of younger fishes to contaminants, since this granulometric class presented a positive correlation with heavy metal accumulation. If smaller fishes will actively ingest particles of this size range, they will potentially retain more contaminated sediment. The present study shows that Hortas salt marsh should be of lesser concern, given the low contamination levels. Other salt marshes of the Tagus estuary, on the other hand, have been reported with accumulation of Cu and Ni above the ERL and Zn and Pb above the Effects range-median (ERM) level

(Caçador *et al.*, 1996b). Concentrations between the ERL and the ERM level represent a range within which effects on the biological communities would occasionally occur, and above the ERM represent a range within which effects on the biological communities would frequently occur (Long *et al.*, 1995).

CONCLUSION

Contamination levels in the superficial sediments are greatly dependent of the estuary physiography and circulation patterns, and contaminants distribution will depend not only of the proximity of a possible source, but also of these factors, among others. The thin-lipped grey mullet, along with known habits of filtering in the water–air interface, grazes on the topmost layer of the sediment. This is where we will most likely find recent origin metal contamination, not related with background/natural levels. Anthropogenic sources of trace metals are still available in the Tagus estuary. Although the Nature Reserve area manifests lower levels of metal contamination, the thin-lipped grey mullet feeds along the estuary, moving in shoals and following the tide; this means that it will probably graze on more contaminated areas than the present study location in some point of the tidal cycle. Direct consequences of the mullets feeding behaviour on bioaccumulation are not completely known.

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BIOACCUMULATION OF TRACE METALS IN THIN-LIPPED GREY MULLET (*LIZA RAMADA*): RELATIONSHIP WITH SIZE AND ECOLOGICAL REPERCUSSIONS

ABSTRACT

Trace metals (Cd, Cu, Hg, Ni, Pb and Zn) accumulation in *Liza ramada*'s gills, liver, muscle tissue and stomach contents, was investigated in different size specimens from the Tagus estuary (Portugal). The metabolic elements Zn and Cu stood out from the other elements, being among the most abundant metals in both tissues and stomach content samples. The liver registered the highest concentrations for Cd, Cu, Hg and Zn, while Pb was higher in the gills and Ni had identical accumulation levels in both organs. Sediment quality guidelines were exceeded in some cases in stomach contents, with potentially hazard situations being found in some samples for Hg, Ni and Zn. This reflects the fact that trace metals hotspots are still present in the Tagus estuary and grey mullets may feed on those locations. Our results were also indicative of common environmental sources for most of the investigated metals. Bioaccumulation evidence was found for Hg in the muscle and the liver, while Pb and Zn in muscle, Cu, Ni and Zn in gills and Cu and Zn in liver decreased significantly with the specimens' size. The negative correlations between size and metal accumulation point towards: 1) a more efficient regulation of metals by the larger specimens; 2) a growth dilution-effect; and/or 3) a positive selection of less contaminated particles by larger specimens. The ecology of this species together with the accumulation pattern for metals indicates that *L. ramada* could be a potential vector of contaminants dispersal within and between estuarine systems.

Keywords: Trace elements; accumulation; regulation; tissues; stomach contents; Mugilidae

INTRODUCTION

The thin-lipped grey mullet, *Liza ramada* (Risso, 1827) (Mugilidae), is a pelagic, catadromous species that spends a great part of its life cycle in estuaries. In the Tagus estuary, the largest Portuguese estuarine system and among the largest estuaries of Western Europe, this species occurs in great abundance. Estuaries, due to their privileged location, are under enormous urban and industrial pressure, and that tends to be reflected in the pollutants load, as it is observable in the multitude of anthropogenic wastes that ended in the shallow estuarine and coastal marine waters during the last century (McLusky and Elliott, 2004). Of these pollutants, trace metals are of major concern due to their potential toxicity to the biota. Trace elements accumulation by fish tissues depends on several aspects, such as fish species (Türkmen *et al.*, 2005), feeding habits (Pourang, 1995), ontogenic development (Farkas *et al.*, 2002, 2003), or the physical and chemical characteristics of the surrounding environment (Dallinger *et al.*, 1987). The pathways by which metal uptake may occur are diverse, the most common being the gills, the food ingested, the skin and the water intake, the latter being common in marine and estuarine fishes (Heath, 1995).

Benthic fish species will generally present higher concentration of pollutants than pelagic species (Roméo *et al*., 1999) because of their proximity to the bottom sediments. The reason for this is that metals tend to adsorb more readily to the bottom sediments than to remain in the water column. *L. ramada* feeds on suspended particulate matter (SPM) and grazes on the bottom sediments of estuaries, going additionally through intertidal mudflats and salt marsh creeks, while feeding on diatoms, detritus, decaying organic matter or even small macrofaunal organisms (Almeida, 1996; Laffaille *et al.*, 2002). The close proximity with the sediment, implied in the feeding behavior of this species, makes it of particularly interesting for the study of metal contamination in estuarine systems and how that could be reflected in biological accumulation. Regarding metals accumulation in fish tissue, a decrease is expected with size/age because feeding is considered to be the primary pathway for metal uptake by marine/estuarine fishes, and the feeding rate of fish diminishes with growth (Dang and Wang, 2012). All this implies that younger fish will potentially show higher accumulation of metals in their tissues (Farkas *et al.*, 2003). In this work, the levels of trace metals found in tissues of *L. ramada* and in their stomach contents were evaluated, and a size dependence relationship was investigated.

MATERIAL AND METHODS

Sampling

The Tagus estuary (38°44'N, 9°08'W) is a semi-diurnal mesotidal estuary with ca. 4 m of tidal range located in the West coast of Portugal (Fig.1). The estuary is composed of a deep and narrow inlet channel and a shallow bay differentiated in salt marsh areas, sand islands, and mudflat areas.

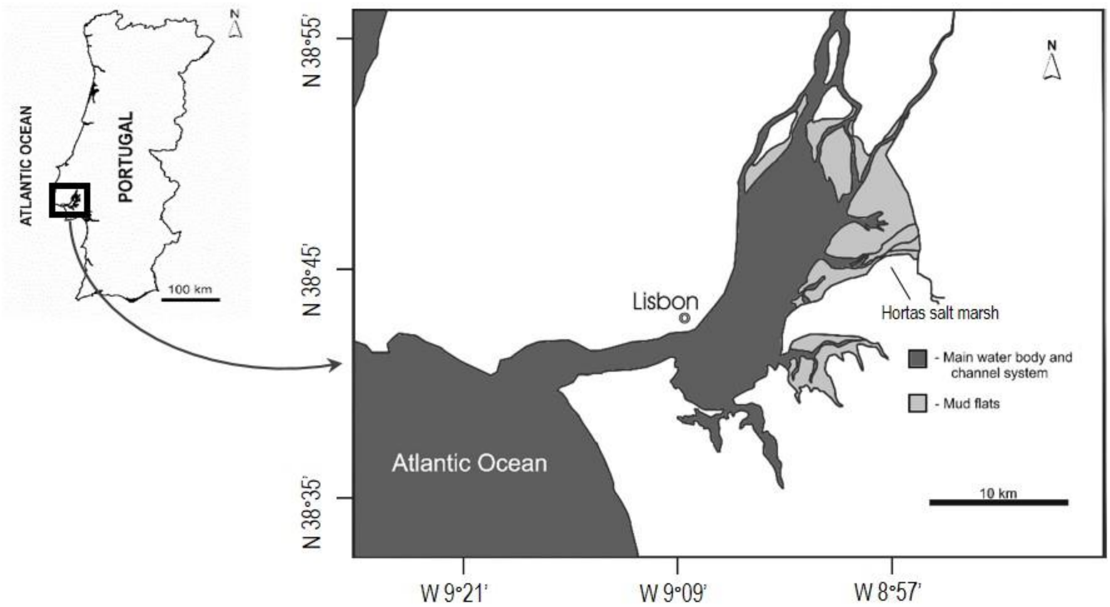

Figure 1 – Tagus estuary and Hortas salt marsh location.

Sampling was conducted in the southern part of the estuary in the extensive mudflats near Hortas salt marsh. Samples of *L. ramada* were obtained using a trammel net (30 to 40 mm knot-to-knot mesh size) in surveys made in 2006 and 2007. Fishes (N=58) were measured to the nearest 1 mm, weighed to the nearest 0.1 g and samples of muscle, liver and gills were frozen at -20°C for metal analysis. Liver and gills were completely removed and a portion of the skeletal muscle was cut from the flank below the first dorsal fin. Stomach contents of the cardiac portion of 76 specimens captured between 2007 and 2009 were also collected for trace metals analysis. Standard length (SL) of the specimens varied between 117 and 326 mm (age class from 1+ to over 8+). All samples were freeze-dried and ground with an agate mortar and pestle to homogenize.

Trace element analysis

All laboratory material used was decontaminated of any adsorbed ions by soaking in 0.25 M nitric acid (HNO_3) for 24 h and 0.25 M hydrochloric acid (HCl) for 48 h, and rinsing three times with deionized water (Reverse Osmose, Elga Purelab Prima) to avoid cross-contaminations. Samples (stomach contents and tissues) were freeze dried (Cryodos-50, Telstar Life Science solutions, Spain) prior to processing for metal extraction, and ground with an agate mortar and pestle prior to chemical treatment.

Stomach contents samples were acid digested (approximately 0.1 g) using 2 ml of a mixture of 65% HNO_3 (Panreac, p.a.) and 37% HCl (Carlo Ebra, p.a.) (3:1, v/v) at 110°C during 3 h. Fish tissue samples (muscle, liver and gills) were digested using 2 ml of a mixture of 65 % HNO_3 and 60% perchloric acid ($HClO_4$) (Panreac, p.a.) (9:1, v/v), in Teflon® vessels, at 110°C for 2 h (Julshamn *et al.*, 1982). The digestion solutions were cooled at room temperature, filtered through Whatman 42 filters (90 mm diameter; <2.5μm pore size) and made to 10 ml with ultrapure water (Type I, 18MΩ/cm, Elga Purelab Classic). Metal determinations (Cd, Cu, Ni, Pb and Zn) were done with inductively coupled plasma mass spectrometry (ICP-MS) using a Termo X Series with detection limits of 0.1 ppm (Cd, Ni), 0.5 ppm (Pb), 1.0 ppm (Cu) and 5.0 ppm (Zn). Total mercury was analyzed on freeze-dried samples by atomic absorption spectrometry (AAS) with thermal decomposition and gold amalgamation, using an Advanced Mercury Analyzer (AMA) LECO 254 (Costley *et al.*, 2000). The accuracy and precision of the analytical methodology for elemental determinations were assessed by replicate analysis of certified reference materials, BCR-277R (IRMM) for sediments and TORT-2 (NRCC) for fish tissues. Blanks and the concurrent analysis of the standard reference material were used to detect possible contamination/losses during analysis and to ensure the accuracy and precision of the analytical method. Measured values for total elements analysis were in agreement with certified reference values.

Statistical treatment of the data

The relationship between SL and metal concentration of fish tissues and stomach contents was assessed with Spearman's correlation coefficient. The same analysis was used to determine correlations between trace elements within tissues and within stomach contents. Kruskal-Wallis test (H) was used to compare trace elements concentrations found in muscle, liver and gills (Zar, 1999). Multiple comparison tests were applied to the analyses that evidenced statistical significant differences ($p < 0.05$) among groups. All analyses were carried on STATISTICA 9.0 analysis pack (StatSoft, 2008).

Median concentrations of metals in stomach contents were compared with established sediment quality guidelines from Long *et al.* (1995), namely to the Effects-Range Low (ERL) and Effects-Range Median (ERM) levels. These guidelines correspond to the lower 10th percentile (ERL) or to the 50th percentile (ERM) of the effects data for each element.

RESULTS

Trace elements content on gills, liver and muscle of *Liza ramada* presented different magnitudes, with Zn and Cu being among the most abundant elements in all tissues (Table 1).

Table 1 – Metal concentrations (mean ±sd) in gills, liver, muscle tissue and stomach contents (S.C.) of *Liza ramada* from the Tagus estuary (μg.g^{-1}, dry weight); n.d. – below detection limit

Tissue	Cd	Cu	Hg	Ni	Pb	Zn
Gills	0.04 ± 0.18	14.67 ± 9.18	0.05 ± 0.01	1.80 ± 0.86	28.25 ± 11.86	170.17 ± 69.43
Liver	2.18 ± 1.65	1060.07 ± 804.37	2.75 ± 1.02	1.75 ± 1.22	10.38 ± 8.96	239.73 ± 146.39
Muscle	n.d.	2.82 ± 1.42	0.16 ± 0.04	0.14 ± 0.11	0.14 ± 0.36	31.88 ± 25.01
S.C.	0.18 ± 0.13	24.51 ± 9.40	0.49 ± 0.19	17.78 ± 5.82	35.73 ± 11.66	153.89 ± 52.80

Liver displayed the highest values of accumulation for most elements. Exceptions occurred for Pb, with gills showing the highest concentration (28.25 ± 11.86 µg.g^{-1}), and Ni, which had similar concentrations in liver and gills (1.75 ± 1.22 µg.g^{-1} and 1.80 ± 0.86, µg.g^{-1} respectively). Muscle tissue had the lowest values for all elements except for Hg (0.16 ± 0.04 µg.g^{-1}). Gills, muscle and liver showed statistically significant differences with each other regarding Cu, Hg and Pb concentrations (Fig. 2).

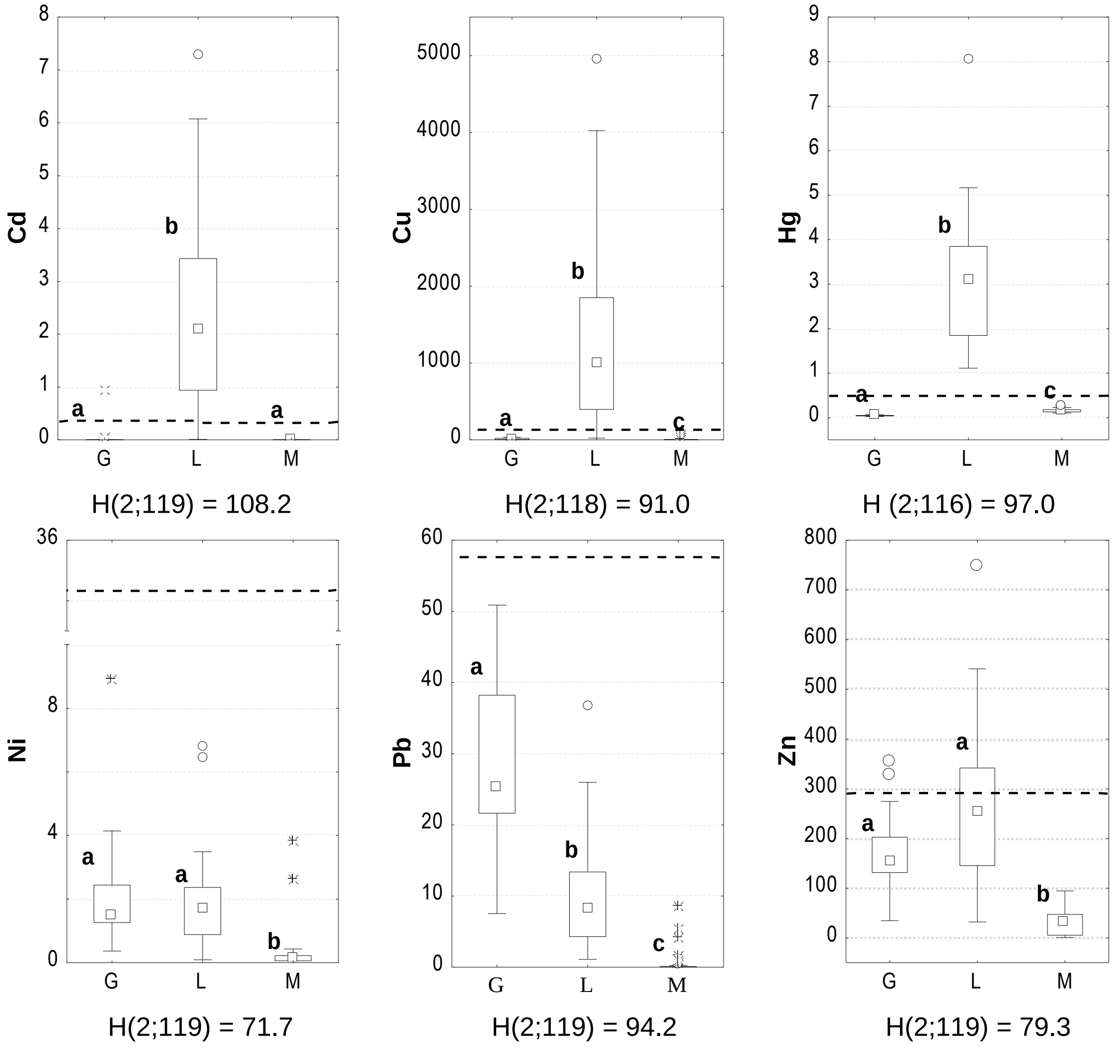

Figure 2 – Box-Whisker plots of trace elements concentration (µg.g^{-1}, dry weight) on gills (G), liver (L) and muscle (M) of *Liza ramada*. ▫ Median, ☐ 25-75%, ⌶ Non-outlier range, ○ Outliers, ∗ Extremes. Dashed line: average stomach contents concentration. Kruskal-Wallis (H) test results are given below each plot. Different lower case letters: significant differences between the tissues ($p < 0.05$).

Of these elements, all except Pb, as mentioned, were higher in the liver. Liver and gills presented significantly higher concentrations of Ni and Zn than those verified in the muscle ($p < 0.05$), but did not differ from each other ($p > 0.05$);

Cd concentration in the liver (2.18 ± 1.65 $\mu g.g^{-1}$) was significantly higher than both gills and muscle, with concentration in the latter two tissues being nearly zero for all samples (Fig. 2).

The average metal concentrations found in the stomach content samples appeared in the following decreasing order of magnitude: Zn > Pb > Cu > Ni > Hg > Cd (Table 1). The comparison of the metal concentrations in stomach contents with sediment quality guidelines showed several metals above the Effects-Range Low concentration (Long *et al.*, 1995) (Fig. 3).

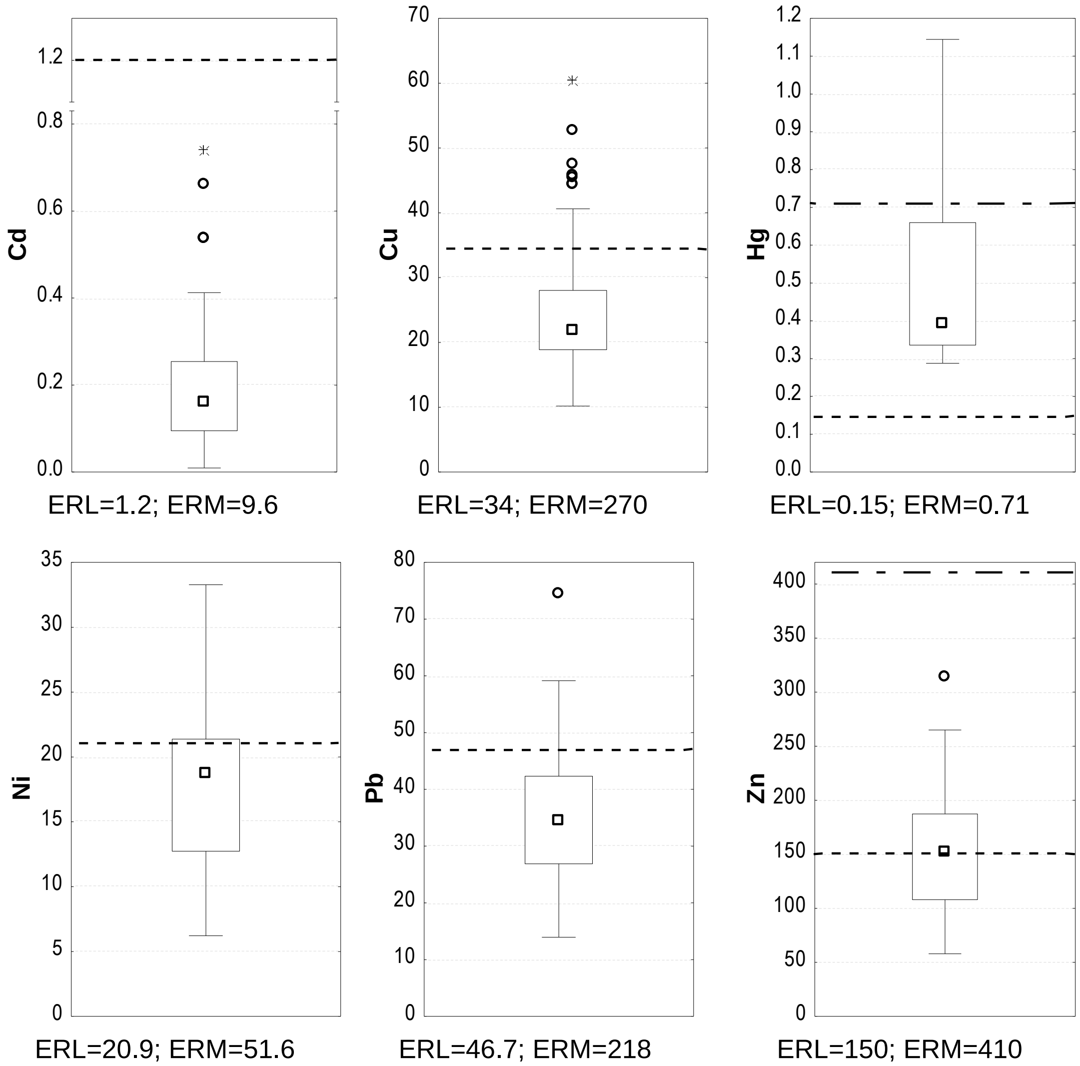

Figure 3 - Concentrations of trace elements ($\mu g.g^{-1}$ dry weight) in stomach contents of *L. ramada*. □ Median, ⎕ 25-75%, ⌶ Non-outlier range, ○ Outliers, ∗ Extremes. - - - Effects Range-Low (ERL), — - — Effects Range-Median (ERM) (Long *et al.*, 1995).

The Effect-Range Median concentration was exceeded in some samples in the case of Cu, Hg, Ni, Pb and Zn concentrations. Zinc showed significant negative correlation with standard length (SL) for all tissues (Table 2). The same significant negative correlation with SL was found regarding Ni accumulation in the liver and gills, and regarding Cu accumulation in the gills. The accumulation of Pb only showed significant correlation with the fishes' size in the muscle and, as it was observed in the previous cases, that correlation was negative. Finally, Hg concentration showed an inverse trend compared with the remaining metals: the accumulation of this metal in the muscle and liver showed a significant increasing trend with the fishes' length.

The analysis of the metal burden present in stomach contents according to the size of the fishes (Table 2) has evidenced significant positive correlations for Cd, Cu, and Hg ($p < 0.01$), i.e., for these metals the concentration in the stomach contents tended to increase in larger animals. The correlation between the remaining elements and the fishes' standard length was not statistically significant.

Table 2 – Spearman's correlation coefficient (R) between standard length of *L. ramada* and metal concentrations in gills, liver, muscle and stomach contents (S.C.); n.d. – below detection limit

R	Cd	Cu	Hg	Ni	Pb	Zn
Gills	-0.03	-0.40*	-0.24	-0.44*	0.18	-0.55**
Liver	-0.08	-0.32	0.55**	-0.43*	0.05	-0.49**
Muscle	n.d.	-0.17	0.30*	-0.09	-0.28*	-0.45**
S.C.	0.62**	0.31**	0.55**	0.09	0.18	0.22

* $p < 0.05$; ** $p < 0.01$

Significant positive correlations ($p < 0.05$) were found between several trace elements within the gills, the liver and the muscle tissue (Table 3). In the gills (Table 3a), almost every element presented significant correlation with one or more elements, with Cd being the only exception. The highest significant correlation in the gills was found between Ni and Zn (R > 0.8), and all other significant correlations were positive, with coefficients varying between 0.46 (between Cu and Zn) and 0.62 (between Ni and Pb).

Liver concentrations of Cd, Cu, Ni, Pb and Zn showed significant positive correlations with each other (except for Cu and Pb, which were not correlated) (Table 3b). Correlation coefficients varied between 0.42 (Pb and Cd) and 0.69 (Cd and Cu). Mercury did not show significant correlation with any metals in liver. The concentration of Hg in the muscle was not correlated with the concentration of the other metals in that tissue either. Copper, Pb and Zn concentrations were positively correlated in the muscle, while Ni only showed significant positive correlation with Zn (Table 3c).

Regarding stomach contents (Table 3c), the correlation analysis revealed very close relationships between Cu, Ni, Pb and Zn (R≈1). Cadmium correlation with other elements was moderate to strong, and significant, with correlation coefficients between 0.32 and 0.53, and R=0.70 in the case of the correlation with Hg. This was in fact the only significant correlation found regarding Hg in stomach contents.

Table 3 – Spearman's correlation coefficient between trace metals within gills, liver, muscle and stomach contents (S.C.) of *Liza ramada*; Each diagonal matrix represents the results within a tissue or stomach contents; n.d. – below detection limit

Gills (a) / Liver (b)	Cd	Cu	Hg	Ni	Pb	Zn
Cd		0.01	0.22	0.11	0.26	0.17
Cu	0.69**		0.47*	0.32	-0.07	0.46*
Hg	0.26	0.09		0.14	-0.08	0.29
Ni	0.54**	0.61**	-0.03		0.62**	0.88**
Pb	0.42*	0.31	-0.01	0.56**		0.44*
Zn	0.48**	0.55**	-0.19	0.57**	0.56**	
Muscle (c) / S.C. (d)						
Cd		n.d.	n.d.	n.d.	n.d.	n.d.
Cu	0.51**		-0.15	0.22	0.56**	0.55**
Hg	0.69**	0.13		-0.05	-0.11	-0.26
Ni	0.29**	0.90**	-0.1		0.25	0.31*
Pb	0.40**	0.97**	0.01	0.95**		0.47**
Zn	0.41**	0.97**	0	0.94**	0.98**	

* $p < 0.05$; ** $p < 0.01$

DISCUSSION

Fish tissues

Metal concentrations found in the muscle, liver and gills of *Liza ramada* were found to be within the range described for this species by other authors (Table 4), and in some cases concentrations were similar to those reported in considerably polluted estuaries (e.g. Blasco *et al.*, 1999; Durrieu *et al.*, 2005). The accumulation levels found in the liver, gill and muscle tissue are in agreement to what is usually found in mugilids and other teleost fishes (Durrieu *et al.*, 2005; Fernandes *et al.*, 2007; Vicente-Martorell *et al.*, 2009).

Contrary to the high levels generally expected for liver and gills, trace metals concentration described for muscle tissue is usually low (Kalay *et al.*, 1999; Karadede and Unlu, 2000; Karadede *et al.*, 2004; Yilmaz, 2005; Chouba *et al.*, 2007) strengthening the idea that muscle is not an active accumulation tissue. The liver, however, is undoubtedly the major accumulation organ analyzed in this work. This is one of the most important organs in detoxification mechanisms and its metabolic importance makes it a primary organ for accumulation of xenobiotics (Heath, 1995; Olsson *et al.*, 1998). The accumulation of metals in the liver may have an important role in the regulation of these contaminants, other than excreting them (Buckley *et al.*, 1982). High levels of Cu, and also Zn, in the liver are also justifiable by the presence of the hepatic metallothioneins (Bunton and Frazier, 1989), low molecular weight proteins that have the capacity to bind both physiological and xenobiotic metals.

Gill anatomy and function makes it exposed to pollutants dissolved in the water, this way becoming a target organ for metal accumulation as well (Olsson *et al.*, 1998). The gill membrane is a complexing ligand on itself, negatively charged, and metals are expected to bind them (Playle, 1998). This organ is the primary uptake point for waterborne metals, and the elements addressed in this study are no exception (Grosell, 2011; Hogstrand, 2011; Kidd and Batchelar, 2011; Mager, 2011; McGeer *et al.*, 2011; Pyle and Couture, 2011).

Table 4 – Concentration of trace elements in muscle, liver and gill of *Liza ramada* (mean values, µg.g^{-1}). Comparison of present work with data from literature. GUA – River Guadalquivir, Spain; GI – Gironde estuary, France; LM – Lake Manzala, Egypt; Med. – Mediterranean Sea; MG – Mersin Gulf, Turkey; RA – Ria de Aveiro, Portugal; TE – Tagus Estuary, Portugal; bold values in muscle exceeded maximum allowable concentrations for edible parts of fish species (legislation available for Cd, Hg and Pb (*OJEC, 2006*); dw – dry weight, ww – wet weight

Species	Orig	Cd	Cu	Hg	Ni	Pb	Zn	References
				Muscle				
L. ramada (dw)	TE	0.0	3.2	0.2	0.3	0.14	30.7	This work
L. ramada (dw)	TE	0.4 – 0.9	0.9 – 9.3			1.5 - 3.7	22.5 – 46.9	*França et al., 2005*
L. ramada (dw)	LM	0.6 – 0.8	3.5 – 4.5			1.5 - 2.2	15.6 – 30.7	*Bahnasawy et al.,2009*
L. ramada (dw)	MG		1.2 – 1.6				21.7 – 27.3	*Kalay et al., 2008*
L. ramada (dw)	GI	0.03	1.7	0.5			20.9	*Durrieu et al., 2005*
L. ramada (ww)	TE	0.0	0.7	0.03	0.07	0.03	6.5	This work
L. ramada (as *M. capito) (ww)*	MED		0.9	0.05	1.1	0.05	6.5	*Storelli et al., 2006*
L. ramada (ww)	GUA	0 – 0.003	0.1 – 1.0		0.07 – 0.26	0.03 – 0.06	4.4 – 21.5	*Blasco et al., 1999*
				Liver				
L. ramada (dw)	TE	1.8	1448.5	2.98	2.50	3.26	239.9	This work
L. ramada (dw)	MG		875.0 – 2066.0				161.5 –	*Kalay et al., 2008*
L. ramada (dw)	GI	40.0	3642.0	2.79			225.0	*Durrieu et al., 2005*
L. ramada (dw)	GUA	0.6 – 7.0	561.0 – 4110.0		1.45 – 3.95	0.36 – 2.16	220.0 –	*Blasco et al., 1999*
L. ramada (ww)	TE	0.5	405.4	0.84	0.71	0.96	69.4	This work
L. ramada (as *M. capito) (ww)*	MED		177.8	0.11	3.59	0.42	49.20	*Storelli et al., 2006*
				Gill				
L. ramada (dw)	TE	0.03	16.78	0.04	2.64	8.61	163.6	This work
L. ramada (dw)	LM	2.30 – 5.20	11.6 – 15.4			5.6 – 10.1	54.3 – 103.6	*Bahnasawy et al.,2009*
L. ramada (dw)	GI	0.97	8.9	0.09			109.0	*Durrieu et al., 2005*
L. ramada (ww)	TE	0.01	4.53	0.01	0.75	2.37	45.2	This work
L. ramada (as *M. capito) (ww)*	MED		2.43	0.05	4.48	2.48	36.9	*Storelli et al., 2006*

In addition to the potential waterborne metals exposure, the thin-lipped grey mullets have their gills exposed to another source of contaminants, the sediment, since they filter the suspended and bottom sediment particles for feeding purposes, as they move through the estuary during each tidal cycle (Almeida *et al.*, 1993). Higher levels of metals are thus expected in the gill of *L. ramada*, especially since the Tagus estuary has received important inputs of contaminants of anthropogenic origin (*i.e.* urban, industrial and from agriculture) for several decades, with high levels of trace metals among those contaminants. Although emissions from industries have diminished and effluent treatment has been improved, the sediment still shows elevated concentrations of those elements (Canário *et al.*, 2005; Vale *et al.*, 2008). Not only elevated concentrations of several elements were found in the gills, namely Ni, Zn and Pb, as the significant positive correlation between those elements reinforces the idea of a common anthropogenic source for them. The amount of anthropogenic origin Pb and Zn, and also Cd, was estimated as being over 80% of the total amount found in surface sediments for Tagus estuary, while Ni and Cu were between 57 to 68% (Vale *et al.*, 2008).

The high concentrations of metals in gills must be interpreted with cautious, though. The metals determined there may not only be associated with the gill tissue itself, since gill filaments and lamellae are lined with protective mucus (Arillo and Melodia, 1990; Shephard, 1994). When analyzing the metal burden in this organ it is very difficult to dissociate metals associated with the mucus from the aforementioned gill tissue contamination. High metal accumulation in the gills can, thus, also be explained by the complexation of the metallic cations with the negatively charged mucus (Shephard, 1994) present in the outer surface of gills. Mucus will simultaneously enhance the bioaccumulation of trace elements in the gills and also act as a barrier to their uptake into the fish (Heath, 1995). Nevertheless, the gill is undoubtedly an uptake route for metals, as already referred, specifically for those that are waterborne (Mager, 2011), as opposed to dietary intake. The uptake of Pb, for example, is likely to follow the Ca^{2+} route across the gill, which is among the tissues that are known to bioconcentrate[4] Pb, along with the kidney and the intestine (Mager, 2011).

Metals like Cd and Pb did not increase with age or size. Taking again the previous example of Pb, this metal mimics essential elements, like Ca^{2+}, and that makes it to be sequestered in the calcifications of the organisms, such as the skeleton. As fishes size/age increases, the contribution of the calcified structures will have less importance when compared to that of the muscle mass, and this will translate into a growth dilution effect (Mager, 2011). On the contrary, Hg concentration increased with the fishes' size. It is possible to find several examples of this increasing size-dependence for Hg accumulation in the literature (e.g. Storelli *et al.*, 2002; Adams and Onorato, 2005; Branco *et al.*, 2007; Staudinger, 2011). An elegant laboratory study conducted on biokinetic parameters' effects on positive allometric concentrations of Hg in juvenile blackhead sea bream (*Acanthopagrus schlegeli*) showed that the growth and Hg efflux rates were probably the key drivers for increasing Hg burdens with increasing body size, and that assimilation efficiency inorganic mercury also increased with size (Dang and Wang, 2012). Additionally to the described bioaccumulation of Hg with age/size, this element has also been described as being the only metal for which studies on marine trophic webs actually show biomagnification (Gray, 2002), i.e., a greater body burden is acquired from being at a higher trophic level (Heath, 1995; Gobas and Morrison, 2000). Biomagnification of Hg has been described for several fish species, particularly for deep-water fishes (Afonso *et al.*, 2007) or top predators (Escobar-Sanchez *et al.*, 2011). This is not the case of *Liza ramada*. This species is a primary consumer, displaying a considerable feeding plasticity; the main food items found in stomach contents are detritus, different microalgae groups (particularly diatoms), copepods, and nematodes (Laffaille *et al.*, 2002; Almeida, 2003). Being at a very low trophic level, it is not expected to find biomagnification of Hg, but the results obtained with the present work support the bioaccumulation referred to Hg, since significant positive correlation with standard length was

[4] Unlike bioaccumulation, in which the chemical' concentration in the organism results from all possible routes of exposure (dietary absorption, transport across the respiratory surface, dermal absorption…), bioconcentration results of exposure to the waterborne chemical only (Gobas and Morrison, 2000; Gray, 2002).

found in both liver and muscle; as in the present results, other species of mugilids have evidenced bioaccumulation of Hg (e.g. Marcovecchio, 2004).

The correlation coefficient in liver was higher than in muscle, probably reflecting the protective role of the liver when the capacity to excrete the metal is somehow exceeded. One of the possible strategies to this is the increase in the metallothioneins (MT) concentration. Metallothioneins often sequester non-essential metals, such as Hg, in order to reduce their toxicity to the surrounding cellular environment (Heath, 1995).

Contrary to Hg, otherwise significant negative relations were found for Pb and Zn in muscle, Cu, Ni and Zn in gills and Cu and Zn in liver. These results could be explained by a more efficient regulation of metals in question by the larger specimens, a growth dilution-effect, in which the gain in body mass surpasses the incorporation rate of the metals (Cronin *et al*., 1998; Lin *et al.*, 2001), and/or by a positive selection of less contaminated particles by larger specimens. Metabolic rates are usually higher in smaller individuals of the same species (Sims, 1996; Dang and Wang, 2012) which reflects in smaller fishes accumulating substances like metals more rapidly than larger ones (Newman and Mitz, 1988; Farkas *et al.*, 2003), but the same applies to depuration rates (Newman and Mitz, 1988; Dang and Wang, 2012). If positive selection of less contaminated particles occurs in larger individuals (Pedro *et al.*, 2008), the negative correlation between metals and the fishes' standard length is also expected.

Stomach contents

The analysis of the stomach contents regarding the size of the fish yielded partially contradictory results to those previously found by the authors (Pedro *et al.*, 2008). Three of the six metals under study in the stomach contents (Cd, Cu and Hg) showed significant positive correlations with standard length, meaning that the larger animals have probably fed onto more contaminated areas. The surface sediment contamination in the Tagus estuary is not uniform (Canário *et al*., 2005; Pedro *et al*., 2008; Vale *et al.*, 2008); in fact, highly contaminated pools only represent about 15% of the estuary total area (Vale *et al.*, 2008), with the remaining presenting substantially lower levels of contamination. This patched distribution of metal contamination is the probable cause for two of the observed results: (i) the fact that the correlation analysis between elements evidenced two distinct groups of metals with a likely common origin (Cu, Ni, Pb and Zn, $R \geq 0.9$; Hg and Cd, $R = 0.69$; $p < 0.05$); and (ii) the difference found in stomach contents regarding the results obtained by Pedro *et al.* (2008). According to that work, negative correlations between standard length of fishes and metal concentration in stomach contents were to be expected, but that was not verified; it is possible that different size fishes may have fed in distinct areas while moving through the estuary during the tidal cycles, thus explaining divergences from the previous work's results in what concerns the metals concentration in the stomach contents.

The strong correlation found among Cu, Ni, Pb and Zn in stomach contents (Spearman's $R \geq 0.9$, $p < 0.05$) corroborates the fact that a large percentage of the sediment has common anthropogenic sources (Vale *et al.*, 2008). The common source hypothesis gains strength if we consider that stomach contents of *L. ramada* are essentially constituted by particles which are either from the sediment surface or from suspended particulate matter in the water column, thus of relative recent origin. Five of the studied elements (i.e. Cu, Hg, Ni, Pb and Zn) were above the ERL and three of these (Hg, Ni and Zn) were even above the ERM levels (Long *et al.*, 1995). Concentration ranges below the ERL level are rarely associated with adverse effects on the biological communities in marine and estuarine sediments; between the ERL and ERM levels, concentrations are occasionally associated with those adverse effects; and above the ERM concentrations will be frequently associated with adverse effects in the biological communities. Our results indicate that the stomach contents levels of most elements are in accord with concentrations capable of inducing adverse effects on biological communities, particularly if a significant fraction of those metals is available for biological uptake. The ingestion of contaminated sediments by deposit feeders may be an important pathway of metal bioaccumulation (Luoma, 1989), and metal available from the sediment may in fact be reflected in fish tissues (Vicente-Martorell *et al.*, 2009).

CONCLUSION

The thin-lipped grey mullet, a cosmopolite and catadromous species, is a key element in establishing an ecological connection between different areas of the estuary. This species has a long residence time in the estuarine ecosystem, throughout the year and also its lifespan, given that it only leaves the estuaries to spawn. This aspect of the grey mullets' life cycle, combined with the direct contact with the surface sediment and particulate matter (SPM) that their feeding habits promote, enables a persistent exposure to the pollutants associated with these estuarine 'compartments'. This is a species that can easily thrive in ecologically stressed environments. The long distances travelled by thin-lipped grey mullets allow for the specimens to transport metals associated to sediment particles and SPM over a wide area. The relevance and extent of the consequences of this transport will depend, for example, on sediment egestion rates or metal efflux rate from the organism.

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CHAPTER 3

METAL SPECIATION IN SALT MARSH SEDIMENTS AND INTERTIDAL MUDFLATS

METAL SPECIATION IN SALT MARSH SEDIMENTS: INFLUENCE OF HALOPHYTE VEGETATION IN SALT MARSHES WITH DIFFERENT MORPHOLOGY

ABSTRACT

Salt marshes provide environmental conditions that are known to affect metal speciation in sediments. The elevational gradient along the marsh and consequent differential flooding are some of the major factors influencing halophytic species distribution and coverage due to their differential tolerance to salinity and submersion. Different species, in turn, also have distinct influences on the sediment's metal speciation, and its metal accumulation abilities. The present work aimed to evaluate how different halophyte species in two different salt marshes could influence metal partitioning in the sediment at root depth and how that could differ from bare sediments. Metal speciation in sediments around the roots (rhizosediments) of *Halimione portulacoides*, *Sarcocornia fruticosa* and *Spartina maritima* was determined by sequentially extracting operationally defined fractions with solutions of increasing strength and acidity. Rosário salt marsh generally showed higher concentrations of all metals in the rhizosediments. Metal partitioning was primarily related to the type of metal, with the elements' chemistry overriding the environment's influence on fractionation schemes. The most mobile elements were Cd and Zn, with greater availability being found in non-vegetated sediments. Immobilization in rhizosediments was predominantly influenced by the presence of Fe and Mn oxides, as well as organic complexes. In the more mature of both salt marshes, the differences between vegetated and non-vegetated sediments were more evident regarding *S. fruticosa*, while in the younger system all halophytes presented significantly different metal partitioning when compared to that of mudflats.

Keywords: Halophytes; *Halimione portulacoides*; *Sarcocornia fruticosa*; *Spartina maritima*; metal partitioning; salt marshes; mudflats

INTRODUCTION

Salt marshes play important roles in the estuarine ecosystem, like nutrient cycling or shoreline stabilizers. They are considered natural sinks for pollutants transported in the ecosystem (Caçador *et al.*, 1993; Doyle and Otte, 1997; Caçador *et al.*, 2000), functioning as buffers. The usual proximity to densely populated and/or heavily industrialized areas leaves salt marshes facing important discharges of such pollutants. The idea that estuaries had the ability to dilute and disperse pollutants led to urban and industrial discharges into estuarine waters without pretreatment of wastes. Together with agricultural and road runoff, urban and industrial discharges added up to increase the pollutant load in these environments, namely metals (Williams *et al.*, 1994). Metals are not naturally removed or broken down, and end up accumulating in the estuarine environment, of which salt marshes are a part (Doyle and Otte, 1997). Halophytes influence the concentration of metals in salt marsh sediments, with increasing concentrations in the sediment between roots when compared to bulk sediments (Caçador *et al.*, 1996a; Doyle and Otte, 1997; Reboreda and Caçador, 2007a). Species distribution in salt marshes is influenced by the typical elevational gradient along the marsh and consequent differential inundation periods (Sanchez *et al.*, 1996). The differential plant zonation will in turn influence a variety of physical, chemical and biological processes (Williams *et al.*, 1994) and ultimately affect the sediment's metal accumulation capacity (Reboreda and Caçador, 2007a). Plants promote these changes by several ways. The pumping of atmospheric oxygen by the root system (Koop-Jakobsen and Wenzhöfer, 2014), for example, is responsible for oxidizing the sediment, causing shifts in the sediment redox potential, thus potentially affecting mobility and availability of metals (Williams *et al.*, 1994). Another example involves plant detritus: plant litter actively draw metals from the water, which can immobilize metals in the salt marsh sediments, making them less available to surface waters (Lyngby and Brix, 1989). Metal uptake by plants does not usually reflect total metal concentrations in sediments (Caçador *et al.*, 2009). Instead, it is the form of metals, i.e., the geochemical fraction to which they are bound, that will influence their bioavailability for plant uptake (Reboreda and Caçador, 2007a). Exchangeable and water-soluble forms are more bioavailable, while metals associated to the crystalline lattice of minerals are potentially unavailable to biota (Weis and Weis, 2004). Parameters such as soil texture or organic matter content combine to increase availability or immobilize metals (Greger, 2004). Plants themselves not only change the sediment's ability to accumulate metals, as mentioned above, but also exert influence on metal speciation, and consequently in metal mobility (Caçador *et al.*, 1996b; Reboreda and Caçador, 2007b; Reboreda *et al.*, 2008).

Three of the most abundant halophytes in Mediterranean salt marshes were chosen to investigate the influence of vegetation on metal mobility and availability in salt marsh sediments. A detailed fractionation scheme was used in two different salt marshes and adjacent areas of intertidal mudflats.

MATERIAL AND METHODS

Study area and sampling

Sampling occurred in two salt marshes in the left margin of the Tagus estuary (Fig. 1), in the spring of 2010. Hortas salt marsh (38° 45.571' N; 8° 54.451' W) is located in the vicinity of Alcochete, in the middle estuary, next to an area that comprises the Tagus Estuary Natural Reserve. Rosário saltmarsh (38° 40.161' N, 9° 00.198' W) is located in the lower estuary, next to an area with higher urban and industrial pressures, in the surroundings of densely populated cities (e.g. Montijo). Rosário is a mature marsh with dense and well established vegetation, while Hortas is a young marsh still accreting and presenting the typical sparse vegetation stands of a young marsh (Duarte *et al.*, 2013a)

Both salt marshes are dominated by three halophyte species: *Spartina maritima* Fernald (Poales, Poaceae) in the lower marsh, followed by *Halimione portulacoides* (L.) Aellen (Caryophyllalles, Chenopodiaceae) in the mid-upper marsh, and *Sarcocornia fruticosa* (L.) A.J. Scott (Caryophyllalles, Chenopodiaceae) in the upper marsh (Caçador *et al.*, 1996a; Caçador *et al.*, 2013). Sediment cores were sampled beneath pure stands of each species, and in the adjacent non-vegetated area. Samples from 5-8 cm deep (higher root density) were sliced for further analysis. All samples were quickly transported to the laboratory in plastic bags within refrigerated boxes. Rhizosediments were cleared from plant material and debris with the aid of tweezers.

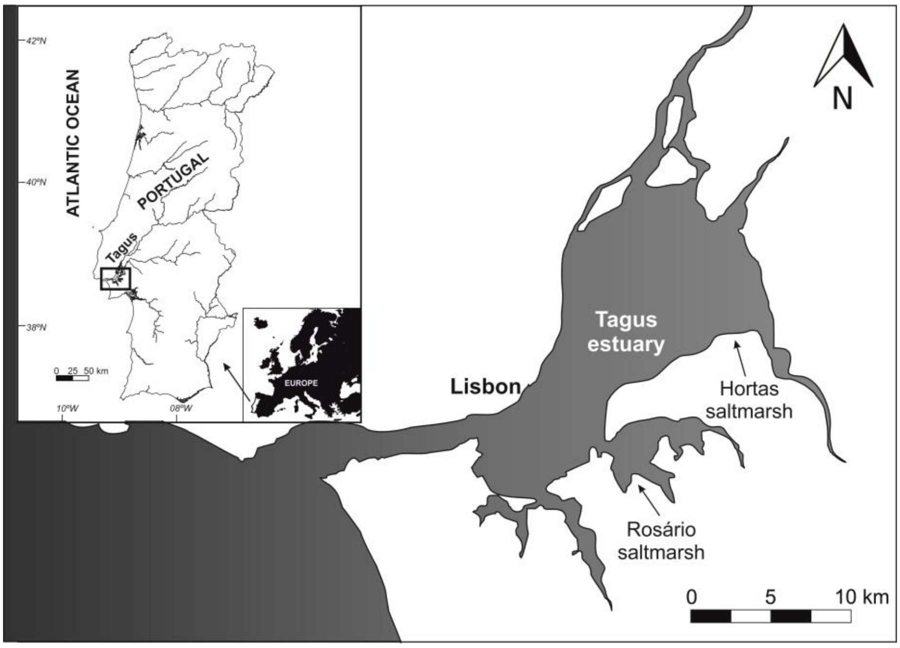

Figure 1 – Tagus estuary, with indication of Hortas and Rosário salt marshes position.

Organic matter content and particle size distribution

Samples were used to determine total organic matter (TOM) content as loss on ignition (LOI), by ashing 1.0 to 5.0 g of sediment (dry weight), at 550 °C for 4h. For particle size distribution, samples were dried to constant weight in an oven at 60 °C for 72 to 120 h and then different fractions were determined by sieving sediment samples with 5.0 to 100.0 g (dry weight) through an AFNOR type column of sieves, with calibrated mesh size. A total of three particle size classes were considered: gravel >2000 µm, sand [63 – 2000 µm[and silt/clay particles [0 – 63 µm[.

Trace metals determination

All samples were stored at -80°C and freeze dried (Laboratory Freeze Dryer Cryodos-50, TELSTAR) for 48 h at -50 °C prior to processing for metal extraction. All laboratory material used was decontaminated of any adsorbed ion by soaking in 0.25 M nitric acid (HNO_3) for 24 h and 0.25 M hydrochloric acid (HCl) for 48 h, and rinsing three times with deionized water to avoid cross-contaminations. (Reverse Osmose, Elga Purelab Prima) to avoid cross-contaminations.

Metal speciation was determined according to the method described in Forster (1995), using 1.0 g of dry sediment. Samples were extracted with sequential solutions of increasing strength and acidity. In Fraction 1 (exchangeable metals, that are unspecifically adsorbed - F1), 25 ml of 1 M ammonium nitrate (NH_4NO_3) were added to the sample, and shaking was performed at 180 min^{-1} for 24h. Samples were centrifuged at 2500 rpm and then decanted. The supernatant was filtered through Whatman 42 filters (90 mm diameter; <2.5µm pore size). In Fraction 2 (metals bound in carbonates, and that are specifically sorbed, occluded near oxide surfaces - F2), 25 ml of 1 M ammonium acetate (NH_4OAc) pH 6.0 were added to the residue from F1. Shaking, centrifugation and decanting were performed as in step 1. The residue was redissolved in 12.5 ml of 1 M NH_4NO_3, shaken for 10 min, centrifuged and decanted, and combined with the preceding extract. In Fraction 3 (metals bound in Mn oxides - F3), 25 ml of hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$) + 1 M NH_4OAc pH 6.0 shaken for 30 min, centrifuged and decanted. The residue was redissolved twice in 12.5 ml 1 M NH_4OAc pH 6, shaken for 10 min, centrifuged and combined with the preceding extract. In Fraction 4 (organic complexes of increased strength - F4), 25 ml of 0.025 M NH_4-EDTA pH 4.6 were added to the residue from F3. Shaking was performed for 90 min, the supernatant was decanted and the residue was redissolved in 12.5 ml 1 M NH_4OAc pH 6, acidified with concentrated acetic acid (CH_3COOH) pH 4.6, shaken for 10 min and combined with the preceding extract. In Fraction 5 (metals bound to amorphous Fe oxides - F5) the residue was redissolved in 25 ml 0.2 M ammonium oxalate ($(NH_4)_2C_2O_4$) pH 3.25. Shaking was performed in the dark for 60 min, followed by centrifugation and decantation; the residue was redissolved in 12.5 ml 0.2 M (($NH_4)_2C_2O_4$ pH 3.25, shaken for 10 min, centrifuged and combined with the preceding extract. In Fraction 6 (metals bound in crystalline Fe oxides - F6), 25 ml of 0.1 M ascorbic acid ($C_6H_8O_6$) + 0.25 M $(NH_4)_2C_2O_4$ pH 3.25 were added to the preceding residue, and kept at 96 ± 3°C in a water bath for 30 min. Samples were allowed to cool, after which they were centrifuged and decanted; 12.5 ml of 0.2 M $(NH_4)_2C_2O_4$ pH 3.25 was used to redissolve the residue, shaking the solution for 10 min in the dark. The supernatant was then combined with the preceding extract. Lastly, in Fraction 7 (total residual metals, e.g. bound to silicates - F7) the sediment was transferred to Teflon containers and digested with 15 ml of concentrated perchloric acid ($HClO_4$) and concentrated HNO_3, for 2 h at 120 °C. Fractions 1 to 4 were stabilized by adding 0.5 ml 65% HNO_3.

Trace metals (Cd, Cu, Ni, and Zn) were determined by Flame Atomic Absorption Spectrometry (FAAS, SpectraAA 50, VARIAN). Total concentrations of Cd, Cu, Ni, and Zn were calculated as the sum of the seven fractions. Instrumental recalibration and analytical blanks were used for quality control. Detection limits of the method were as follow (ppm): Cd – 0.03; Cu – 0.03; Ni – 0.15; Zn – 0.33.

Statistical analysis

Two-way analysis of variance (ANOVA) (Sokal and Rohlf, 1995) was used to test for differences in total concentrations of metals and organic matter percentage (TOM), considering sampling site (Hortas and Rosário) and species (*H. portulacoides*, *S. furticosa*, *S. maritima)*. Tukey’s HSD was used to compare groups means where significant differences were found. ANOVA’s assumptions of normality and homoscedasticity were verified with Kolmogorov-Smirnov test, with Lilliefors correction (normality), and Cochran's C test (homoscedasticity). When the criteria were not met, variables were log transformed (Log_{10} [M], where M is the concentration of a given metal), or arcsine transformed in the case of TOM ($\arcsin \sqrt{p}$, where *p* is the percentage value for TOM).

Non-metric multidimensional scaling (nMDS) (Clarke, 1993) was used in combination with permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001; McArdle and Anderson, 2001) to test for differences between the sediment samples regarding the type of vegetation cover (or lack of it). A similarity percentage analysys (SIMPER) was used to determine the average contribution of each variable (metal fraction) to the differences between the sediment groups (Clarke, 1993).

The statistical packages STATISTICA 12.0 (StatSoft Inc., 2013) and Primer v.6 & PERMANOVA (Clarke and Gorley, 2006) were used for data treatment and statistical analysis.

RESULTS

Total organic matter content (TOM) and particle size distribution in sediments from both sampling sites is presented in Table 1. A significant effect of *Site* [F(1,16)=215.3, $p < 0.001$], and of the interaction between *Site* and *Sediment group* [F(3,16)=58.7, $p < 0.001$] was observed in TOM content in the sediment. Organic matter, silt and clay particles were generally higher in Rosário salt marsh sediments (Fig. 2).

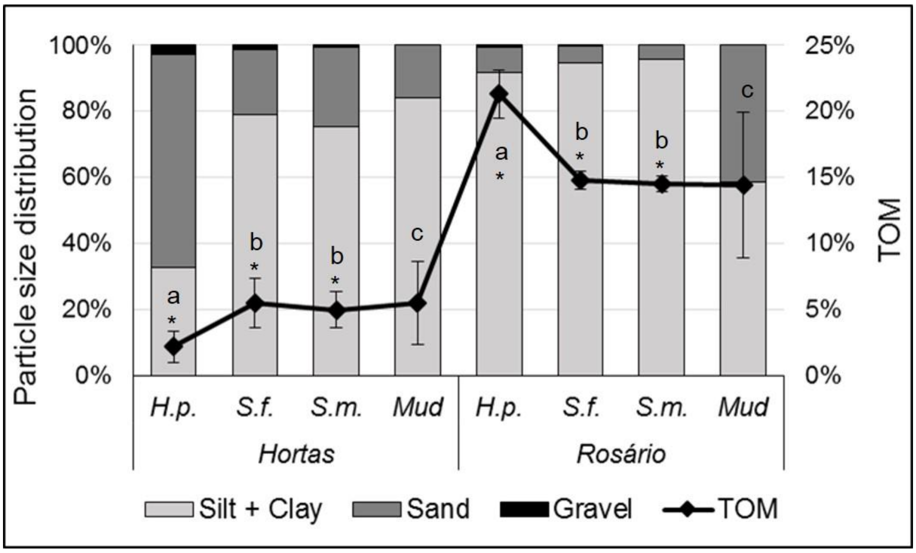

Figure 2 – Particle size distribution and organic matter content (TOM, average ± sd), at root depth and mud flat sediments. *H.p.* – *Halimione portulacoides*; *S.f.* – *Sarcocornia fruticosa*; *S.m.* – *Spartina maritima*; *Mud* – bare sediments. Different letters: significant differences in LOI among species; *: significant differences in LOI between salt marshes ($p < 0.05$), N=24.

Sediments between the roots of *Halimione portulacoides* had the highest TOM in Rosário salt marsh (21.3 ± 1.8%), and the lowest in Hortas salt marsh (2.2 ± 1.2%). Significant differences were found within Hortas salt marsh between TOM of the sediments beneath *H. portulacoides* and the remaining groups (*Sarcocornia fruticosa* and *Spartina maritima*, $p < 0.05$, and bare sediments, $p < 0.001$). In Rosário saltmarsh, TOM in the sediments of *H. portulacoides* was significantly higher than those of *S. fruticosa*, *S. maritima* and bare sediments ($p < 0.001$). No significant differences were found between TOM beneath pure stands of *S. maritima* and *S. fruticosa* within either salt marsh ($p > 0.05$). The comparison of TOM within each species' sediments between salt marshes, on the other hand, was always significant ($p < 0.001$). Bare sediments (*Mud*) presented statistically significant differences from *H. portulacoides* ($p < 0.001$), *S. maritima* (Hortas, $p < 0.05$; Rosário, $p < 0.001$) and *S. fruticosa* (Hortas, $p < 0.01$; Rosário $p < 0.001$), but despite TOM in *Mud* from Rosário being higher than Hortas salt marsh, differences were not statistically significant ($p > 0.05$).

Particle size distribution (Fig. 2) was reasonably consistent among samples from Rosário salt marsh. Finer particles were predominant beneath the three halophytes, whereas bare sediment was almost proportionally divided into sand and fine particles. In Hortas saltmarsh, *H. portulacoides* rhizosediments showed a larger proportion of sand than those beneath *S. fruticosa* and *S. maritima*, which had higher contents of silt and clay. Sand content in bare sediments from Hortas salt marsh was the lowest in the salt marsh. Generally, the sediments in Hortas salt marsh were coarser grained than in Rosário.

Total Concentration of Metals

Total concentration of metals in sediments (Table 1) showed statistically significant differences ($p < 0.05$) regarding Cd, Ni, and Zn, but not in the case of total Cu. ANOVA results showed a significant effect of *Site* [$F(1,16)=23.6$, $p < 0.001$], *Sediment Group* [$F(3,16)=4.5$, $p < 0.001$], and of the interaction of both factors [$F(3,16)=6.2$, $p < 0 .05$] in Cd concentration in the sediments and rhizosediments. The concentration of Ni was significantly affected by *Site* [$F(1,16)=30.9$, $p < 0.001$] and by the interaction of *Site* and *Sediment Group* [$F(3,16)=13.6$, $p < 0.001$], but not by the latter on its own. Samples from Rosário salt marsh showed higher concentrations of Ni than those from Hortas (except *Mud* samples). Lastly, the concentration of Zn was only significantly affected by *Site* [$F(1,16)=70.9$, $p < 0.001$], with all samples from Rosário presenting higher concentrations of Zn than the samples from Hortas saltmarsh. Tukey's HSD results are reported next to metal concentrations in Table 1.

Table 1 – Average metal concentrations (μg.g^{-1} dry weight ± sd) in rhizosediments (c. 5-8 cm) and mud flat sediments. *Halimione portulacoides* (*H. p.*), *Sarcocornia fruticosa* (*S. f.*), *Spartina maritima* (*S. m.*) and bare sediments (*Mud*), in Hortas and Rosário salt marshes. Different superscript lower case letters (^{a,b}) represent statistically significant differences among species within a salt marsh; asterisks (*) represent statistically significant differences within a species between salt marshes (p < 0.05); N=24

Site	Sediment group	Cd	Cu	Ni	Zn
Hortas	*H. p.*	1.1 ± 0.1	18.8 ± 14.1	4.2 ± 1.1^{a}*	36.1 ± 39.6*
	S. f.	2.2 ± 0.8	60.7 ± 44.0	12.8 ± 6.4*	52.9 ± 3.8*
	S. m.	1.6 ± 0.2*	68.4 ± 60.8	12.1 ± 4.0	80.3 ± 17.9*
	Mud	2.3 ± 0.1	8.8 ± 0.2	29.5 ± 4.5^{b}	197.3 ± 5.7
Rosário	*H. p.*	2.5 ± 0.3	54.9 ± 3.4	32.3 ± 2.2*	322.4 ± 82.9*
	S. f.	2.0 ± 0.6^{a}	58.0 ± 11.0	32.8 ± 4.5*	328.4 ± 55.5*
	S. m.	3.9 ± 0.8^{b}*	63.5 ± 37.1	32.4 ± 22.9	527.3 ± 195.8*
	Mud	2.8 ± 0.6	19.4 ± 3.2	16.3 ± 3.3	384.2 ± 56.4

Metal Speciation

Metal associations to the seven operationally defined fractions in the sediments between roots and in bare sediments are shown in Figure 3. Globally, the association to the most labile fractions, particularly to the carbonates fraction, was higher in Cd (up to 56%) and Zn (up to 40%); these two elements were the most variable in their geochemical partitioning, considering the high mobility observed within the several fractions. The partitioning into the residual phase was more abundant for Cu (7 out of the 8 groups had between 50 and 77% of Cu associated to this fraction) and Ni (whose partitioning into the residual fraction was between 60 and 80% in 7 out of the 8 groups of samples). The remaining fractions were varied among metals and type of sediments.

The least variable element regarding geochemical partitioning was Cu: the organic fraction was the second most abundant fraction for Cu (13 to 37%), after the residual fraction (23 to 77%). Only *H. portulacoides* rhizosediments from Hortas salt marsh presented a slightly different pattern regarding Cu speciation, with a higher partitioning into the carbonates phase (36%), followed by the residual fraction (24%) and organic complexes (23%). The most variable elements regarding geochemical partitioning were Cd and Zn, given the high mobility observed within the several fractions.

Cadmium was more readily available at Rosário salt marsh (25.4%) than at Hortas (8.3%), considering the first two fractions (exchangeable + bound to carbonates) (Fig. 3).

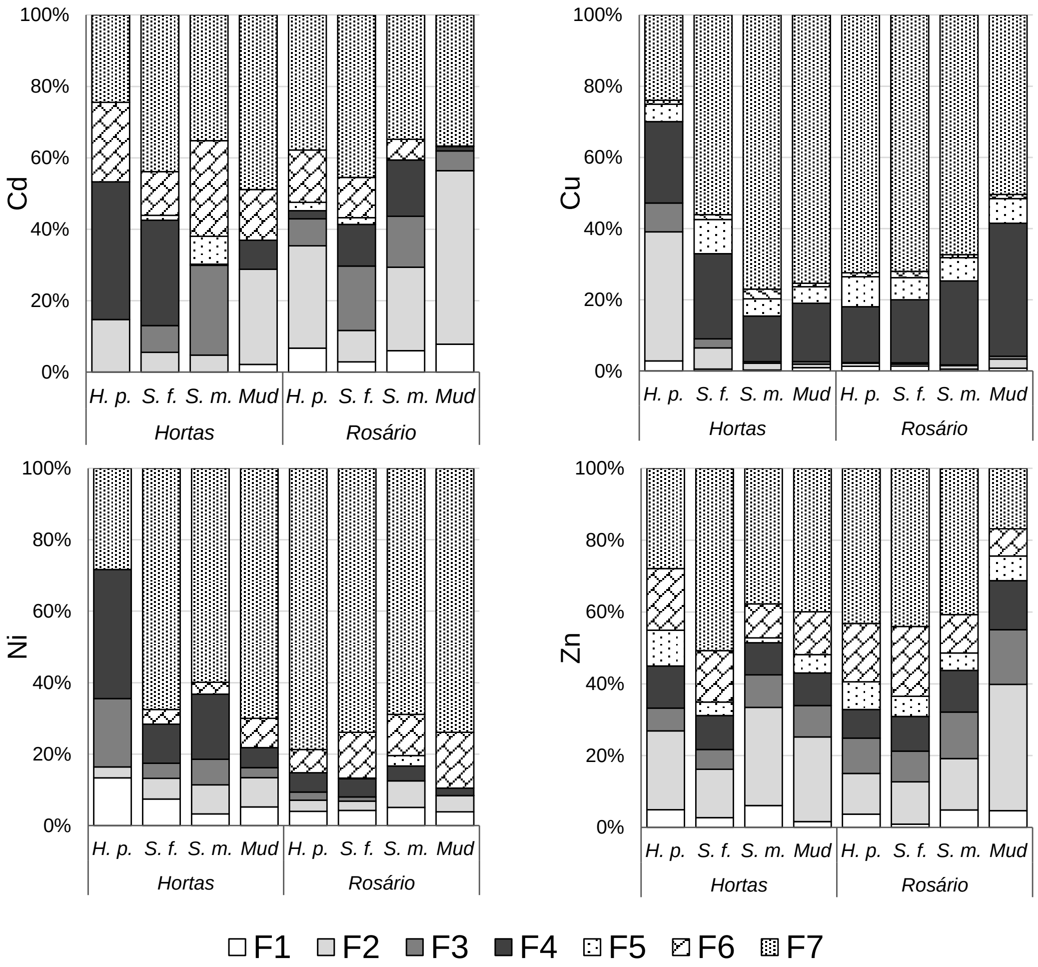

Figure 3 – Metal partition in the rhizosediments of *Halimione portulacoides* (*H. p.*), *Sarcocornia fruticosa* (*S. f.*), *Spartina maritima* (*S. m.*) and bare sediments (*Mud*), in Hortas and Rosário salt marshes (N=24). □ F1 – Easily exchangeable metals; □ F2 – Bound to carbonates; ■ F3 – Bound to Mn oxides; ■ F4 – Organic complexes; ⬚ F5 – Bound to amorphous Fe oxides; ⧄ F6 – Bound to crystalline Fe oxides; ▦ F7 – Residual metals.

Cadmium was more readily available at Rosário salt marsh (25.4%) than at Hortas (8.3%), considering the first two fractions (exchangeable + bound to carbonates) (Fig. 3). Within each salt marsh, Cd was more readily available (F1+F2) in bare sediments (29% in Hortas salt marsh, 56% in Rosário salt marsh), followed by *H. portulacoides* rhizosediments (14% in Hortas, 36% in Rosário). Zinc availability was similar in Hortas salt marsh between sediments with and without vegetation cover. In Rosário salt marsh, bare sediments exhibited greater availability of Zn (40%) than sediments beneath either of the three halophytes (average 15.6 ± 3.3%).

Multivariate analysis was applied to each element separately, with the geochemical fractions as variables. The nMDS ordination plots (Fig. 4) showed a good representation of the data ordination, as the stress value was smaller than 0.1 (Clarke, 1993).

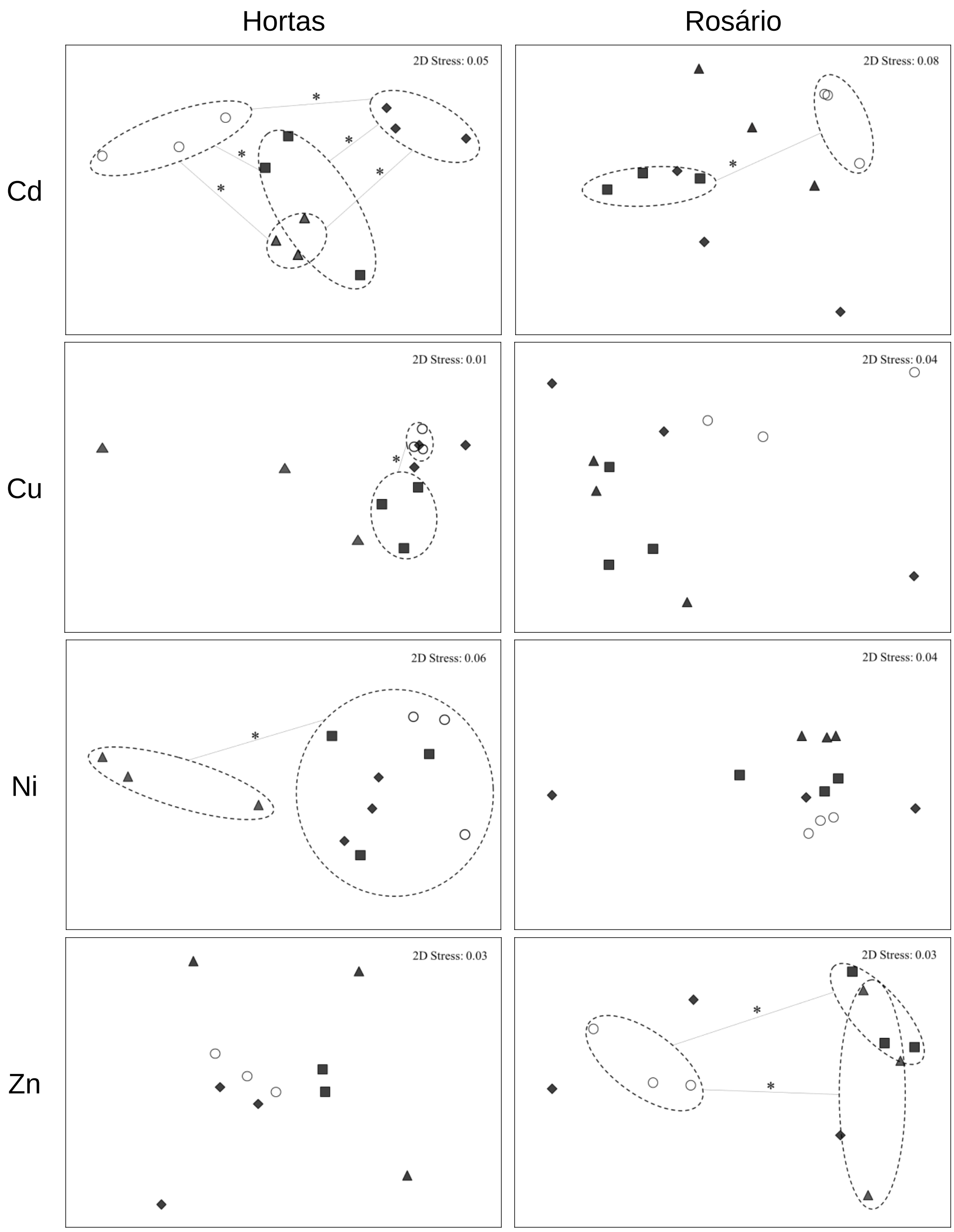

Figure 4 – Non-metric multidimensional scaling (nMDS) ordination plots based on Euclidean distances of each metal partitioning for the rhizosediments of *Halimione portulacoides* (▲), *Sarcocornia fruticosa* (■) and *Spartina maritima* (◆), and bare sediments (○) collected in Hortas and Rosário salt marshes. Permanova pairwise tests results are superimposed on the nMDS ordination plots. * $p < 0.05$.

PERMANOVA results (Table 2) showed the two salt marshes had distinct partitioning of Cd depending on the type of coverage. Bare sediments and vegetated sediments in Hortas salt marsh exhibited significantly different Cd partitioning, as did *S. maritima*'s rhizosediments and sediments covered by the other two halophyte species ($p < 0.05$). In Rosário salt marsh, *H. portulacoides* and bare sediments both differed from sediment between the roots of *S. fruticosa* ($p < 0.05$).

Table 2 – PERMANOVA analysis of trace metals partitioning in sediment between roots of *Halimione portulacoides* (*H. p.*), *Sarcocornia fruticosa* (*S. f.*), *Spartina maritima* (*S. m*.) and bare sediments (*Mud*), in Hortas and Rosário salt marshes. Pseudo-F: pseudo-F statistic, *p*: *p*-value; Perm: number of permutations

	Pseudo-F	*p*	Perm.	Pairwise tests
Hortas				
Cd	8.8	0.000	7330	*Mud* ≠ (*H. p.***; *S. f.**; *S. m.***) *S.m.* ≠ (*H. p.***; *S. f.**)
Cu	3.9	0.000	7361	*S. f.* ≠ *Mud**
Ni	6.5	0.001	7341	*H. p.* ≠ (*Mud***; *S. f.**; *S. m.**)
Zn	1.4	n.s.	7291	
Rosário				
Cd	3.7	0.001	7298	*S. f.* ≠ *Mud***
Cu	1.6	n.s.	7357	
Ni	1.6	n.s.	7296	
Zn	4.0	0.015	7277	*H. p.* ≠ (*Mud***; *S. f.***)

n.s.: non-significant ($p > 0.05$); * $p < 0.05$, ** $p < 0.01$

Different fractionation was also found among sediment groups for Cu and Ni in Hortas sediments, and Zn in Rosário salt marsh. Bare sediments and *S. fruticosa* exhibited significant differences in Cu partitioning ($p = 0.02$), whereas Ni fractionation was distinct in *H. portulacoides* and the remaining groups ($p < 0.05$). Regarding Zn in Rosário salt marsh, significant differences were found between non-vegetated sediments and both *H. portulacoides* ($p = 0.01$) and *S. fruticosa*.

SIMPER analysis (Table 3) highlighted the geochemical fractions that were more important in the distinction among sediment groups. Some variation was observed, but otherwise common tendencies were noticed. For example, Fe/Mn oxides were largely responsible for the differences found in Cd partitioning between *S. maritima* and the other sediment groups, whereas Cd in the labile fractions (Exchange and Carbonates) was more important in the separation between non-vegetated sediments and rhizosediments.

Table 3 –SIMPER results listing the highest contributing fractions for the distance between sediment groups (rhizosediments from *Halimione portulacoides* (*H. p.*), *Sarcocornia fruticosa* (*S. f.*), *Spartina maritima* (*S. m.*) and bare sediments (*Mud*), in Hortas and Rosário salt marshes; N=24); Cut off for low contributions: 90.00%. Metal-association fractions: Exch – Exchangeable; Carb – Carbonates; MnOx – Mn oxides; Org – Organic complexes; FeOx – Fe oxides; Res – Residual fraction

Groups	Contribution of individual fractions (%)				
Hortas					
Cd					
H. p. - Mud	Exch (34)	> Res (27)	> Org (21)	> Carb (11)	
S. f. - Mud	Carb (34)	> Exch (30)	> Org (15)	> Res (14)	
S. m. - Mud	MnOx (26)	> FeOx (23)	> Carb (23)	> Exch (21)	
S. m. - H. p.	MnOx (37)	> Org (32)	> FeOx (13)	> Carb (9)	> Carb (9)
S. m. - S.f.	FeOx (37)	> Org (29)	> MnOx (19)	> Res (14)	
Cu					
S. f. - Mud	FeOx (40)	> Org (23)	> Res (18)	> MnOx (14)	
Ni					
H. p. - Mud	Org (20)	> MnOx (19)	> Res (18)	> FeOx (17)	> Exch (13)
H. p. - S.f.	MnOx (24)	> Res (23)	> Org (20)	> Carb (12)	> Exch (11)
H. p. - S. m.	Exch (30)	> Res (19)	≈ MnOx (19)	> Carb (14)	> Org (11)
Rosário					
Cd					
S. f. - Mud	Carb (32)	> FeOx (20)	> MnOx (19)	> Org (14)	> Exch (10)
Zn					
H. p. - Mud	Carb (25)	> Org (19)	> FeOx (17)	> Res (16)	> MnOx (14)
S. f. - Mud	Carb (24)	> FeOx (23)	> MnOx (18)	> Res (16)	> Org (12)

In Rosário salt marsh, labile Cd, Fe/Mn oxides, and organic bound metal were important in the distinction between *S. fruticosa* and both *H. portulacoides* and bare sediments. Generally, metals bound to either carbonates or the exchangeable fractions showed important contributions to the separation between bare sediments and those between roots, particularly in Rosário salt marsh, but also verified regarding Cd partitioning in Hortas salt marsh, as referred above (Table 3).

DISCUSSION

Metal concentrations reported in this work were generally in the same order of magnitude of those reported for the Tagus estuary salt marshes across the literature (e.g. Caçador *et al.*, 1996a; Reboreda *et al.*, 2008; Vinagre *et al.*, 2008; Caçador *et al.*, 2009). Variation in the sampling years, sampling season and core depths may account for the variability found in the several works, and apart minor exceptions, e.g. considerable higher Zn concentration in the vegetated sediment in Caçador *et al.* (1996a), no other striking differences were noticed. On an overall basis, the sediment biogeochemical environment is mostly associated to the maturity of the marshes, with young marshes showing low organic matter contents and high sand percentages, contrarily to mature marshes. This is in accordance with previous findings where the biogeochemical environment of the marshes can be distinguished based on its characteristics (Duarte *et al.*, 2013a). This environment will condition not only the metal retention in its overall extension, but also the forms in which metals will be retained. If this is true for the overall differences between marshes, some differences arise within each marsh among different halophyte stands (Duarte *et al.*, 2008; Duarte *et al.*, 2009).

Higher concentrations of Cd, Ni and particularly Zn were generally found in Rosário salt marsh samples (even though not all comparisons were found to be significant). The two areas present distinct characteristics that influence a higher metal enrichment in Rosário, compared to Hortas, namely higher TOM and greater proportion of finer particles. Close proximity to higher urban and industrial pressures in Rosário salt marsh also influence the observed differences in metal accumulation in the sediments (Caçador and Vale, 2001; Reboreda *et al.*, 2008). Organic matter concentration and particle size distribution also contribute to some differences in metal burden found in sediments beneath different types of vegetation cover. The sediments colonized by *Spartina maritima* presented higher concentrations of all metals than the rhizosediments of *Halimione portulacoides* (except for Ni, which had similar concentrations beneath the two species in Rosário salt marsh). When higher percentages of organic matter and finer particles (clays and silt) are present, there is an increase in the metal binding capacity, with higher cation exchange capacity, enhanced by the negatively charged clay particles and their large surface area (Ujevic *et al.*, 2000). On the contrary, sediments with higher percentage of sand and low organic matter content usually present lower levels of metal retention (Williams *et al.*, 1994). In Hortas salt marsh, *S. maritima* rhizosediments were dominated by finer particles, and had significantly higher TOM, while beneath *H. portulacoides* stands they were dominated by sand. However, this characteristic was not enough to explain the differences in the metal burden beneath the two species in Rosário salt marsh. There, the three species grow in sediments with similar particle size distribution (Caçador *et al.*, 2009) and TOM was higher in *H. portulacoides* rhizosediments. As for *Sarcocornia fruticosa*, TOM and particle size distribution were generally closer to those observed in *S. maritima*. Metal content, on the other hand, was not consistent between salt marshes: it was closer to *H. portulacoides* in Rosário salt marsh, but approached the concentrations of *S. maritima* in Hortas. Sediments with vegetation cover usually present higher metal content than bulk sediments. Again, organic matter appears to exert a certain influence in obtaining such results, but Doyle and Otte (1997) found that Fe oxides may be more important in binding metals like Zn than organic matter itself.

When observing the geochemical partitioning of the four metals, two main tendencies were revealed: Cd and Zn, having analogue chemical behavior (Smolders and Mertens, 2013), showed greater mobility among the several operationally defined fractions, while Ni and Cu did not. The latter, above all, was particularly less mobile, given its partitioning being observed largely into the residual and organic fractions. Hortas salt marsh sediments showed greater variability in metal fractionation than that observed in Rosário salt marsh. The morphology of the two sites is distinct, and the results may be influenced by such differences. Rosário salt marsh is a mature system (Valiela *et al.*, 2000; Duarte *et al.*, 2013a), with extensive vegetation cover and a complex branched system, while Hortas is considered a young salt marsh (Valiela *et al.*, 2000; Duarte *et al.*, 2013a), with considerable open areas and sparser vegetation cover, and with a less complex channeling system than Rosário.

Sediments at Rosário salt marsh are more uniformly distributed, particularly rhizosediments, being mostly constituted by finer particles. Reboreda and Caçador (2007a) observed that physical and chemical characteristics varied little in the sediments between the roots (5 to 15 cm deep) of *S. maritima* and *H. portulacoides* in this salt marsh; Caçador *et al.* (2009) found a slightly reductive environment in the top 25 cm beneath *S. fruticosa* whereas similar and more oxidative Eh values were accounted beneath *S. maritima* and *H. portulacoides*; the same authors reckoned identical pH values beneath every species considered in the study. None of those differences (Eh and pH) were statistically significant. This low variation among different species may therefore help to explain why metal partitioning in the solid phase of Rosário's sediments exhibited similar trends regardless of the vegetation cover, especially for Cu and Ni. Cd and Zn, on the other hand, were more labile/weakly adsorbed in non-vegetated sediment than beneath *H. portulacoides* (Zn) or *S. fruticosa* (Cd and Zn). On the absence of vegetation cover, Cd and Zn seem to be predominantly bound to carbonates, whereas between roots the partitioning favors the association with Fe/Mn oxides, organic matter and the residual fraction, thus reflecting the influence of plant roots in the immobilization of metals. These immobilized metals may be relatively inert over long periods of time (years), only responding to slow changes like mineral weathering or organic decomposition (Young, 2013). Metal partitioning in sediments from Hortas salt marsh was more variable than in Rosário sediments, but nonetheless similar trends were observed. Cadmium partitioning in the rhizosediments of the three halophytes differed from non-vegetated sediment. Similarly to what was observed for Rosário's sediments, the partitioning into the labile fractions was more noticeable in bare sediments, pointing like before to greater availability in the absence of vegetation cover. Middle and upper marsh vegetation differed from *S. maritima* predominantly regarding Cd bound to Fe and Mn oxides and to organic complexes. Iron and Mn oxides were more abundant in sediments colonized by *S. maritima,* while Cd bound to organic complexes was more abundant in *H. portulacoides* and *S. fruticosa* rhizosediments. These species have higher root densities than *S. maritima* (Duarte *et al.*, 2010). Organic complexing agents, like humic substances, released by the roots will favor the complexation with Cd, since this metal has a higher sorption strength to humic acids than to Fe oxides (Smolders and Mertens, 2013). *Spartina* cordgrasses have a highly developed aerenchyma (Maricle and Lee, 2002), which is paramount in surviving waterlogging for a low-marsh species. Aerenchyma is thus responsible for supplying the oxygen for the roots' metabolic demands (Maricle and Lee, 2002). Once metabolic demands are satisfied, oxygen not required for respiration is lost into the sediments, supplying microorganisms' demands for oxygen and oxidizing reduced components (Sundby *et al.*, 2005). Cadmium is typically very mobile in the estuarine environment (Förstner and Kersten, 1988). The larger proportion of Cd and Zn in the labile fractions of the sediment, (exchange and carbonates) showed these metals were generally more available than Cu and Ni.

Copper was predominantly bound to the residual and organic fractions, as already pointed, except beneath *H. portulacoides* rhizosediments in Hortas salt marsh. In this case, almost 40% Cu was associated to the labile fractions, pointing towards increased availability of this metal. A combination of factors helps explaining this observation: the high root biomass of *H. portulacoides* (Duarte *et al.*, 2010), together with the larger proportion of sand particles and low organic matter content found in its rhizosediments, would justify an increased oxygen pumping into the sediments, ultimately originating a more oxidative environment. The oxidation of sulfides may explain the increase in the labile Cu found beneath *H. portulacoides* in Hortas salt marsh. As long as metal binding is not facilitated by high organic matter content, metal mobility may increase because metals are released from sulfides but not immediately adsorbed onto Fe oxides (Jacob and Otte, 2003). As a result, leaching of Cu from the sediment may occur, enhanced by oxidative pumping by tidal drainage (Förstner and Kersten, 1988). Additionally, it may also increase the bioavailability of metal to halophytes, which was actually observed (Duarte *et al.*, 2013b), and particularly high concentrations of Cu were found in *H. portulacoides* roots in Hortas salt marsh (5 and 10-fold the concentrations in *S. maritima* and *S. fruticosa* – own unpublished data). Regarding Ni partitioning, *H. portulacoides* rhizosediments in Hortas showed a distinct fractionation scheme from the other samples. However, if we consider the proportion of available metal (exchangeable and bound to carbonates), there was little variation among species, with labile Ni being considerably low. Caetano *et al.* (2008) registered a weak removal of Ni from the sediment by plant roots, which was also related with the low affinity that the soluble forms of this element have to oxides. The observed increase in the Ni bound to organic complexes is also expected to decrease the rate of uptake to plant tissues due to the stability inherent to such complexation mechanisms. All these data come to enlighten the retention processes among young and mature marshes. Rosário salt marsh, previously classified as a mature marsh (Duarte *et al.*, 2013a) with high amounts of bioavailable metals (Duarte *et al.*, 2013b) has an overall higher capacity for metal retention due to its higher silt and organic matter contents. On the other hand young marshes like Hortas (Duarte *et al.*, 2013a) have low total and bioavailable metal concentrations (Duarte *et al.*, 2013b), conditioned by the comparatively low availability of organic and high affinity ligands for metal binding.

CONCLUSIONS

With this work, general trends were observable in metal partitioning in salt marsh sediments: Cd and Zn present more mobility within the solid phase fractionation while Ni, and particularly Cu, are predominantly unavailable for plant uptake. These general trends occur regardless of the area or sediment vegetation cover, meaning that the metal chemistry is the most important factor in these processes. Notwithstanding those common tendencies, a closer look within each marsh type showed that metals with greater mobility are also more influenced by the type of vegetation cover (or its absence). In agreement with the literature, Cd and Zn are more available in bare sediments, evidencing the important role of vegetation in stabilizing metal contamination in salt marsh sediments. Salt marsh morphology or colonizing species are clearly of minor importance in the mobilization of Ni and Cu. Nonetheless, the presence of *Halimione portulacoides* in low organic matter and high sand content seems to favor the mobility of the latter within different salt marsh compartments, i.e., Cu is eventually mobilized from the sediment into the pore water, later becoming available for plant uptake. Overall, although the metal's characteristics conditions its chemical binding forms within the sediment matrix, the marsh maturity must also be accounted as a major factor modulating the sediment composition and thus the availability of binding forms. Thus, it seems evident that the marsh maturation process develops side by side with the marsh capacity as a sink for contaminants.

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METAL PARTITIONING AND AVAILABILITY IN ESTUARINE SURFACE SEDIMENTS: CHANGES PROMOTED BY FEEDING ACTIVITY OF *SCROBICULARIA PLANA* AND *LIZA RAMADA*

ABSTRACT

Several works have evidenced in the past the importance and influence of plants and terrestrial invertebrates in metal availability in soils and sediments through changes in metal speciation. In contrast, the impact of estuarine invertebrates and fishes in this process has been poorly explored. The partition of metals in estuarine surface sediments was studied in a controlled environment according to four operationally defined fractions. Sediments were analyzed before and after the passage through the gut of two detritivorous species. *Scrobicularia plana* feeds on the bottom and suspended sediment particles through the inhalant siphon. *Liza ramada* is an interface feeder, filtering the superficial layer of the sediment and suspended particles in the water column. Cd, Cu and Ni bound to carbonates increased in the pellets of *S. plana*, compared with the ingested sediment, as did exchangeable Zn. Similarly, Cd and Zn bound to carbonates have also increased in the pellets of *L. ramada*; on the contrary, a decrease of Ni was observable in the pellets of this fish. The outcome of the controlled experiments pointed to a potential increase in some metals’ availability in the estuarine environment, as a result of the more mobile metal forms in the excreted fecal pellets. This draws the attention to a relevant impact of the trophic activity of both species, alongside with the potential enhancement brought to it by the bioturbation promoted by them, in the role that the estuary itself has as a contaminants’ buffer.

Keywords: Metals; sequential extraction; bioavailability; fecal pellets; estuarine sediment.

INTRODUCTION

Metals in the estuarine ecosystem and in estuarine sediments in particular, have become a subject of increasing interest among ecologists in the last decades, and that resulted in a massive number of publications on the subject (Sun *et al.*, 2012). One of the concerns raised by the presence of metals in these ecosystems is the permanent character of such pollutants, as they are not degradable and therefore persist in the environment (Wood, 2011).

Estuarine sediments may either act as a sink or as a source of metals to the ecosystem, depending on the metals' predominant flux (Mason *et al.*, 2006). Stakeholders are often faced with the necessity of deciding if the occurrence of high concentrations of metals in the sediments are a synonym of ecological risk, but it can be very difficult to predict environmental impacts based on total concentration of metals in the sediment (Ankley *et al.*, 1994), despite the efforts made in that this direction (e.g. Long *et al.*, 1995; Crommentuijn *et al.*, 2000). Accordingly, it is mostly agreed nowadays that the total concentration of metals in the ecosystem does not provide a real image of their availability or, more importantly, of their toxicity to the biota (Tessier *et al.*, 1979; DiToro *et al.*, 1990). It is the bioavailable metal fraction (Harmsen, 2007), i.e. the total amount of a metal that, within a given period, is either available or can be made available to be taken up by organisms (Peijnenburg and Jager, 2003), that will determine the degree of toxicity, instead of the total concentration of that metal. This bioavailability is dependent, among other factors, on metal speciation (the physical and chemical forms among which the metal may be distributed). Metal speciation is intrinsically connected to sediment geochemistry, since the way in which a metal is bound to the sediment particles will affect its mobility, and ultimately the fraction of the metal that is available for biological uptake and its potential toxicity.

Several works to date have showed that organisms, beside sediment geochemistry, may as well modify metal partition in the sediment, from microorganisms (Duarte *et al.*, 2008), to plants (Caçador *et al.*, 1996; Reboreda and Caçador, 2007), and animals (Udovic *et al.*, 2007; Sizmur *et al.*, 2011a). The activities of benthic organisms, like feeding and borrowing, increase the turbidity and physical disturbance in the water-sediment interface, which may either affect metal partitioning and toxicity in muddy sediments (Green and Chandler, 1994; Ciutat and Boudou, 2003), or cause no evident alteration in that metal toxicity (Fleeger *et al.*, 2006). It has been shown, for example, that the presence of some terrestrial invertebrates alters the speciation of metals and metalloids in soil and manure (Li *et al.*, 2009; Sizmur *et al.*, 2011a; Sizmur *et al.*, 2011b). Gut chemistry is usually pointed as responsible for metal alterations by macrofauna, as the reducing conditions and pH variations found therein may induce changes in metal speciation and be responsible for differences in the assimilation efficiency of those metals (Plante and Jumars, 1992; Ahrens and Lopez, 2001; Griscom *et al.*, 2002), hence responsible for changing the bioavailability of such elements.

Based on the principle that differences in physicochemical properties among sediments can result in differences in the bioavailable fraction of metals (Luoma, 1989), metal partitioning studies are often used to make an attempt in predicting such bioavailability. Although sequential extraction protocols have recognized limitations (Bordas and Bourg, 1998) and are not free of criticism (Nirel and Morel, 1990; Bacon and Davidson, 2008), these methods are generally used with the purpose to indirectly obtain what is likely to be released in solution under different environmental conditions (Tessier *et al.*, 1979). Methods may include more or less manipulative and time consuming consecutive steps. These techniques use sequential “selective” extractions with increasingly strong reagents under specific conditions to extract metals associated with various sized particles, and used as a proxy for metal associations with various geochemical, albeit operational, fractions e.g. ammonium oxalate at a low pH and in the dark to extract metals bound in the amorphous Fe oxides of the sediment (Forster, 1995).

The present work investigated the alterations in metal partition promoted by the passage of estuarine sediment through the gut of two species that, although belonging to different taxonomic groups, feed on detritus from the bottom sediments. The premise considered here was that differences between the gut chemistry and the environment conditions could influence contaminants bioavailability (Ahrens and Lopez, 2001). Thus, the main objective of this work was to understand the extent to which metal mobility/availability could be affected as a direct consequence of the presence of two abundant species in the estuarine environment: the peppery furrow shell *Scrobicularia plana* (da Costa, 1778) (Bivalvia: Semelidae) and the thin-lipped grey mullet *Liza ramada* (Risso, 1827) (Actinopterygii: Mugilidae).

MATERIALS AND METHODS

Sampling

The sampling took place in the Tagus estuary intertidal mudflats. The Tagus (38°44’N, 9°08’W) is a semi-diurnal mesotidal estuary with ca. 4 m of tidal range located in the West coast of Portugal (Fig. 1). The estuary is composed of a deep and narrow inlet channel and a shallow bay differentiated in salt marsh areas, sand islands, and mud flat areas.

Scrobicularia plana, is a widely distributed species in the Northeast Atlantic estuaries (from the Norwegian Sea into the Mediterranean and southward to Senegal) (Tebble, 1976), highly tolerant to saline and temperature variations during tidal cycles (Bryan and Hummerstone, 1978). This bivalve is very common in the intertidal mudflats of the Tagus estuary, living buried at about 5 to 20 cm deep. It feeds on the bottom sediment particles and although it also ingests suspended particles through the inhalant siphon, it is considered a deposit feeder (Hughes, 1969). The location of these clams is easily recognizable during low tides by the star-shaped marks left on the sediment where they fed.

Liza *ramada*, is a catadromous fish with a wide distribution (Mediterranean, Black Sea, Azov Sea and Eastern Atlantic from Cape Verde and Senegal to southern Baltic and British Isles) (Freyhof and Kottelat, 2008), frequently prevailing in polluted waters. It feeds on the extensive mudflats of estuaries, scraping the superficial layer of the sediment and also suspended particles in the water column (Almeida, 1996), presenting a great feeding plasticity (Bruslé, 1981). The thin-lipped grey mullets are responsible for stirring a large amount of surface sediment during the ample movements made while feeding (Almeida *et al.*, 1993). The amount of stirred and ingested sediment is proportional to the thin-lipped grey mullets' body length (Almeida, 2003) and small parallel grooves are visible during low tides in the mudflats where the mullets fed in the previous tide (P.R. Almeida, *pers. com*.). Stomach contents primarily include microalgae, meiofauna, organic debris and inorganic sediment particles (Laffaille *et al.*, 2002; Almeida, 2003). The latter function as a grinding paste to break cell walls in the pyloric portion of the mullets stomach (Odum, 1968), a muscular gizzard similar to the one found in birds. Finer particles are preferentially selected by the mullets (Almeida, 2003; Pedro *et al.*, 2008).

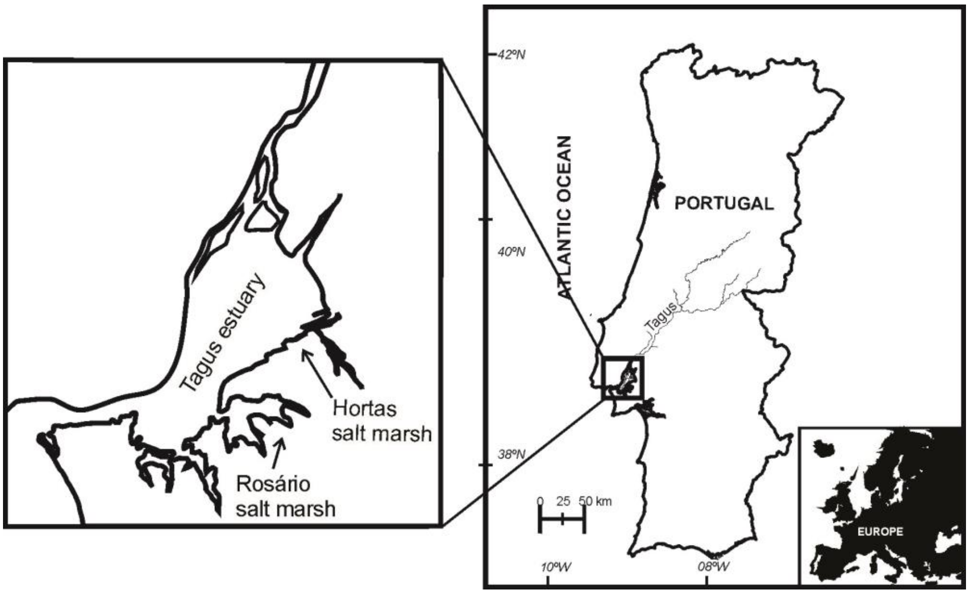

Figure 1 - Tagus Estuary detail with the location of Rosário and Hortas salt marshes.

Scrobicularia plana specimens were captured in a low-contamination intertidal mud flat (Pedro *et al.*, 2008) in Hortas salt marsh (Fig. 1). In the considered sampling area, mean density of *S. plana* varies from approximately 810 ind./m^2 to 1087 ind./m^2 (França *et al*., 2009). Specimens of similar size (N=420; approx. 2.5 cm shell width) were used in the experiments after a 7 day minimum period of depuration and acclimation in artificial saltwater, with approximate salinity of 25 at 17°C.

Liza ramada specimens were captured with trammel nets next to the area of the Tagus estuary Nature Reserve (Hortas salt marsh area, Fig. 1). Eight specimens of *L. ramada* of similar size (approx. 25 cm standard length) were kept in a 300 L tank for acclimation and to allow the elimination of stomach contents. The experimental conditions regarding salinity and temperature were maintained approximately similar to the ones found in the location used to collect the sediment samples (17-19°C and salinity of approximately 25).

Experimental Design

To obviate the inconvenience that low metal content poses during detection, surface sediment samples (top 0.5 cm) were collected in a mud flat with higher concentration of metals (Reboreda *et al.*, 2008), in the Rosário saltmarsh (Fig. 1). Sediment particles larger than medium and coarse grain sand (> 250 µm) were removed from the samples by sieving through a calibrated nylon mesh on arrival at the laboratory facilities.

Scrobicularia plana

The sieved sediment (initial sediment samples, henceforth referred as “Sediment”) was homogenized by mixing the water-logged sediment in a container, and distributed in different holding tanks with artificial saltwater, with 8 to 10 cm of sediment in each tank. Constant water temperature was assured by keeping the holding tanks (33 L capacity aquariums, approx. 50x25x32 cm each) in a cold bath (ca. 17°C). The cold bath was achieved by placing each individual tank inside an 800 L approximate capacity tank (180x90x50 cm^3) with 250 - 300 L of cooled water (HAILEA HC-500a water chiller and EHEIM-1260 pump). Redox potential (Eh) and pH were also monitored (HANNA pH/mV, HI 9025); 420 specimens were added to the tanks immediately after turbidity of the water had diminished - ca. 70 specimens per tank - and left in the tanks for a 48 h period to allow them to feed; after that period, they were transferred into new tanks with artificial saltwater to expel the fecal pellets. Fecal pellets were collected with a disposable pipette (2.5 ml capacity, one per tank) during the following two days. The control sediment samples consisted of sediment in contact with water but not with the specimens during the trial. The procedure described above (depuration, feeding period and collection of pellets) was repeated twice with the same specimens to obtain enough mass of fecal pellets for the chemical analysis. No mortality was observed in the first trial, and less than 5% mortality was observed in the second trial. All samples were freeze-dried prior to analysis.

Liza ramada

The specimens were anesthetized in a 2-phenoxyethanol solution at a concentration of 0.4 ml L^{-1} before the experiment. Each specimen was fed with the sieved sediment by using a probe while anesthetized. The probe was specifically developed for this study, and consisted in a polypropylene tube with approximately 2.5 mm diameter, with epoxy resin molded at the tip to create a smooth tapered end. The probe's efficiency was tested in thin-lipped grey mullets cadavers (bought from local fishermen) prior to the experiment, to ensure the animals welfare during the trials. After the probe-feeding step, the specimens were left to recover from anesthesia and placed in individual tanks during approximately 30-45 min. Fecal pellets were collected during the following 6 h ("Pellets"). Control samples were obtained by leaving the same sediment used to feed *L. ramada* specimens in tanks without fishes ("Control"). To achieve the amount of sample necessary to the sequential and total extraction procedures, the experiment was repeated three times, with an interval of ca. 5 days between each test.

Trace metal analysis

All laboratory material used was decontaminated of any adsorbed ions by soaking in 0.25 M nitric acid (HNO_3) for 24 h and 0.25 M hydrochloric acid (HCl) for 48 h, and rinsing three times with deionized water to avoid cross-contaminations. (Reverse Osmose, Elga Purelab Prima) to avoid cross-contaminations.

Total concentration of metals was obtained by digesting 0.1 g of each sample (Sediment, Pellets and Control) with 2 ml of *Aqua regia* (HNO_3/HCl, 1:3 v/v), for 3h at 110°C. A sequential extraction procedure, described by Tessier *et al.* (1979) and modified by van Hullebusch *et al*. (2005), was used to determine trace metals partitioning (Cd, Cu, Ni, Pb and Zn) in the samples. The method consisted of consecutive extraction of 1.0 g of sample through the following steps: easily exchangeable/available fraction (EXCH) – 10 ml of 1 M ammonium acetate (NH_4OAc), shaking at 150 rpm for 1 h at room temperature, followed by centrifugation for 10 min at 4000 rpm; bound to carbonates fraction (CARB) – 10 ml of 1 M acetic acid (CH_3COOH), shaking at 150 rpm for 1 h at room temperature, followed by centrifugation as described before; bound to organic matter/sulfide fraction (OM/S) – 5 mL of a 30% solution of hydrogen peroxide (H_2O_2) (brought to pH 2 with HNO_3) and shaken at 35°C and 150 rpm for 3 h; residual fraction (RES) – 10 ml of *Aqua regia* (HNO_3/HCl; 1:3, v/v), digested for 3 h at 110°C in PTFE closed vessels. Obtained solutions (supernatant from centrifugations and digestion product from *Aqua regia*) were made to 10 ml with ultrapure water. Metal determinations (Cd, Cu, Ni, Pb and Zn) were done with inductively coupled plasma mass spectrometry (ICP-MS) using a Termo X Series, with detection limits of 0.1 ppm (Cd, Ni), 1.0 ppm (Cu) and 5.0 ppm (Zn). The efficiency of the sequential extraction procedure was obtained by comparing total concentration of trace metals with the sum of the four individual fractions. The accuracy and precision of the analytical methodology for total elemental determinations were assessed by replicate analysis of certified reference materials, BCR-277R (IRMM) for sediments. Blanks and the concurrent analysis of the standard reference material were used to detect possible contamination/losses during analysis and to ensure the accuracy and precision of the analytical method.

Statistical analysis

Wilkoxon signed-ranks test (Wilcoxon, 1992) was used to compare total concentration of metals with the sum of the four fractions from the sequential extraction, in order to ascertain the efficiency of the latter. Kruskal-Wallis (H) test, followed by Simultaneous Test Procedures (STP) (Siegel and Castellan, 1988) was used to compare trace metals partition into each sedimentary phase among the three groups of samples (Pellets, Sediment and Control). Non-metric multidimensional scaling (nMDS) (Clarke, 1993) was used to ordinate the similarity data (Euclidean distance) obtained for the three groups of samples regarding the partitioning of all metals into the different geochemical fractions considered. The obtained nMDS ordination plot allowed for an immediate visual interpretation of the metal speciation among groups. An Analysis of similarities (ANOSIM) routine was performed on normalized data to examine statistical significance between the groups (Clarke and Gorley, 2006). Similar percentages (SIMPER) test was used to determine which specific variables contributed to overall differences, i.e., which elements' fractions had more influence on dissimilarities among groups (Clarke, 1993).

The statistical packages SPSS© Statistics 20.0 (IBM, 2011), and Primer v.6 & PERMANOVA (Clarke & Gorley, 2006) were used for data treatment and statistical analysis.

RESULTS

Total concentration of metals was compared to the sum of the four fractions sequentially extracted (EXCH, CARB, OM/S and RES). The differences between the sum of fractions and the total concentration of metal in each group of samples were not statistically significant ($p > 0.05$), indicating an efficient recovery rate of metals with the sequential extraction methods (sum of the four fractions, divided by the total extraction). For *Scrobicularia plana*, the recovery rates of metals varied between 72% and 105% of the total metal concentrations; regarding *Liza ramada*, the recovery rates were between 88% and 109%.

Trace metal partitioning

Scrobicularia plana

Chemical associations of Cu and Ni were largely dominated by the RES fraction (70 to 91%), followed by the OM/S fraction (7 to 30%), the CARB fraction (0.2 to 4%) and finally by the metals in the EXCH fraction (0.1 to 1%) (Fig. 2). The partitioning of Cd and Zn was slightly different, with less weight of the RES fraction of both metals in the three groups of samples (36 to 61%). The order of abundance of the fractions was identical to the previous metals, but in a different magnitude. The OM/S fraction assumed a more important role in metal partitioning, representing 32 to 62% of the associations, while the CARB and EXCH fractions combined represented less than 20% (2 to 19%) of Cd and Zn partitioning.

When comparing the total concentration among the three groups (pellets, sediment and control) no significant differences were found ($p > 0.05$). The partitioning into the CARB fraction of all metals was generally higher in the pellets, with significant differences found in the case of Cd, Cu and Ni ($p < 0.05$) (Table 2). Significant differences were also found regarding the residual fraction of Cd and Zn, with pellets showing higher concentrations than the control group ($p < 0.05$). Although the difference between pellets and the sediment was not statistically significant, the concentration of the latter was very similar to the one of the control samples, which was in fact observed in all the above comparisons. Also the differences between pellets and sediment samples regarding Ni in the OM/S fraction and Zn in the EXCH fraction proved to be statistically significant ($p < 0.05$).

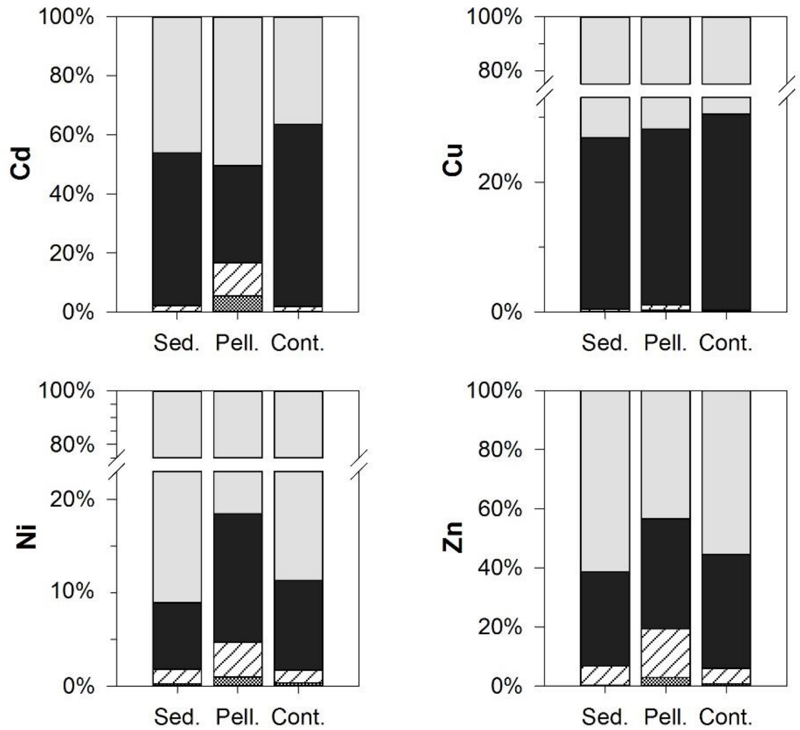

Figure 2 – Metal partition in the pellets, sediment and control samples from the experiences with *Scrobicularia plana*. EXCH – Exchangeable fraction; CARB – Carbonates-bound fraction; OM/S – Organic matter-bound fraction; RES – Residual fraction.

Liza ramada

Chemical associations in the *Liza ramada* experiment were dominated by the RES fraction (68 to 95%) regarding all metals (Fig. 3). Generally, the association of metals to the remaining fractions was considerably variable. The ORG fraction followed in decreasing order in most cases, varying between 2.6 and 23%, but the CARB fraction was relatively similar regarding Cd and Zn.

The Cu association to the OM/S fraction represented less than 1% in all groups, and the same was observed regarding Ni in the sediment. The EXCH fraction was the least abundant for most metals and groups. The exception to this was verified for Cd in the control group (10% of total Cd), and Ni in sediment and control samples (4% and 13%, respectively).

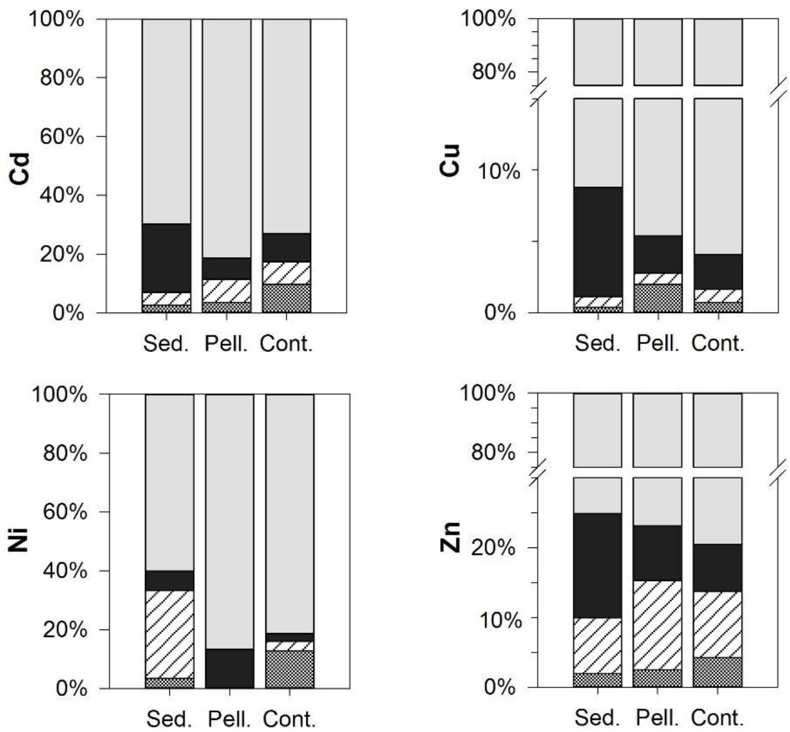

Figure 3 – Metal partition in the pellets, sediment and control samples from the experiences with *Liza ramada*. EXCH – Exchangeable fraction; CARB – Carbonates-bound fraction; OM/S – Organic matter-bound fraction; RES – Residual fraction.

Significant differences were found between pellets and control samples in the case of total Cd ($p < 0.01$), and between pellets and both sediment and control samples in the case of total Zn ($p < 0.01$). In both situations, the concentration in the pellets group were higher. Total Cu and Ni did not differ among groups. Considering metal partitioning significant differences were found in the EXCH fraction of Cu, Ni and Zn (Table 2). Pellets had higher concentration of Cu associated with the EXCH fraction than the other groups, and higher concentrations of Ni ($p < 0.01$) and Zn ($p < 0.05$) were found in the control samples. Regarding the CARB fraction of Cd, Ni and Zn. significant differences were found between pellets and sediment samples (Table 2). Sediment samples had higher concentration of Ni in this fraction than the pellets ($p < 0.01$), while the opposite was observed for the other two elements ($p < 0.05$ in the case of Cd, and $p < 0.01$ in the case of Zn).

Table 2 – Kruskal-Wallis test (H) results for the chemical associations of metals in the sediment (S), pellets (P) and control (C) samples from the *Scrobicularia plana* and *Liza ramada* experiments. EXCH – Exchangeable fraction; CARB – Carbonates fraction; OM/S – Organic matter and sulfides fraction; RES – Residual fraction

		Scrobicularia plana			*Liza ramada*		
		$H_{(2,N=9)}$	*p*-level	Post-hoc comps.	$H_{(2,N=16)}$	*p*-level	Post-hoc comps.
Cd	EXCH	6.161	n.s.		4.740	n.s.	
	CARB	6.489	*	P > C	7.474	*	P > S
	OM/S	3.289	n.s.		2.945	n.s.	
	RES	6.489	*	P > C	11.046	**	P > S,C
Cu	EXCH	5.422	n.s.		12.941	**	P > S
	CARB	7.200	*	P > C	2.647	n.s.	
	OM/S	5.956	n.s.		4.041	n.s.	
	RES	5.600	n.s.		7.522	*	P > S
Ni	EXCH	5.422	n.s.		12.223	**	C > P
	CARB	6.489	*	P > C	10.904	**	S > P
	OM/S	7.200	*	P > S	10.741	**	P > C
	RES	5.422	n.s.		7.371	n.s.	
Zn	EXCH	7.200	*	P > S	6.463	*	C > S
	CARB	5.600	n.s.		11.581	**	P > S
	OM/S	5.956	n.s.		3.898	n.s.	
	RES	6.489	*	P > C	10.006	**	P > S

n.s.: non-significant ($p > 0.05$); * $p < 0.05$, ** $p < 0.01$.
Post-hoc comps.: multiple comparisons tests results

The OM/S fraction only differed between groups regarding Ni association, with pellets presenting higher concentration of Ni in this fraction than the control samples ($p < 0.01$). Finally, Cd, Cu and Zn concentrations in the RES fraction were higher in the pellets, when compared with the sediment samples. In the case of Cd, this difference was also observed between the pellets and the control samples. Although the RES fraction of Ni yielded significant differences in the Kruskal-Wallis test, multiple comparisons *a posteriori* were unable to find differences among the three groups of samples.

The multivariate analysis on the metal partitioning in the *S. plana* experiment showed tight clusters for the samples belonging to the pellets group, and for the sediment and control samples together (Fig. 4, left plot). Considerably more scatter was observable in the nMDS ordination plot for *L. ramada*, especially within the sediment samples (Fig. 4, right plot). A certain degree of overlapping was apparent between the sediment and control samples in this case. Although two separate clusters are showed for the pellets samples, this group still appeared completely separated from the other two. The goodness of fit estimate for the nMDS ordination plots, given by the stress value, showed that the ordination of the plot could be considered a good representation of the data ordination, with no real prospect of misinterpretation (Clarke, 1993).

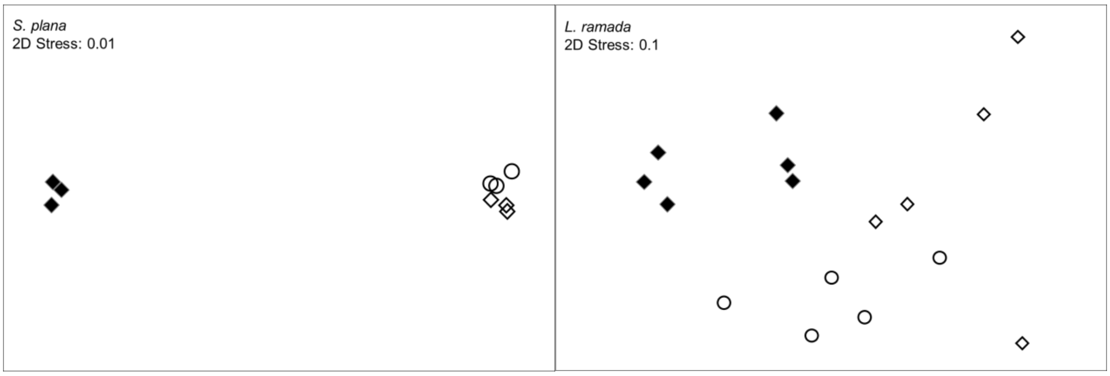

Figure 4 – Non-metric multidimensional scaling (nMDS) ordination plot based on Euclidean distances of the metal partitioning data of the three groups from the experiments with *Scrobicularia plana* (left plot) and *Liza ramada* (right plot) . ◆ Pellets; ◇ Sediment; ○ Control.

The global R statistics from ANOSIM were 0.844 for *S. plana* ($p < 0.05$) and 0.643 for *L. ramada* ($p = 0.01$), respectively, which means that the overall distances between the pellets, sediment and control samples were statistically significant. Pairwise comparisons failed to detect differences when comparing groups from *S. plana* ($p = 0.10$), but the comparison of the *L. ramada* groups showed significant differences between pellets and both sediment (R = 0.845, $p = 0.02$) and control samples (R= 0.704, $p = 0.02$), but also between sediment and control samples (R = 0.38, $p = 0.024$). Although the R value indicated that sediment and control samples were more similar to each other than to the pellets, both groups were still significantly different when considering metal partitioning for all elements together.

SIMPER analysis showed that in *S. plana* the control and sediment samples were closer (average squared distance, $d^2 = 11.08$) than the pellets with either sediment (d^2 = 56.12) or control samples (d^2 = 54.70). None of the variables (metal partitioning fractions) stood out in the SIMPER analysis when comparing the pellets with the other groups, with relatively low individual contributions to the dissimilarity (<9%). Cadmium associated with the organic phase and Ni in the residual fraction showed the highest contributions for the dissimilarity between sediment and control samples (34.35 and 31.64 %, respectively). All other metal fractions contributed with less than 9% for the dissimilarity of the two groups, with the labile fractions of Zn and Cd (exchangeable and carbonates) showing the smallest contribution (<1% each). In *L. ramada*, the average squared distance between the groups was similar, although slightly higher between the pellets and the sediment samples (d^2 = 44.23, against an average squared distance of 33.17 and 34.14 between the control samples and the pellets and sediment, respectively). The individual contribution of each variable to the observed distances was low, with the highest values staying below 13%.

DISCUSSION

Analyzes undertaken in this work have showed that after ingestion of the sediment by *Scrobicularia plana* and *Liza ramada* some alterations occurred in the partitioning of several trace metals, which were enough for the three groups to be considered distinct by multivariate analysis. Deposit feeders are known to modify sediment geochemistry through as they change physic-chemical parameters of the sediment with their activity e.g., burrow construction, irrigation or molecular diffusion (Green and Chandler, 1994). In the present work, however, sampling constraints did not allow to evaluate if changes in metal partitioning were accompanied by changes in the physical and chemical characteristics of the samples, like organic matter content, particle size distribution, Eh or pH, mostly due to the difficulty in obtaining enough fecal pellets material.

The most noticeable differences observed in both experiments (*S. plana* and *L. ramada*) were related to changes in the carbonates and, in some cases, in the exchangeable fractions. In *S. plana,* Cd, Cu, Ni and Zn bound to EXCH and CARB were higher in the pellets. The increase in the concentration of exchangeable metals may be related to the increment in the acidity of the gut environment. The pH in the digestive diverticula of this species may be as low as 5.6 (Payne and Thorpe, 1993), which could be enough to destroy some carbonates and this way contribute to change the original partitioning of metals in the sediment, releasing metals that could eventually be more easily extractable. The increase in the acidity causes increased solubility (Förstner, 1993), since additional protons will compete with metal cations for the same binding sites. The simultaneous increase in the carbonates-bound fraction points to an interaction between the sediment passing through the gut of the clam and the gut environment, which may have led to the remobilization of the particle bound metals in this fraction. Regarding *L. ramada*, the concentrations of Cd, Cu and Zn in the EXCH and/or CARB fractions in the pellets were higher than in the ingested sediment (although statistical significance was not achieved for all the cases), and Ni partitioning into the carbonates phase significantly decreased in the pellets. The increase in the exchangeable Zn may be related to the increase that was observed in the total concentration of this metal in the pellets, although this relationship is recognized to a greater extent in Cd (Wang *et al.*, 2002; Chakraborty *et al.*, 2012). The increase in the concentration of total Cd in the pellets, when compared to the initial sediment, could be enough to influence the geochemical partitioning of this element after passing through the mullets' gut (Chakraborty *et al.*, 2012). Within the gut, the ingested food suffers the action of enzymes, changes in pH, abrasion, all of this working together towards breaking down organic matter. Compounds previously complexed with organic matter may be released in the gut by this process, and together with everything that is not taken up in the gut are transformed into fecal pellets and released in the aquatic environment (Wotton and Malmqvist, 2001). A process that can explain the significant increase of Zn in the pellets in the one involved in Zn excretion through intestinal sloughing to maintain Zn homeostasis in teleost fish (Bury *et al.*, 2003). The pH in the stomach of grey mullets vary from acidic (3.5 – 4) to slightly alkaline (7 – 8.5), depending on the species (Payne, 1978). *Liza ramada*'s gut pH has not been described so far, but given its feeding ecology it is expected to be predominantly acidic. In such case, the acidic environment may cause the destruction of carbonates, similarly to the described for *S. plana*, and also increase Ni solubility (Gonnelli and Renella, 2013). The fact that a correspondent increase in Ni associated with the pellets exchangeable fraction was not observed is likely a consequence of increased assimilation of Ni bound to the sediment labile fractions (Baumann and Fisher, 2011).

The partitioning of Ni into the organic matter/sulfides phase showed higher concentrations in the fecal pellets of both species. This may seem counterintuitive, as the digestive process of both species is expected to break down the organic matter, decreasing its content in the egested sediment. It was not possible to assess the organic matter content in the pellets, and for that reason the incorporation of organic matter by the animals could not be quantified. Nickel has a high affinity with organic matter; low molecular weight organic ligands (LMWOL), humic substances or particulate matter can easily form complexes with Ni in soils (Gonnelli and Renella, 2013). It is likely that metals associated with the OM/S phase of the sediment were bound to more refractory organic compounds, which would decrease the assimilation efficiency (AE) of those metals. A predominance in labile organic compounds (like LMWOLs), on the other hand, would enhance the AE of metals bound to organic matter (Baumann and Fisher, 2011). A depletion in labile organic matter in the ingested sediment, together with further complexation of metals with the lining mucus that involves the fecal pellets (Wotton and Malmqvist, 2001), can thus help explaining the increase in metals associated with the OM/S phase in the pellets.

CONCLUSION

The results showed that in the experimental conditions of this work the activity of *Scrobicularia plana* and *Liza ramada* potentially favored the mobility of Cd, Cu, and Zn in the sediments. It is possible that the underlying increase in the acidity in the gut of the animals, compared to the nearly neutral pH of the surrounding environment, is involved in the solubilization of the metals, making them more available to the estuarine environment or to be more easily assimilated by the organisms. Evident changes in the metal partitioning were evidenced in the abovementioned trials and should be taken into consideration. Although there is a high degree of similarity of the underlying mechanisms, some specificity was also detected. This pointed to a differential role of the different trophic mechanisms carried out by different animals and the consequent bioturbation implied. The processes that lead to changes in metal partitioning seem to be almost metal-independent mechanisms, being mostly associated to the gut chemistry rather than to the metal chemistry. All these findings point out to a new role for the deposit feeders as key players in metal biogeochemistry, and thus in the estuary depuration function.

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CHAPTER 4

ESTUARINE BIOTA AS SENTINEL ORGANISMS FOR SEDIMENT METAL CONTAMINATION: A CASE STUDY

THE LUSITANIAN TOADFISH AS BIOINDICATOR OF ESTUARINE SEDIMENT METAL BURDEN: THE INFLUENCE OF GENDER AND REPRODUCTIVE METABOLISM

ABSTRACT

Early diagenetic processes and anthropogenic activities are responsible for metal enrichment in estuarine sediments. The Tagus estuary (Portugal) is no exception, and as a result of past and present pressures, surface sediment contamination is still an issue in some areas. Since such metal loads may be incorporated by benthic organisms, this study analyzed the accumulation of trace metals in the Lusitanian toadfish (*Halobatrachus didactylus*) in the Tagus estuary. In order to determine the role played by the seasonal reproductive cycle of the Lusitanian toadfish in the bioaccumulation process of trace metals in its tissues, the concentrations of Cd, Co, Cr, Cu, Ni, Pb and Zn were determined in the liver of male and female specimens captured during reproductive and non-reproductive periods. The results showed that metal accumulation in the liver was related simultaneously with gender and season, with females having higher levels of Cd, Cu and Zn during the reproductive period. The metabolic roles of Cu and Zn in embryonic development may explain such results, as both metals accumulated in the female liver to be transported to the gonads later on. Cd, on the other hand, does not have a metabolic role, and the higher concentrations of this metal found in spawning females could be related to the high affinity of Cd to vitellogenin, which is produced in the liver. To assess the species' potential as an indicator of metal contamination, the concentrations of the seven elements were compared in the muscle tissue of adult, type I males (age ≥ 5), from two areas with distinct sediment metal loads. Non-essential metals in the muscle reflected the same differences between areas that were found in the sediment samples, evidencing *H. didactylus* as a potential indicator of those elements bioavailability from the sediment. The results showed that the muscle tissue of adult specimens of a relatively sedentary species such as *H. didactylus* is a useful indicator of long term accumulation of trace metals. On the contrary, liver concentrations of trace metals showed variation according to the reproductive status, which could lead to overestimate of the environmental status concerning trace metals bioavailability. Spawning season and liver tissue should thus be avoided in biomonitoring studies targeting this benthic fish.

Keywords: *Halobatrachus didactylus*; Bioindicator; Trace elements; Muscle; Liver; Spawning season

INTRODUCTION

The continuous growth of human populations around coastal areas and the constant demand for their natural resources is a long standing and increasing phenomenon (Tilman *et al.*, 2001). This resulted from the fact that, within coastal areas, estuaries are among some of the most valuable ecosystems, when considering the average (per hectare) of the estimated values of the services they provide (Costanza *et al.*, 1997). In the last two to three decades, a growing environmental awareness of the negative impacts of anthropogenic pressures led to the emergence of an increasing number of studies concerning pollution in estuaries (Sun *et al.*, 2012), focusing e.g. on the contaminant determination in these ecosystems (Chapman *et al.*, 2013). More specifically, a growing awareness has been observed regarding metals in the aquatic ecosystems (Zhou *et al.*, 2008), particularly due to their non-degradable and persistent character (Wood, 2011), and the impacts they may promote on the biota.

The uptake of metals in aquatic organisms may occur via direct uptake from water by gills or skin, by ingestion of contaminated suspended particles, or by ingestion of contaminated food items (van der Oost *et al.*, 2003), and a relationship between metal levels in tissues and those in water, sediment or food items may be found (e.g. Bervoets *et al.*, 2001). Fishes are among the organisms that are usually described as pollution indicators (e.g. Marcovecchio, 2004; Birungi *et al.*, 2007; Caçador *et al.*, 2012; Zrnčić *et al.*, 2013), with several reasons contributing to this interest. Some studies address commercial and/or public health interest on the fish species (Burger and Gochfeld, 2005; Wang *et al.*, 2005; Pedro *et al.*, 2014), while others are more concerned with the species ecology and the potential usefulness as indicators of metal bioavailability (Jørgensen and Pedersen, 1994; Caçador *et al.*, 2012). Metal accumulation in fish tissues is affected by several aspects, among which are the trophic ecology (Pourang, 1995), ontogenic development (Farkas *et al.*, 2002, 2003), or body size (Heath, 1995). The latter is not consensual, as some studies found positive relations between tissue metal burdens and size (e.g. Zyadah, 1999), while others found negative (Canli and Atli, 2003; McKinley *et al.*, 2012) or no relationship (Canpolat and Calta, 2003) between the two.

In metal accumulation studies involving fish tissues, two of the most commonly addressed are the liver and the muscle. The primary role of liver in the accumulation, biotransformation and excretion of contaminants (Heath, 1995; Pereira *et al.*, 2010), justifies the prominent interest of this organ in bioaccumulation studies. This is particularly true in situations of chronic (e.g. for Cd) and acute (e.g. Cu) exposures, given that a prompt response to the contaminant levels is found in accumulation levels in the liver (Sorensen, 1991). However, variations due to different reproductive status of the specimens throughout the year (Miramand *et al.*, 1991; Monsefrad *et al.*, 2012) may encourage using concentration of metals in the muscle as an alternative to the liver, despite the fact that the muscle generally concentrates metals to a lesser degree than other tissues (Miramand *et al.*, 1991). Nevertheless, the muscle can be considered as a long-term storage tissue, reflecting persistent contamination, such as it is observed in mollusks' mantle, for example (Langston *et al.*, 1998). It also has the clear advantage of being easy to obtain, both quantitatively and in terms of the reduced possibility to occur contamination from other tissues during the harvesting.

The Lusitanian toadfish *Halobatrachus didactylus* (Bloch and Schneider, 1801), is a mainly marine species, but in the northern limit of its distribution (Iberian Peninsula) it is also common in brackish waters (Costa and Costa, 2002). It can be found buried in the sediment or under rock crevices (Roux, 1986), feeding on clams, crabs, shrimps and fishes (Costa *et al.*, 2000; Costa *et al.*, 2008), with sediment also being found in its stomach contents. Regardless of its ability to perform ample movements in the estuary (Campos *et al.*, 2008), it is a relatively sedentary species. Distinct behavior between genders was previously observed during the reproductive season, with type I (“normal” morphotype) males building nests and presenting parental care for eggs, larvae and juveniles, while females and type II males (“alternative” morphotype) do not (Modesto and Canário, 2003; Pereira *et al.*, 2011).

The Lusitanian toadfish has been, to a certain degree, overlooked regarding biomonitoring studies. Considering its ecology and feeding habits, a relationship between metal levels in the surrounding environment and the accumulation in fish tissues should be expected. *H. didactylus* was used in this work to investigate if the muscle tissue of could be a good indicator of distinct levels of trace metals in the bottom sediment. A second objective of this work was to assess if differences in the concentration of metals in the liver of *H. didactylus* would arise considering the reproductive metabolism of the species. While males need to build up large energy reserves to endure the time they will spend guarding the nests, females high energetic necessities are directed towards the gonadal development for the production of the species characteristic large eggs (Costa, 2004), leading necessarily to distinct hepatic metabolisms. Thus, gender and season (reproductive/non-reproductive) should yield different metal accumulation in the liver.

To accomplish the first objective, i.e., the bioindicator quality of *H. didactylus*, sediment and fish samples were collected from two distinctly disturbed areas in the same estuary. The second objective was addressed by analyzing male and female specimens collected in the more contaminated of the two areas, during and after the reproductive season.

MATERIAL AND METHODS

Study area

This study was carried out in the Tagus estuary, Portugal (Fig. 1), one of the largest estuaries on the west coast of Europe (38°44′ N, 9°08′ W), and the largest transitional water area in Portugal, covering 320 km^2. It is a semi-diurnal mesotidal estuary (ca. 4 m tidal amplitude), with a deep and narrow inlet channel and several large and shallow bays, with almost 40% of the estuarine area comprising intertidal mudflats and extensive areas of salt marshes, particularly in the southern and eastern shores. The tidal influence of this estuary reaches 80 km upstream from Lisbon, and under normal hydrological conditions salinity reaches 0 roughly 50 km upstream from the river mouth, at Vila Franca de Xira (Costa *et al.*, 2007).

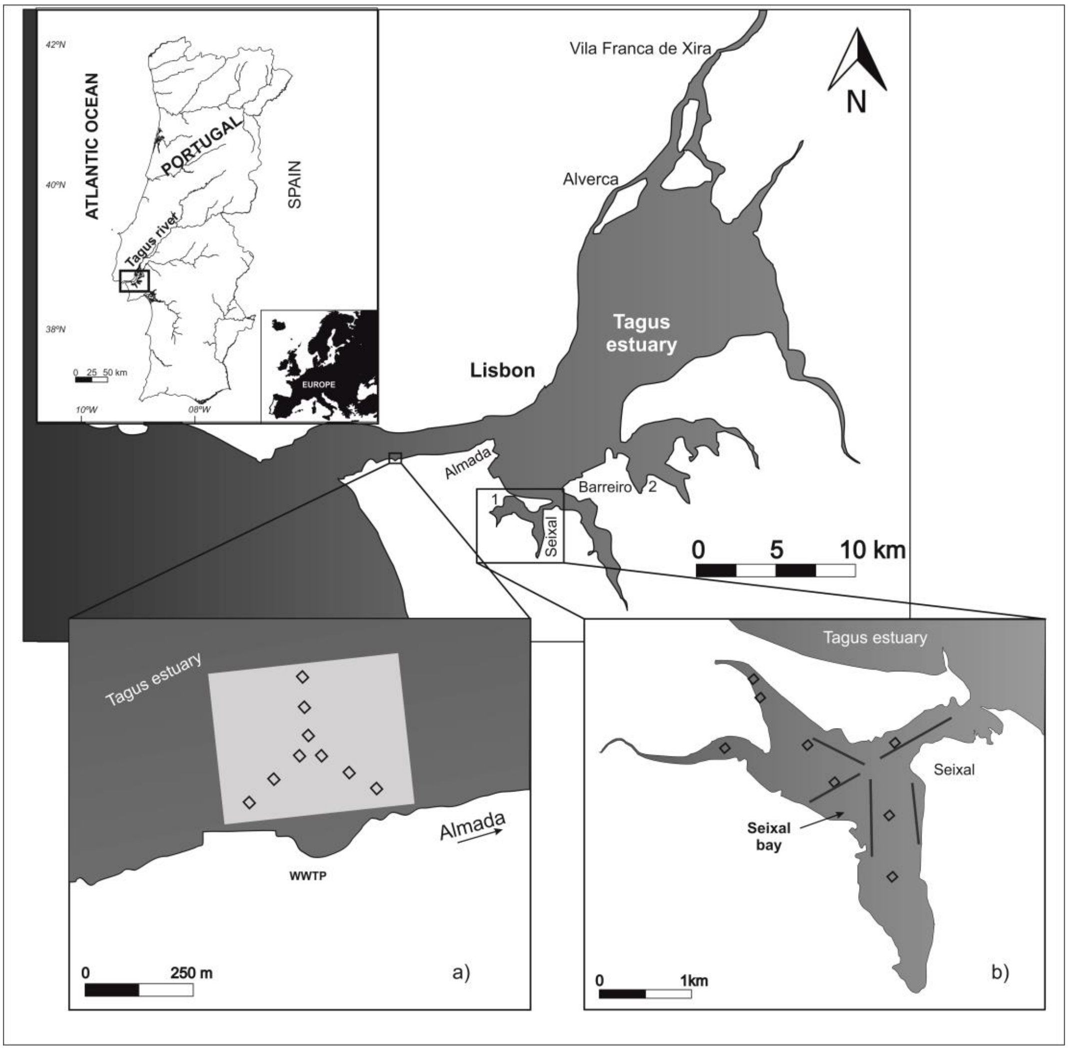

Figure 1 – Tagus estuary (Portugal), showing Almada (a) and Seixal bay (b) sampling areas in detail. 1 - Corroios salt marsh; 2 - Rosário salt marsh; ▭ in (a): *Halobatrachus didactylus* sampling area with gillnets; —— in (b): beam-trawl transepts for *H. didactylus* sampling; ◇: sediment sampling sites in both areas; WWTP – Wastewater treatment plant.

As in many other areas, the anthropogenic pressure over the Tagus estuary is intense and diverse (Rilo *et al.*, 2012), leading to the deposition of a large array of contaminants. Agricultural runoff and urban and industrial effluents are discharged into the estuary, with the most important agricultural areas located primarily in the NE region of the left bank, and the two major industrial areas being comprised within Vila Franca de Xira – Alverca and Seixal – Barreiro regions (Fig. 1).

Because of the size and high diversity of habitats, the Tagus estuary has an important role as a nursery area for many fish (Cabral and Costa, 1999), notwithstanding the human pressures. *Halobatrachus didactylus*, a resident species, assumes a key ecological role in this ecosystem due to its abundance, longevity, large maximum size reached, and for being a top predator in the estuarine food web (Pereira *et al.*, 2011).

The Seixal Bay is an area with high potential spawning grounds for the Lusitanian toadfish, *H. didactylus*, given its relatively closed and shallow nature. This favors warmer water conditions in late spring and early summer, when this subtropical species spawns, which is evident from data reporting high species abundance and recruits density in the bay and its vicinity (Cotter *et al.*, 2013; unpublished data). On the contrary, in the Almada area larger specimens are found, with smaller densities (Cotter *et al.*, 2013).

The Almada municipality estuarine front (Fig. 1a) is located near the Tagus estuary mouth. It is a highly hydrodynamic, exposed area, with depths that may be greater than 25 m. Untreated domestic effluents were previously discharged in the area, but a wastewater treatment plant (WWTP) began to operate in 2003. This area benefited from the natural conditions, never having shown significant degradation of its waters. Nonetheless, ecological improvements were observed since then (Costa *et al.*, 2010). The Seixal Bay (Fig. 1b) is a relatively small water body (ca. 4.2 km^2 total surface) with intertidal areas composing approximately 95% of the bay. The anthropogenic pressure in this bay was very relevant, with less than 50% of domestic effluents treated until recently. After 2011, the Seixal WWTP allowed for 100% rates of domestic effluent treatment. Notwithstanding the fact that issues such as eutrophication were addressed with this improvement, contamination with metals cannot be solved through classic wastewater treatment. Consequently, their bioavailability is still a matter of great concern in this area, as reported by Caçador *et al.* (2012), who showed that several vertebrate and invertebrate *taxa* reflected sediment and suspended particulate matter contamination, particularly of non-essential metals. A few familiar farms and small factories (tannery, fertilizers and pesticides production) are still operating in the area, as well as two aquaculture units. The Seixal Bay has also harbored in the past industrial fish processing and naval construction and repairing, with only some shipyards currently still in business (unpublished data, source Seixal Municipality). The continuous monitoring of metal contamination in this area remains a current necessity, particularly given the ecological importance of the bay.

Sampling

Fish sampling

A total of 44 specimens of *H. didactylus* were sampled during low-tide with a beam-trawl (width ≈ 1.5 m; mesh size of 5 mm) in the Seixal Bay (SXL) (Fig. 1b), in the spring (mid April) and summer (late July) of 2010 [spring: N=34 (14 males, 20 females); summer: N=10 (7 males, 3 females)]. Additionally, a total of 6 male specimens with L_T ≥ 300 mm (age class >5 (Pereira *et al.*, 2011)) were captured in Almada (ALM) (Fig. 1a) during the summer of 2010, using gillnets. Data on body total length (L_T) were registered for each fish. Muscle and liver samples were collected and stored at -80°C. Gonads were used to differentiate gender.

Sediment sampling

Sediment samples were collected at SXL and ALM (Fig. 1) using a modified van Veen grab (0.05 m^2 attack area) and a modified Day grab (0.1 m^2 attack area), respectively. Distinct grabs were used to ensure optimal operational conditions at both sites, considering each location depth and hydrodynamics. A total of 17 samples were collected (SXL, N=8 and ALM, N=9), each with 3 replicates. Only the uppermost 7-10 cm of the dredged sediment were stored for analysis, using for that purpose polyethylene vials filled directly from the surface of the dredged sediments, and deep frozen on arrival to the laboratory (-80°C). Organic debris and larger particles (e.g. small pebbles, broken shell pieces) were removed prior to trace metals determination.

Trace metals determination

All samples (tissues and sediment) were freeze dried (Cryodos-50, Telstar Life Science solutions, Spain) prior to processing for metal extraction. After complete dehydration, about 0.1 g of grinded, homogenized dry tissue was acid digested in Teflon® vessels with 2 ml of a mixture of HNO_3 (65%, Panreac, p.a.) and $HClO_4$ (60%, Panreac, p.a.) (9:1, v/v), during 2 h, in an electrical oven at 110°C. Approximately 0.1 g of homogenized dehydrated sediment was acid digested in Teflon® vessels with 2 ml of a mixture of HNO_3 and HCl (37%, Carlo Ebra, p.a.) (3:1, v/v), during 3 h, in an electrical oven at 110°C. The resulting solutions were allowed to cool at room temperature before being filtered through Whatman 42 filters (90 mm diameter; <2.5μm pore size) and diluted to 10 ml with ultrapure water (Type I, 18MΩ/cm, Elga Purelab Classic). All laboratory material used was decontaminated of any adsorbed ion by soaking in 0.25 M nitric acid (HNO_3) for 24 h and 0.25 M hydrochloric acid (HCl) for 48 h, and rinsing three times with deionized water to avoid cross-contaminations. (Reverse Osmose, Elga Purelab Prima) to avoid cross-contaminations.

Trace metals (Cd, Co, Cr, Cu, Ni, Pb and Zn) were determined by Flame Atomic Absorption Spectrometry (FAAS, SpectraAA 50, VARIAN). Detection limits of the method were as follow (ppm): Cd – 0.03; Co – 0.13; Cr – 0.15; Cu – 0.03; Ni – 0.15; Pb – 0.32; Zn – 0.33. The accuracy and precision of the analytical methodology for elemental determinations were assessed by replicate analysis of certified reference materials, TORT-2 (NRCC) for fish tissues and BCR-146 (IRMM) for sediments. Blanks and the concurrent analysis of the standard reference material were used to detect possible contamination/losses during analysis and to ensure the accuracy and precision of the analytical method. The obtained values of the reference materials were within 80 – 110% of the certified concentrations.

In line with other recent studies about Tagus metal contamination (Duarte *et al.*, 2013; Duarte *et al.*, 2014), enrichment factors were calculated using as background levels, the metal concentrations from the upper crust (Turekian and Wedepohl, 1961). Previous studies showed that the application of the Earth's crust values is adequate for this type of studies, due to the large dimension of the Tagus estuary and its spatially heterogeneous industrial history (Duarte *et al.*, 2013).

Statistical analysis

Mann-Whitney U-test was used to compare the accumulation of the seven metals in fish muscle between the two sampling areas (SXL and ALM), using adult type I males (age ≥ 5 years) collected in both areas during summer. The same test was used to compare trace metal concentrations in sediment samples from the two locations. Principal Component Analysis (PCA) (Hair *et al.*, 1998) was conducted to assess how sediment and muscle samples were distributed in the Euclidean space when all metals were considered. Data were normalized by logarithmic transformation prior to being analyzed. Permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001; McArdle and Anderson, 2001) was used to assess differences between the two areas under study considering all metals.

Analysis of variance (ANOVA) (Zar, 1999) was used to test differences in the total length of specimens according to gender and season. Linear regression was used to assess the relationship between the fishes' body size and metal concentrations in the liver of SXL specimens. PCA (Hair *et al.*, 1998) was used to identify potential groups according to gender and/or season using the concentrations of the different metals in the liver of *H. didactylus* as variables. Statistical significance among gender and season groups was assessed with PERMANOVA.

The statistical packages SPSS v.20 (IBM, 2011) and PRIMER-e +PERMANOVA (Clarke and Gorley, 2006; Anderson *et al.*, 2008) were used for data treatment and statistical analysis.

RESULTS

The average concentration of metals in the sediment samples of both sites followed different patterns: Seixal Bay (SXL) samples showed Zn > Pb > Cu > Cr > Co > Ni > Cd, while metals in Almada (ALM) sediment samples followed the order Zn > Pb > Cr > Ni > Co ≈ Cu > Cd. The highest values for all elements in sediments were found in SXL (Fig. 2), with significant differences between the metal burden of the two areas for all the studied metals ($p < 0.05$ for Cd; $p < 0.001$ for the remaining elements). Trace metal concentrations in muscle samples from both areas also showed different patterns. In SXL the order was Zn > Pb > Cu > Ni > Co > Cd > Cr, while in ALM the order was Zn > Cu > Cd > Cr > Co > Ni > Pb. The levels of the trace metals in *H. didactylus* muscle were generally higher in Seixal Bay (Fig. 2), as it was observed in sediment samples, except for Cr, which was higher in ALM, and Cu, that showed similar concentrations in both locations. Significant differences between the two areas were found regarding Cd, Co, Ni and Pb concentrations ($p < 0.01$). Ni and Pb in muscle tissue from ALM area were below the detection limits (0.15 and 0.32 ppm, respectively).

The PCA based on the concentrations of the seven metals under study showed a clear separation between SXL and ALM, for both sediment and muscle samples (Fig. 3). The first two principal-components (PC1 and PC2) for the sediment accounted for 84.4% and 12.0% of the variation, respectively. All variables, except Cd, contributed similarly to the variability of PC1 (eigenvectors, $\lambda \approx -0.4$, Cd $\lambda = -0.3$). Regarding PC2, Cd and Cu were the most important variables contributing to the overall variation (λ: Cd = -0.8, Cu = 0.3).

In muscle analysis, PC1 and PC2 accounted for 56% and 28% of total variation, respectively. The most contributing variables in PC1 were Cd ($\lambda = 0.4$), Co, Ni and Pb ($\lambda \approx 0.5$), while in PC2, that was observed for Zn ($\lambda = -0.6$), Cr and Cu ($\lambda \approx -0.5$). The PERMANOVA results (Table 1) showed that in both cases (sediment samples and *H. didactylus* muscle), the differences between the two areas were statistically significant ($p < 0.001$).

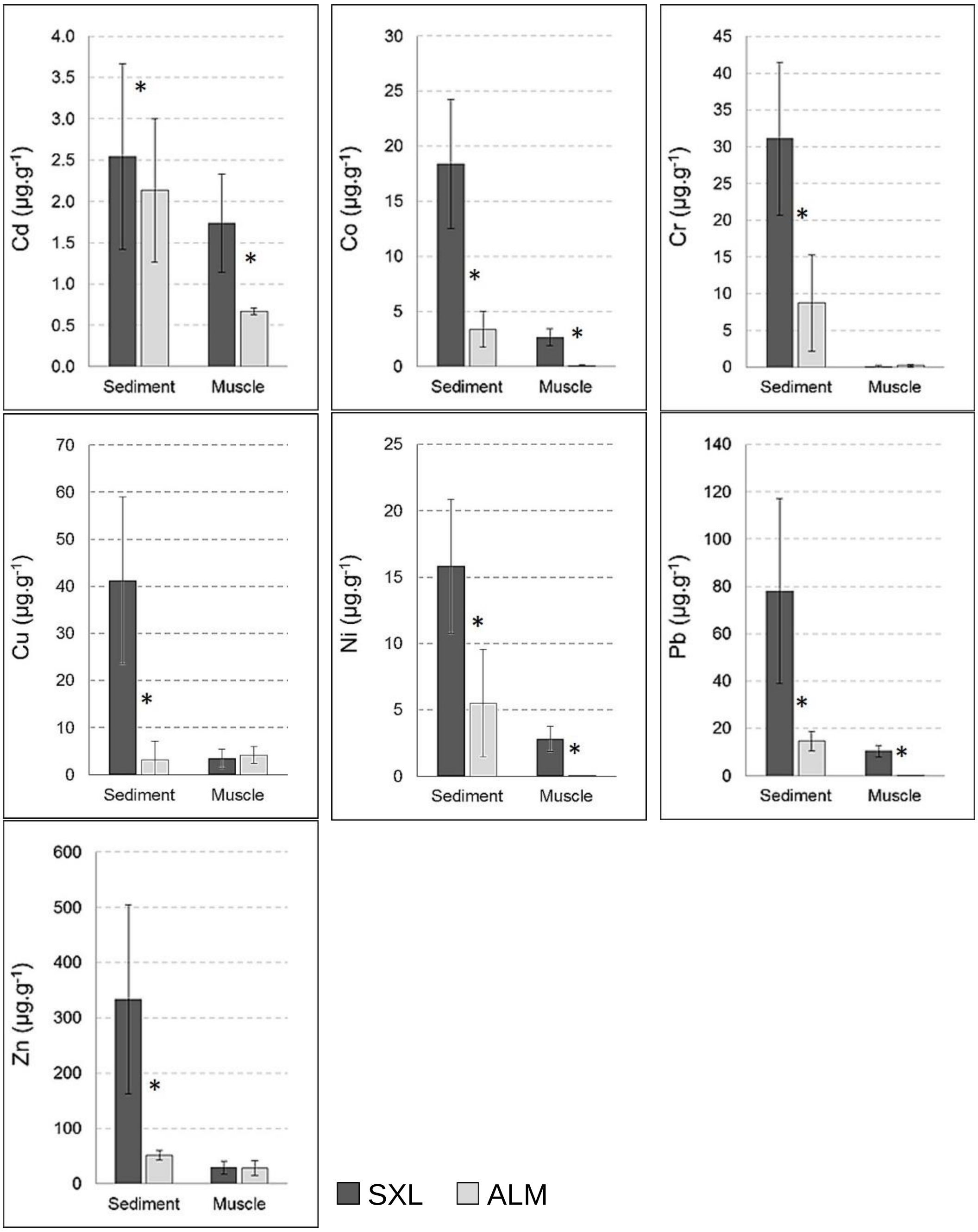

Figure 2 – Trace metals concentration (µg.g^{-1} dry weight, $\bar{x}$ ± sd) in sediment samples from Almada, ◻, ALM (n=9) and Seixal Bay, ◼, SXL (n=8), and in *Halobatrachus didactylus* muscle (adult males) from the same areas (ALM: n=5; SXL: n=8). * Significant differences between areas (p < 0.05).

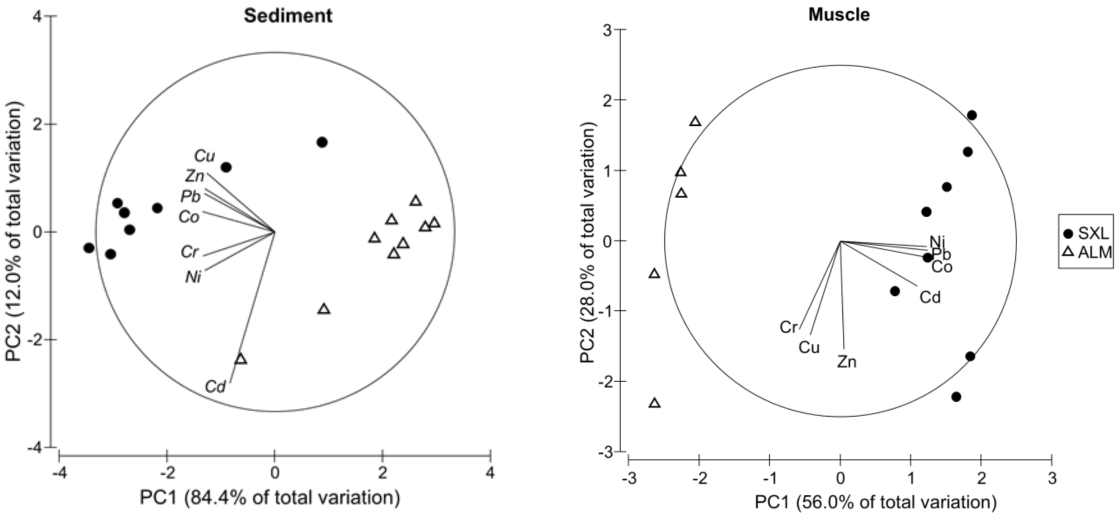

Figure 3 – PCA diagrams (1st and 2nd components), based on metal concentrations in whole sediment samples (left) and *Halobatrachus didactylus* muscle samples (right), from Almada (ALM, △) and Seixal bay (SXL,●). Number of sediment and muscle samples as in Fig. 2.

Males' total length (TL) ranged between 151 mm and 409 mm, while females TL ranged between 155 mm and 426 mm. The difference in TL of males and females was not statistically significant ($F_{(1,43,0.05)}$ = 0.200, p = 0.657). The relationship between metal concentrations in *H. didactylus* liver and fish size was tested and a significant positive correlation (r^2_{adj} = 0.542, p < 0.001, N = 23) was only found between TL and Co concentration in female specimens. None of the other metals yielded significant results in the regression analysis, and the same absence of significance (p > 0.05) was found for all metals when considering the male specimens in the regression analysis. Considering this result, and given the almost generalized lack of relationship between fish length and metal concentrations for both male and female specimens, total length was not included as a factor in the subsequent multivariate analysis.

Table 1 – PERMANOVA analysis, testing differences between areas (Almada and Seixal Bay) for trace metals accumulation profile (Cd, Co, Cr, Cu, Ni, Pb and Zn) in sediment and *Halobatrachus didactylus* muscle samples. Locations and sample numbers as described in Fig. 1 and Fig. 2. Pseudo-F: pseudo-F statistic, *p*: p-value; Perm: number of permutations

	Source	**Pseudo-F**	***p***	**Perm**
Sediment	Area	6.10	0.0001	9945
Muscle	Area	13.20	0.0008	1285

Regarding specimens captured during the spring, the liver of *H. didactylus* females (n=20) exhibited higher concentration of most metals than the liver of males (n=14) (Fig. 4).

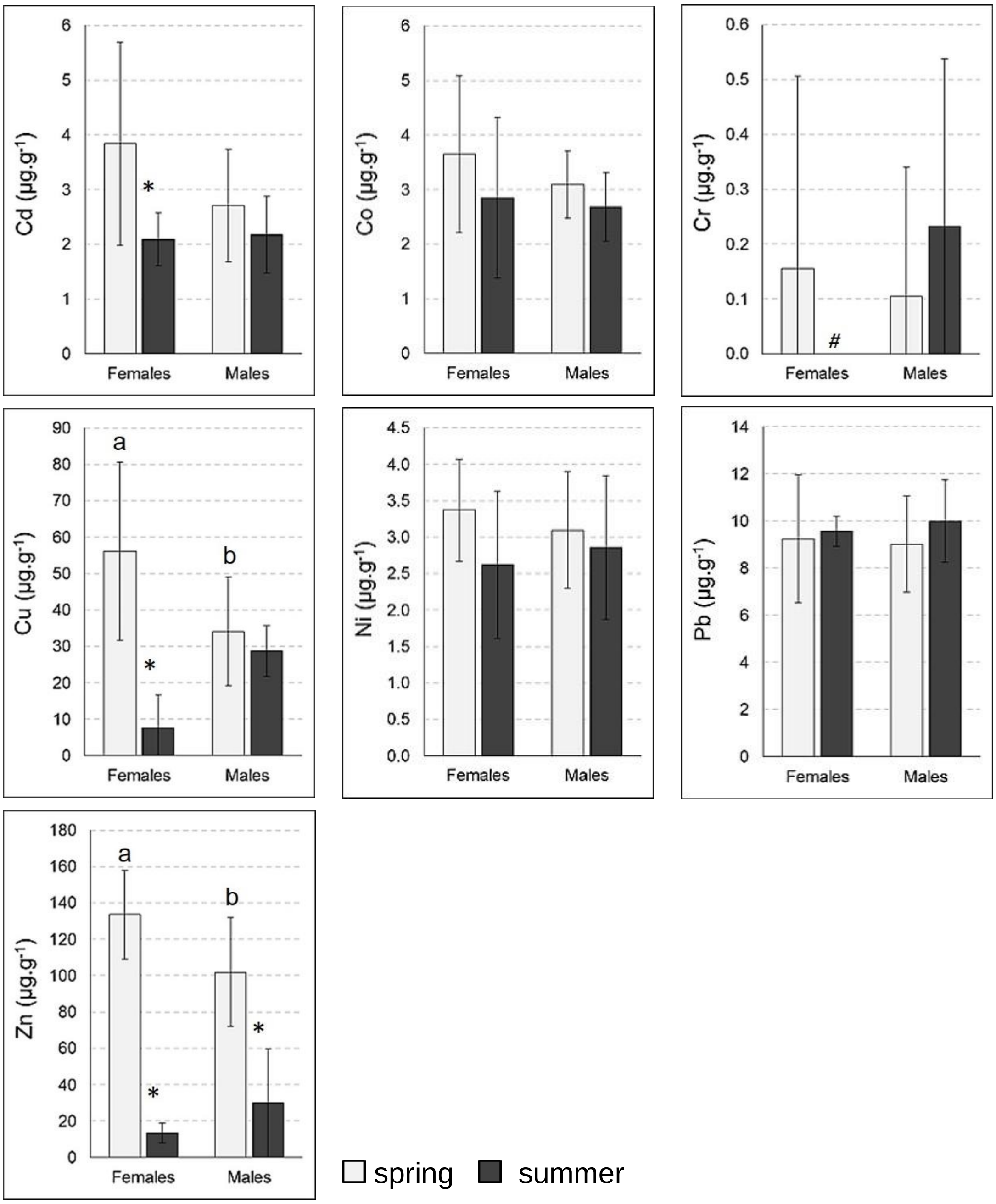

Figure 4 – Trace metals concentration (µg.g^{-1} dry weight, $\bar{x} \pm$ sd) in the liver of male and female *Halobatrachus didactylus* collected in the spring, ☐, and summer, ■, of 2010 in Seixal bay (spring: $n_{(female)}$=20, $n_{(male)}$=14; summer: $n_{(female)}$=3, $n_{(male)}$=7). Different lower case letters: significant differences between males and females (p < 0.05); *: significant differences between spring and summer (p < 0.05). # below the detection limit (0.15 ppm).

Significant differences were found in the case of Cu (males: 34.1 ± 14.9 $\mu g.g^{-1}$; females: 56.2 ± 24.5 $\mu g.g^{-1}$; p = 0.015) and Zn (males: 102.0 ± 30.0 $\mu g.g^{-1}$; females: 133.6 ± 24.5 $\mu g.g^{-1}$; p = 0.011. No significant differences were found (p > 0.05) when comparing the concentrations of metals in the liver of males and females captured during the summer. In what concerns each gender separately (Fig. 4), Zn concentration in the males' liver was significantly different between spring (102.0 ± 30.0 $\mu g.g^{-1}$) and summer (30.0 ± 29.9 $\mu g.g^{-1}$) specimens (p = 0.001). The liver of female specimens presented significant differences between spring and summer concentrations for Cd (p = 0.03), Cu and Zn (p = 0.01) concentrations, with the three elements presenting higher concentrations in the spring.

Most specimens had Cr concentrations in the liver below the detection limit (0.15 ppm). For that reason, Cr was removed from the multivariate analysis. Regarding the remaining trace metals (Fig. 5), *H. didactylus* specimens sampled in spring and summer showed different tendencies for metal accumulation in the liver.

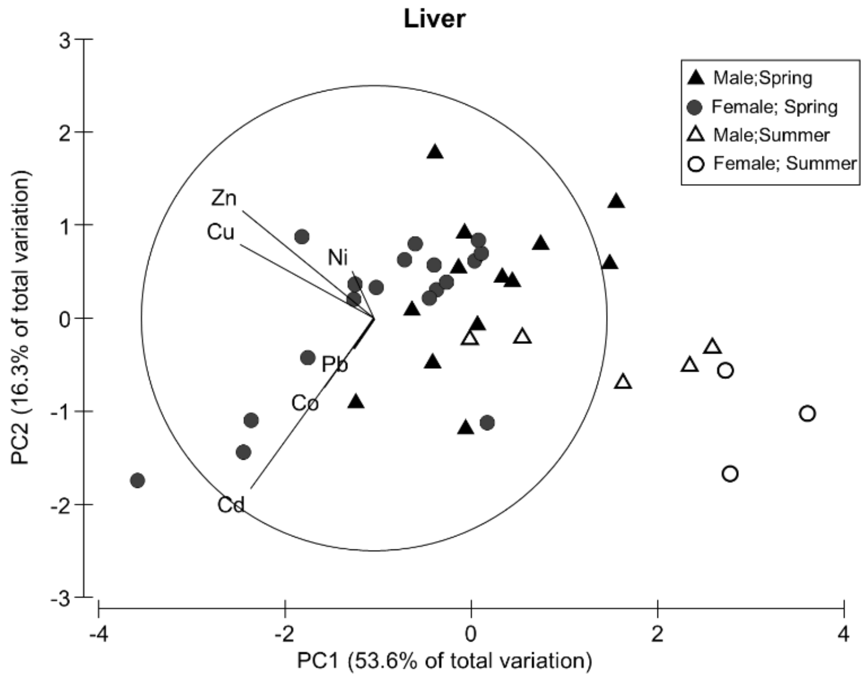

Figure 5 – PCA diagram (1st and 2nd components), based on metal concentrations in the liver of male and female *Halobatrachus didactylus*, collected in the spring (▲,●) and summer (△,○) of 2010 in Seixal bay (SXL). Number of liver samples as in Fig. 4.

The PCA showed that these differences were mostly due to Cu, Zn and Cd variation. In liver analysis, PC1 and PC2 accounted for 53.6% and 16.3% of total variation, respectively. The most contributing variables in PC1 were Cu (λ = -0.6), Zn (λ = -0.6) and Cd (λ = -0.5), and in PC2, the same variables were accounted with the highest contributions (λ: Cd = -0.7, Zn = -0.5 and Cu = 0.3).

PERMANOVA results (Table 2) showed that the differences between male and female specimens were not statistically significant ($p > 0.05$) when season was not considered, but significant differences were found regarding season (p = 0.0001) and considering the interaction of season and gender (p = 0.003). Subsequent pairwise tests (Table 2) showed that, in spring, males and females concentrated metals distinctly in their liver (t = 1.984; p = 0.010). On the contrary, females and males sampled in the summer did not present significant differences (t = 1.769; $p > 0.05$). Specimens sampled in the spring presented statistically significant differences from summer specimens, considering both genders ($p < 0.001$).

Table 2 – PERMANOVA analysis for trace metals (Cd, Co, Cu, Ni, Pb and Zn) accumulation profile in the liver of male and female *Halobatrachus didactylus*; samples collected in the spring and summer of 2010. Location and replicate numbers as described in Fig. 1 and Fig. 4; df: degrees of freedom; Pseudo-F: pseudo-F statistic, *p*: p-value; Perm: number of permutations; Pairwise: significant pairwise tests

Source	**Pseudo-F**	***p***	**Perm**	**Pairwise**
Season	17.83	0.0001	9942	
Gender	1.42	0.2107	9945	
Season x Gender	5.49	0.0032	9957	Spring: males ≠ females* Males: Spring ≠ Summer *** Females: Spring ≠ Summer***

* $p < 0.05$; *** $p < 0.001$

DISCUSSION

Metal concentrations in the surface sediment of Seixal Bay (SXL) and Almada (ALM) exhibited evident differences. These are justified both by the distinct urban/industrial pressure and by the different physical conditions of the inner and outer estuary areas in which SXL and ALM they are located. Greater depths and high hydrodynamics are found in ALM (Fortunato *et al.*, 1997), with low organic matter and finer particles content (Costa *et al.*, 2010), while SXL area surface sediments exhibit greater abundance in finer particles and consequently higher organic matter content (Freitas *et al.*, 1998).

When compared to the upper continental crust (UCC) average concentration (Wedepohl, 1995), the concentration of Cd, Co, Cu, Pb and Zn in SXL sediments are above the documented values, while in ALM only Cd concentration exceeds the UCC concentration, thus supporting the greater importance of the anthropogenic input of metals in the SXL area. On a more regional scale, Zn, Pb, Cu, Cr and Ni concentrations were compared to concentrations considered to represent pre-industrial levels (prior to 1963) (Vale, 1986), and calculated for two saltmarsh areas (Corroios and Rosário, Fig. 1) in the Tagus estuary (Caçador *et al.*, 1996). This showed that SXL sediment samples were above such background values for Zn, Cu and Pb, while ALM sediment concentrations did not exceed them. These results confirm previous studies (Costa *et al*., 2010) which had shown that the ALM area is, in general, relatively poorly impacted with metal contamination in sediments, rocky shore invertebrates, and fishes.

The Seixal Bay area, which had the highest metal concentrations in the sediment, showed the highest levels of Co and non-essential metals (Cd, Ni and Pb) in the muscle of *Halobatrachus didactylus*. Such relationship, where metal levels in fish muscle from different areas reflected the differences found in the sediment's metal concentrations has been described in other studies (Alquezar *et al*., 2006; Usero *et al*., 2003). As a resident estuarine species, of sedentary character and slow growth rates (Costa, 2004), *H. didactylus* type I males may potentially be good providers of persistent metal contamination in sediments.

The gills are usually the preferred route of uptake for waterborne Cd, Co, Pb, and Ni, particularly for the former three, and the uptake occurs primarily using other divalent cations pathways (Blust, 2011; Mager, 2011; McGeer *et al.*, 2011; Pyle and Couture, 2011). Being a benthic and relatively sedentary fish (Campos *et al*., 2008), close contact with the sediment is predominant in this species life cycle. Alongside with the gills pathway, the ingestion of contaminated suspended particles or food items is another preferential route of uptake (van der Oost *et al.*, 2003). Benthic macrofauna in SXL area showed high levels of metal concentration (Caçador *et al.*, 2012), while ALM macrofauna usually shows lower metal burdens (unpublished data). Thus, in SXL, the ingestion route bears higher metal loads through sediment particles intake and preyed organisms. The role of the skin in the uptake of metals, although in most cases less important than other pathways, is not completely discarded (Blust, 2011; Mager, 2011; McGeer *et al.*, 2011; Pyle and Couture, 2011). Batrachoidids in general and *H. didactylus* in particular, have rough skin, profusely covered with a thick layer of mucus (Roux, 1986). Glycoproteins in fish mucus may act as ion exchangers and fish mucus, although acting like an isolating layer from surrounding water, cannot generally provide a significant barrier to diffusion of water, but may have a role in ionic regulation in fish (Shephard, 1994). Contrarily to the present results, (Neto *et al.* (2011)) found that no clear relationship could be established between metal concentrations in the European eel muscle and sediments in the Tagus estuary. Those results were attributed to a great heterogeneity in environmental conditions, unlike the present study, where only two very distinct areas were compared.

Recently, several EU directives have been implemented in all EU-member states, focusing on the improvement of the ecological quality of the water bodies under each country's jurisdiction. The Water Framework Directive (WFD), even though not focusing on the levels of contaminants in the biota, tends to evaluate the ecological quality of the transition and coastal waters by the usage of organisms/communities as proxies (European Comission, 2000). In light of the present results, it is possible to say that *H. didactylus*, being a benthic species, resident and predominantly sedentary in the estuaries in its northern distribution limits, can be used as a proxy of the sediment quality in terms of metal accumulation. Furthermore, the Marine Strategy Framework Directive (MSFD) is far more specific than the WFD, having an ecological quality descriptor focused exactly on the contaminant levels of fish species for human consumption (European Comission, 2010). As mentioned earlier, this is also a marine species (particularly as it gets further from the northern limit of its distribution) and has economic importance (Costa, 2004); hence, this is a possible bioindicator for metal contamination both in estuaries and costal ecosystems.

The concentration of metals in fish tissues may be affected, among other factors, by differences in size. As referred, inverse relations between body length/weight and metal concentration are commonly found (Heath, 1995; Canli and Atli, 2003; McKinley *et al.*, 2012). This is mostly due to distinct metabolic rates between smaller and larger specimens (Sorensen, 1991), with the younger (smaller) having higher metabolic activities and hence displaying correspondently higher metal concentrations. Nonetheless, even though a considerable variation in total length was found within males and females in this study, not only did body size not varied between genders, but the majority of the elements analyzed in the liver showed no relationship with the fish size. Only Co evidenced significant increasing concentration in the liver of females with increasing size. Cobalt is a key component of cobalamin (Blust, 2011), which is essential for fish growth (John and Mahajan, 1979), but a justification for sex specific increasing Co uptake as it was observed could not be found.

In a study conducted in the Tagus estuary, Pereira *et al.* (2011) observed that reproductive specimens (gonadal states IV and V – maturing and mature gonads, respectively) of *H. didactylus* were only found between February and June. Higher Cu and Zn concentrations were found in the liver of specimens captured during spring (April), therefore in the middle of the reproductive period. Although with less expressive (yet significant) differences, Cd concentration was also higher in spring specimens' liver. During the reproductive phase, in response to the requirements for Zn and Cu for gametogenesis, metallothioneins (MT) levels usually increase in the liver (Olsson *et al.*, 1987). This explains the greater liver burdens of these metals in specimens captured in spring, when compared to those captured in the non-reproductive season (in this case, in the summer). MTs are responsible for the detoxification of non-essential metals, like Cd or Hg, and the regulation of metal availability for metal dependent functions, as it happens with Zn and Cu (Roesijadi, 1992). Cu and Zn were not only higher in spring, but females concentrated more metal in their livers than males in that season. Higher metal concentration in female fish tissues have been described before (Al-Yousuf *et al.*, 2000; Alquezar *et al.*, 2006). These differences are likely related to distinct reproductive metabolism between male and female toadfishes. Among many other functions, the liver is responsible for the production of vitellogenin, a protein that is the precursor of the egg yolk (Shackley *et al.*, 1981; Pereira *et al.*, 1993). From there, vitellogenin is taken to the gonads by the blood (Shackley *et al.*, 1981). The toadfish eggs are rather large (up to 5.5 mm), and contain a considerable amount of yolk (Costa, 2004). Cu and Zn, both essential metals, are probably accumulating to a greater extent in the female liver to be transported to the gonads afterwards, given their metabolic roles in the embryonic development (Shackley *et al.*, 1981). The higher concentrations described for Zn in reproductive females when comparing with males and non-reproductive females may also be related to prostaglandin metabolism (Watanabe *et al.*, 1997). Although Cd plays no role in the embryonic development, the liver of reproductive females of *H. didactylus* also presented higher concentrations of this element comparatively to non-reproductive females. This result can be explained by the fact that Cd shares chemical properties with Zn. Like Zn, Cd forms strong binds to metallothioneins, resulting in increasing lipid solubility and bioaccumulation (Sorensen, 1991). Shackley *et al*. (1981) suggested that Cd could have the same uptake and transfer in liver and gonads of the female blenny that Cu and Zn endure during gametogenesis.

Contrary to the reproductive period specimens, the liver of post-spawning females did not exhibit significant differences in Cd, Cu and Zn accumulation when compared to the liver of post-spawning males. Nonetheless, while Cu and Zn concentrations in the liver of post-spawning females are lower than in males, practically no difference is observed in Cd concentration. This could mean that females might be eliminating most of the Cu and Zn accumulated in the liver during the vitellogenesis phase through the gametes during spawning (Deb and Fukushima, 1999), similarly to what was described for mussels (Langston *et al.*, 1998) or barnacles (Rainbow, 1998), but that the same does not occur for Cd. Results from the study with the female blenny (Shackley *et al.*, 1981) suggested that Cu and Cd peaked in the liver during vitellogenesis, and were then incorporated in the oocytes (which will ultimately be shed). Although some authors found little evidence of Cd accumulation in female gonads (Pereira *et al.*, 1993), others showed that Cd can in fact bind to vitellogenin, displacing endogenous metals (Ghosh and Thomas, 1995). The same study showed that Cd incorporation in the ovaries increased when this metal was bound to vitellogenin, indicating this as an important pathway for metal transfer from the liver during vitellogenesis. This could, of course, compromise the viability of the eggs or embryos, depending on the concentration of metal exposure (Jezierska *et al.*, 2009), but it is possible that metal tolerance is transferred maternally instead, and the survival of the offspring is not affected (Lin *et al.*, 2000; Peake *et al.*, 2004). Our results, however, seem to suggest that unlike Cu and Zn, Cd is not likely to be eliminated or incorporated via the liver – gonads – gametes pathway that appears to be possible for the other two metals.

Zinc was the only metal for which differences were found between reproductive and non-reproductive males of *H. didactylus*. Zn burden in the liver of *Mullus barbatus* males was also significantly different between the reproductive and non-reproductive periods, being higher in the former (Miramand *et al.*, 1991). The significant increase in Zn levels in reproductive males is probably related to the fundamental role of this element in the maintenance and regulation of spermatogenesis and sperm motility, as it was suggested by Yamaguchi *et al.* (2009) in their work with a Japanese eel animal model.

Hepatic levels of metals may, in some cases, give a fast response to short-term environmental exposure, particularly in severe situations (Sorensen, 1991). For instance, acute and chronic exposures to Cd and Cu, respectively, promoted a rapid increase of those elements in the liver, and a delayed increase was observed regarding other tissues accumulation, like the muscle (Sorensen, 1991). Liver concentrations of some metals in *H. didactylus* are, however, clearly influenced by the reproductive status of the specimens, particularly in the case of Cu and Zn, and therefore the hepatic tissue should be avoided during the reproductive period from a monitoring point of view. The usefulness of the liver as an indicator of environmental exposure to metals, either regarding non-reproductive specimens, or metals whose accumulation is not affected by the reproductive stage, is yet to be verified in the Lusitanian toadfish.

CONCLUSIONS

The concentrations of trace metals in *Halobatrachus didactylus* tissues must be regarded under different approaches. Our results clearly show that, in the liver, essential elements accumulation is naturally influenced by metabolism and physiology, and that accumulation will most likely not reflect environmental levels during their reproductive period, exhibiting homeostatic regulation. Gender and season influence in Cu and Zn accumulation in the liver were evident, but Cd burden was also affected by these two factors. On the other hand, the muscle of the Lusitanian toadfish appears to be a good indicator of metal deposition in estuarine sediments, mainly concerning the accumulation of non-essential metals (Cd, Ni and Pb). Therefore, this work highlights *H. didactylus* as a useful species to be studied when assessing the efficiency of measures to achieve a good environmental status in aquatic ecosystems, considering both the Water Framework Directive and the Marine Strategy Framework Directive. This species is well established in several estuaries of the Iberian Peninsula, and the population in the Tagus estuary appears to be expanding (Pereira *et al.*, 2011). Also, its relative sedentary behavior enhances the likelihood to provide local responses to contaminant levels.

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CHAPTER 5

FINAL CONSIDERATIONS

This thesis focused on metal speciation and metal availability in salt marsh sediments and adjacent intertidal mudflats, and more specifically on the effect of sediment-organism interactions on such parameters. Reports on sediment quality based on total metal concentration are still common (e.g., Varol, 2011), and will probably continue to be a part of environmental quality assessment studies. Total metal concentrations can give us a picture of environmental modification when compared to pre-industrial values for those elements (Hahladakis *et al.*, 2013), or to the Earth's upper continental crust composition (Wedepohl, 1995), but they ultimately do not provide information on whether those metals are bioavailable, and to what extent. It is in this context that metal speciation becomes important. Total concentrations of metals may, nonetheless, reveal the degree of metal pollution and even be related to the accumulation of metal by organisms (Fan *et al.*, 2014).

In this work it was assessed how organisms could be affected for being in close contact with potentially contaminated sediment (Chapters 2 and 4). Dietary intake is one of the ways by which metals may enter an organism, having particular importance in the case of deposit feeders ingesting metal-bearing sediments (Millward *et al.*, 2001). In Chapter 2, the feeding habits of *Liza ramada*, scraping and filtering the sediment surface, served as an example of a close link between estuarine sediment contamination and dietary intake of metals by a vertebrate. The relationship between the two is not always apparent or observable, since metals may be regulated and/or excreted without assimilation by the organisms (Brown and Depledge, 1998). Younger specimens of *L. ramada* were potentially more exposed to the sediment's metal contamination, given the preference showed by smaller sediment particles in their diet. Smaller particles are characterized by a larger specific surface area, creating a greater adsorption surface for metals. The hypothesis of higher concentration of metals in the tissues of smaller fishes was confirmed, and the higher metabolism of younger animals (Heath, 1995) likely enhanced the potentially greater exposure to metal contamination and subsequent metal assimilation. Stomach contents provided contrary results, but this is most likely a consequence of the lack of homogeneity in estuarine surface sediment, which was in fact described in the first part of Chapter 2. Stomach contents of mugilids thus appear as poor indicators of metal exposure in the estuarine ecosystem. They provide a “snap shot” of the feeding habits, and will not necessarily be reflected in the accumulation of trace metals in the tissues, namely in muscle. Additional insight could have been achieved with metal partitioning analysis or enzymatic studies to provide information on actual metal availability and exposure to metal contamination. Notwithstanding these results, the relationship between metals in sediments and in fish tissues should not be disregarded. As it was observed in Chapter 4, sediment contamination (primarily regarding non-essential metals) can be reflected in long term metal accumulation in the muscle of adult male toadfishes. *Halobatrachus didactylus* is a resident, predominantly benthic species, occupying a high level in the estuarine trophic web (Branco *et al.*, 2008). It has a relative importance for human consumption, and consequently the bioaccumulation displayed by this fish highlights how a top predator from highly impacted estuarine areas can be a source of toxic metals ingestion to human populations.

Chapter 3 dealt with how organisms could affect trace metals cycle in the sediments, specifically by altering their geochemical fractionation and mobility. Salt marsh plants are known to promote entrapment and mobilization of metals in the sediments, modifying the sediment’s chemical and physical characteristics (Caçador *et al.*, 1996; Reboreda and Caçador, 2007; Reboreda *et al.*, 2008). Benthic organisms, on the other hand, affect the dynamic of sediment particles, promoting bioturbation and thus also interfering, directly or indirectly, with the dynamic and mobility of metals (Green and Chandler, 1994; Ciutat and Boudou, 2003). The feeding habits of *L. ramada* promote an increase in the bioturbation of the top layer of bottom sediments. According to some studies, bioturbation by benthic organisms can affect sediment layers down to 20 cm deep, exposing previously anoxic sediments to oxidation and thus promoting electron transfers (Williams *et al.*, 1994). Such great depth is not affected by the movements of *L. ramada*, but it may be observable in the burrowing behavior of *Scrobicularia plana.* The grey mullet’s movements throughout the estuary enable the transport of sediment from distinct areas through great distances, thus making this species a horizontal vector of sediment whose metal partitioning has been altered while passing through the digestive system of the fish. On the other hand, *S. plana* burrowing behavior favors the transport of surface sediment particles into deeper layers of the sediment, bringing oxidized surface sediment into anoxic layers, thus promoting a vertical exchange of sediment particles under different geochemical conditions. The oxidation of previously anoxic sediment also occurs, when particles removed by *S. plana* from its burrows are released at the surface by the exhalant siphon (Hughes, 1969). The oxidation of organic matter and sulfides may result in more soluble forms, especially through the formation of sulfates from the oxidation of sulfides, as the sulfates are more soluble than sulfides (Ngiam and Lim, 2001). Trace metals bioavailability may change as a result of geochemical changes in the sediment, which are usually diverse and metal specific, i.e., generalizations are usually difficult to make (Griscom *et al.*, 2000). Fecal pellets from benthic organisms increase the organic matter content in the bottom sediments, and hence the metal sink associated with it (Duarte *et al.*, 2008). Breaking down of organic matter, on the other hand, increases the area exposed to microbial decomposers. Microorganisms produce exopolymers that play multiple functions, like attachment to substrata, adsorption and retention of nutrients or conservation of exoenzymes (Wotton, 2004). These extracellular polymeric substances (EPS) have great metal binding capacity and form multiple complexes with ions (Bhaskar and Bhosle, 2006). Bacterial EPS may thus function as carriers for metal into the estuarine trophic chain, since many organisms feed on EPS as a supplementary source of carbon. On the other hand, the presence of chloride ions competes with metal binding EPS, (Bhaskar and Bhosle, 2006), hence reducing the extent to which metals adsorbed to these polymers enter food chains. The laboratory trials with the bivalves and fishes carried in this work suggest that the gut chemistry of these animals render some metals more available in the estuarine sediments than they initially were. Together with the possibility of enhanced metal binding to microbial EPS, both *S. plana* and *L. ramada* may be increasing the bioavailability of metals to the trophic web directly and indirectly.

Contrary to the animals' effect on metal partitioning in estuarine sediments, the presence of salt marsh plants render metals potentially less available. Local influence of the vegetation cover type was observable, but the metals' chemical behavior appeared to override site or species effects to metal geochemical partitioning. Two groups were formed regardless of the site or species, based on more or less mobile elements (Cd/Zn and Cu/Ni, respectively). Metals may become more immobilized and accumulate to a higher extent in sediments between the roots of halophytes, and rhizosediments also tend to promote higher accumulation of metals when compared to bare sediments. The higher the specific surface area of the root systems, the more pronounced accumulation of metals surrounding them is expected (Reboreda and Caçador, 2007), as this enhances root-sediment interactions and allows a greater adsorption and/or complexation of metals (Sundby *et al.*, 2005; Duarte *et al.*, 2009). Halophytes can, nevertheless, be a source of metals to the estuarine trophic chain, as decaying material bearing metals sheds and continues to be decomposed, becoming metal-bearing detritus (Caçador *et al.*, 2009; Duarte *et al.*, 2010; Couto *et al.*, 2013). The hydrology and morphology of the Tagus estuary salt marshes create conditions to a rapid flush of those detritus (Caçador *et al.*, 2009). This way, salt marshes, generally regarded as sinks for metal pollution, become a source to the adjacent estuarine areas through plant decay and decomposition. In a scenario of sea level rise due to global climate change, this export of metals is expected to increase (Duarte *et al.*, 2014), consequently affecting the metal fluxes into the estuarine trophic web.

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