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Trace elemental characterization of fish species and their Portuguese coastal systems.

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Trace elemental characterization of fish species and water from Portuguese coastal systems.

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Declaração de autoria de trabalho
(Statement of Authorship)

Trace elemental characterization of fish species and their Portuguese coastal systems.

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

José Carneiro

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Abstract

Estuaries are subjected to multiple anthropogenic stressors, among them enhanced inputs of potentially toxic trace metals. These are commonly derived from human activities, including mining, sewage discharge or fossil fuel burning, displaying high persistence in estuaries and affecting ecosystem health. In this study, multi-elemental concentrations of metals in fish tissues and water from major Portuguese estuaries were determined via Total X-ray Fluorescence Spectroscopy, to assess temporal and spatial variability patterns, which could be linked to environmental contamination, fish species ecology and origin of collection. Temporal changes in muscle and spatiotemporal variations in water elemental composition were assessed in the Mondego estuary and for juvenile *Platichthys flesus*. A multi-taxa and -estuary approach to metal distribution in muscle and liver tissues, and in water, was used to assess spatial variability of trace elements and our ability to accurately determine estuary of origin and inter-species differences. Target species included *Diplodus vulgaris, D. bellottii, D. sargus, Platichthys flesus, Solea senegalensis, S. solea, Dicentrarchus labrax, Sparus aurata* and *Halobatrachus didactylus,* collected from four Portuguese estuaries (Douro, Tejo, Sado and Mira). Results show that metal concentrations in Mondego water and *P. flesus* muscle were generally low, albeit some juveniles had metal loads above food safety thresholds. Multivariate statistical analysis, using canonical ordination methods (CAP), was only able to discern temporal variability of metal signatures in fish. However univariate analysis showed explicit relationships between abiotic factors (salinity and pH) and metals (e.g. Na, Cr, Mn, Sr, Cd and Hg) along the estuarine gradient. CAP analysis of muscle and liver elemental composition evidenced differences between tissues, with higher discretion capability in liver samples. Estuary elemental signatures were separated with a higher accuracy than species, which highlights environmental conditioning and underpins the potential of multi-elemental signatures to identify the estuary of origin and fish species.

Keywords: estuaries, metals, fish tissue, elemental signatures

Resumo

Os estuários estão sujeitos a várias pressões antropogénicas, entre elas, metais potencialmente tóxicos. Estes, derivam frequentemente de atividades humanas, incluindo a extração mineira, a descarga de águas residuais ou a queima de combustíveis fósseis, e possuem uma elevada persistência nos estuários afetando a saúde do ecossistema. Apesar de ocorrerem naturalmente no ambiente, os metais estão presentes em quantidades vestigiais, geralmente com pouco ou nenhum efeito tóxico. Alguns são essenciais à vida, como os micronutrientes (Cu, Zn, Fe, Mn, Co, Mo, Cr e Se) e os macronutrientes (Ca, Mg, Na, P e S), que regulam funções importantes do corpo por meio de reações de redução e oxidação. Contudo, em concentrações elevadas, todos os metais são tóxicos, especialmente porque se bioacumulam em organismos, potencialmente perturbando o crescimento, o metabolismo ou a reprodução de toda a cadeia trófica, incluindo os seres humanos. A poluição de estuários por metais é especialmente preocupante para espécies de peixes devido às suas relações complexas com os ambientes aquáticos de transição e o seu valor socioeconómico para os seres humanos. Ao longo de todo o seu ciclo de vida, muitas espécies de peixes utilizam estuários para fins ontogenéticos, de forma facultativa ou oportunista.

O trabalho atual divide-se em dois capítulos: o capítulo 2 aborda variações temporais de elementos no músculo de juvenis *Platichthys flesus* e espaço temporais na composição elementar das águas recolhidas ao longo de todo o gradiente do estuário do Mondego; e o Capítulo 3, explora uma abordagem multi-taxa e -estuários ao estudo da distribuição de metais em tecidos moles, e em águas, para determinar a variabilidade espacial de elementos químicos em estuários, e adicionalmente se assinaturas elementares têm a capacidade de distinguir com precisão o estuário de origem e diferenças inter-espécies de peixes. A técnica escolhida para medir as concentrações multi-elementares em amostras, foi a espectroscopia de fluorescência total de raios X. Em vários estudos foi estabelecido que esta técnica é altamente eficiente na leitura elementar simultânea e menos trabalhosa em comparação com métodos convencionais.

No capítulo 2, o estuário de Mondego é um sistema bem misturado, com muito poucas pressões antropogénicas, e a espécie de peixe que foi selecionada, estritamente em fases juvenis, a solha europeia (*Platichthys flesus*) é um organismo marinho migrador. Ao considerar uma única espécie de peixe e limitando os indivíduos à mesma idade, pretendemos ter em conta variações na composição dos tecidos metálicos induzidas por diferenças fisiológicas ou metabólicas, e

assegurar que os padrões temporais de concentrações de metais estão fortemente ligados ao ambiente. Além disso, tendo em conta que *P. flesus* é uma espécie marinha migradora, que utiliza o estuário durante a sua fase juvenil e que passa a fase adulta no mar, presume-se que todos os indivíduos têm aproximadamente o mesmo nível de exposição ambiental.

Em relação ao capítulo 3, foram considerados quatro sistemas estuarinos: Tejo é o maior estuário de Portugal (320 km^2), onde se encontram grandes atividades portuárias e de navegação, várias indústrias e escoamento urbano e agrícola, e a sua bacia hidrográfica é uma das zonas mais densamente povoadas do país (região de Lisboa); Sado é o segundo maior estuário (212,4 km^2), seguido do Douro (7,3 km^2), e ambos estão sujeitos a importantes atividades antropogénicas (atividades portuárias e de navegação, urbanização, agricultura), que podem influenciar a abundância e a estrutura comunitária dos peixes; o estuário do Mira é o sistema incluído mais pequeno (4,7 km^2) e é geralmente considerado sob baixa pressão antropogénica, embora tenha sido caracterizado como altamente vulnerável. As espécies-alvo desses estuários incluem: sete espécies marinhas migratórias, *Diplodus vulgaris*, *Diplodus bellottii*, *Diplodus sargus*, *Platichthys flesus*, *Solea senegalensis*, *Solea solea* e *Dicentrarchus labrax*; uma espécie marinha oportunista, *Sparus aurata* e um residente estuarino, *Halobatrachus didactylus*. Os tecidos analisados nas várias espécies foram músculo e fígado. Estes são os órgãos mais estudados na literatura sobre acumulação de metais, e apresentam diferentes oportunidades de relacionar contaminação ambiental com a carga de metais em peixes. O músculo é a principal parte comestível do peixe, o que o torna uma possível ameaça para a saúde humana, e o fígado é o principal local de acumulação responsável por importantes processos de transformação, armazenamento e distribuição de poluentes no organismo.

O objetivo deste estudo é medir e examinar a distribuição de metais em dois tecidos moles metabolicamente diferentes e em água, o que inerentemente avalia a qualidade dos sistemas costeiros em questão, e clarifica a influência de alguns fatores importantes na distribuição de metais no ambiente e na bioacumulação, como diferenças fisiológicas, a dinâmica do estuário e estratégias de vida. Além disso, tentamos destacar a eficácia das assinaturas elementares em tecidos moles na determinação da origem de espécies.

Os resultados mostram que as concentrações de metais na água do Mondego e no músculo de *P. flesus* eram geralmente baixas, embora alguns juvenis tivessem níveis de mercúrio acima dos

limites de segurança alimentar. A análise estatística multivariada, usando métodos de ordenação canónica (CAP), só foi capaz de discernir a variabilidade temporal das assinaturas metálicas em peixes. No entanto, a análise univariada mostrou relações explícitas entre fatores abióticos (salinidade e pH) e metais (por exemplo, Na, Cr, Mn, Sr, Cd e Hg) ao longo do gradiente estuarino. A análise CAP da composição elementar do músculo e do fígado no estudo multi-taxa evidenciou diferenças entre os dois tecidos, e as assinaturas elementares do fígado aparentaram ter uma maior capacidade de discriminação. As amostras de peixes de diferentes estuários foram separadas com uma precisão maior do que as de diferentes espécies, o que destaca o condicionamento ambiental da composição elementar de tecidos e reforça o potencial das assinaturas multi-elementares para identificar o estuário de origem e as espécies de peixes. A análise CAP da água indicou alguma semelhança entre a composição química da água dos vários estuários. Entre os sistemas analisados Mira e Douro apresentaram teores significativamente mais elevados dos metais perigosos Cu e Pb, e Hg e Cd respetivamente. As amostras de água de todos os estuários geralmente ultrapassam o limite de Hg imposto em águas superficiais pela União Europeia. De acordo com os rácios dos metais no músculo, calculados com base nas diretrizes de segurança alimentar, os peixes do Sado e do Tejo foram os mais poluídos por metais e as espécies *D. labrax*, *D. bellottii*, *H. didactylus* e *S. solea* foram as mais afetadas. Em geral, a análise elementar dos componentes abióticos e bióticos dos estuários fornecem informações cruciais sobre o estado do ecossistema, evitando a deterioração da saúde de ambos a biota e dos humanos. Além disso, as assinaturas elementares no fígado dos peixes e no tecido muscular podem vir a ser ferramentas eficientes para determinar a proveniência dos peixes.

Palavras-chave: estuários, metais, composição multi-elementar, peixe, músculo, fígado

Table of Contents

Declaração de autoria de trabalho (Statement of Authorship) ii
Acknowledgements iii
Abstract iv
Resumo v
Table of Contents viii
Index of Figures ix
Index of Tables xii
List of Abbreviations xiii
Chapter 1 1
1.1 General Introduction 1
1.2 References 8
Chapter 2: Spatial and Seasonal variations in trace metal composition of water and European Flounder (*Platichthys flesus*) tissues from a temperate estuary (Mondego). 15
2.1 Abstract 15
2.2 Introduction 16
2.3 Materials and Methods 19
2.4 Results 23
2.5 Discussion 31
2.6 References 38
Chapter 3: Multi elemental signatures in soft tissue of several fish species from Portuguese estuaries 49
3.1 Abstract 49
3.2 Introduction 50
3.3 Materials and Methods 53
3.4 Results 56
3.5 Discussion 72
3.6 References 78

Index of Figures

Figure 2.1 Map showing sampling sites within the Portuguese estuary, Mondego. Water sampling was conducted in sites A (Lower estuary), B (Middle estuary), C and D (both Upper estuary) and fish were gathered from sites C and D. 16

Figure 2.2 Diagram of the Canonical Analysis of Principal coordinates (CAP), showing the distribution of chemical elements found in water considering time and location. Locations: A, B, C and D. Time: Spring, Summer, Autumn and Winter. Lines represent individual metal contribution to the distribution, aiming to their direction of influence and with lengths proportional to their relative importance for distribution. 20

Figure 2.3 Diagram of the Canonical Analysis of Principal coordinates (CAP), showing the distribution of chemical elements found in fish from different seasons. Time: Spring, Summer, Autumn and Winter. Lines represent individual metal contribution to the distribution, aiming to their direction of influence and with lengths proportional to their relative importance for distribution. 24

Figure 2.4 Ratios of toxic metals in fish based on European guidelines established for food safety displayed. 26

Figure 3.1- Map of Portugal showing the location of each studied estuary (Douro, Tejo, Sado and Mira estuaries). 49

Figure 3.2 Canonical Analysis of Principal coordinates (CAP), showing the distribution of chemical elements found in water from different estuaries. Sites:Sado, Tejo, Douro and Mira. 53

Figure 3.3 Canonical Analysis of Principal coordinates (CAP), showing the grouping of species and estuaries based on chemical elements found in fish muscle samples from different estuaries. Sites: Sado, Tejo, Douro and Mira, including the species: *Diplodus vulgaris*, *D. bellottii*, *D. sargus*, *Platichthys flesus*, *Solea senegalensis*, *S. solea*, *Dicentrarchus labrax*, *Sparus aurata* and *Halobatrachus didactylus* *54*

Figure3.4 Canonical Analysis of Principal coordinates (CAP), showing the grouping of *Dicentrarchus labrax* from different estuaries based on chemical elements found in fish muscle samples. Sites: Tejo, Douro and Mira. 54

Figure 3.5 Canonical Analysis of Principal coordinates (CAP), showing the grouping of *Halobatrachus didactylus* from different estuaries based on chemical elements found in fish muscle samples. Sites:, Tejo, Sado and Mira. 55

Figure 3.6 Canonical Analysis of Principal coordinates (CAP), showing the muscle grouping of the species: *Solea senegalensis, Solea. solea, Dicentrarchus labrax, Sparus aurata* and *Halobatrachus didactylus* from Tejo based on chemical elements found in fish muscle samples..... 55

Figure 3.7 Canonical Analysis of Principal coordinates (CAP), showing the muscle grouping of the species: *Solea senegalensis, Diplodus vulgaris, Dicentrarchus labrax* and *Halobatrachus D. sargus, didactylus* from Mira, based on chemical elements found in fish muscle samples. 55

Figure 3.8, Boxplots depicting metal ratios in muscle of all species analyzed, calculated using European Union food safety standards from the COMMISSION REGULATION (EC) No 1881/2006. Dotted line indicates a ratio of 1. Estuaries: Douro, Mira, Sado and Tejo. Species: *D. labrax* (DL), *P.flesus* (PF), *D. sargus* (DS), *D. vulgaris* (DV), *H. didactylus* (HD), *S. aurata* (SA), *S. senegalensis* (SSe), *D. belotti* (DB), *S. solea* (SSo)..... 59

Figure 3.9 Boxplots depicting metal ratios in muscle of fish from all estuaries analyzed, calculated using European Union food safety standards from the COMMISSION REGULATION (EC) No 1881/2006 (European Commission, 2006b). Dotted line indicates a ratio of 1. Estuaries: Douro, Mira, Sado and Tejo. Species: *D. labrax* (DL), *P.flesus* (PF), *D. sargus* (DS), *D. vulgaris* (DV), *H. didactylus* (HD), *S. aurata* (SA), *S. senegalensis* (SSe), *D. belotti* (DB), *S. solea* (SSo). 60

Figure 3.10 Boxplots depicting metal ratios in muscle of fish from all estuaries analyzed, calculated using guidelines from the United Kingdom Ministry of Agriculture, Fisheries and Food (MAFF, 1995). Dotted line indicates a ratio of 1. Estuaries: Douro, Mira, Sado and Tejo. Species: *D. labrax* (DL), *P.flesus* (PF), *D. sargus* (DS), *D. vulgaris* (DV), *H. didactylus* (HD), *S. aurata* (SA), *S. senegalensis* (SSe), *D. belotti* (DB), *S. solea* (SSo)..... 61

Figure 3.11, Boxplots depicting metal ratios in muscle of all fish species analyzed, calculated using guidelines from the United Kingdom Ministry of Agriculture, Fisheries and Food (MAFF, 1995). Dotted line indicates a ratio of 1. Estuaries: Douro, Mira, Sado and Tejo. Species: *D. labrax* (DL), *P.flesus* (PF), *D. sargus* (DS), *D. vulgaris* (DV), *H. didactylus* (HD), *S. aurata* (SA), *S. senegalensis* (SSe), *D. belotti* (DB), *S. solea* (SSo)..... 61

Figure 3.12 Canonical Analysis of Principal coordinates (CAP), showing the grouping of species and estuaries based on chemical elements found in fish liver samples from different estuaries. Sites: Sado, Tejo, Douro and Mira, including the species: *Diplodus vulgaris, D. bellottii, D. sargus, Platichthys flesus, Solea senegalensis, S. solea, Dicentrarchus labrax, Sparus aurata* and *Halobatrachus didactylus* 63

Figure 3.13 Canonical Analysis of Principal coordinates (CAP), showing the grouping of *Halobatrachus didactylus* from different estuaries based on chemical elements found in fish liver samples. Sites:, Tejo, Sado and Mira. 63

Figure 3.14 Canonical Analysis of Principal coordinates (CAP), showing the grouping of *Dicentrarchus labrax* from different estuaries based on chemical elements found in fish liver samples. Sites: Tejo, Douro and Mira. 63

Figure3.15 Canonical Analysis of Principal coordinates (CAP), showing the muscle grouping of the species: *Solea senegalensis, Diplodus vulgaris , Dicentrarchus labrax* and *Halobatrachus didactylus and D. sargus* from Mira, based on chemical elements found in fish liver samples64

Figure 3.16- Canonical Analysis of Principal coordinates (CAP), showing the muscle grouping of the species *Solea. solea, Dicentrarchus labrax,* and *Halobatrachus didactylus* from Tejo based on chemical elements found in fish liver samples 64

Index of Tables

Table 2.1 Means and standard errors (in brackets) of the abiotic factors: Temperature, Salinity, pH and Dissolved Oxygen. Measured in all sampling sites across the estuarine gradient of the Mondego. 19

Table 2.2 Means and standard errors (in brackets) of trace metals concentrations detected in water (μgL^{-1} in dry weight). 21

Table 2.2 Continuation Means and standard errors (in brackets) of trace metals concentrations detected in water (μgL^{-1}). 22

Table 2.3 Length. Weight. Relative condition factor (Kn) and Metal pollution Index means of fish in each studied season (standard errors in brackets). 23

Table 2.4 Means and standard errors (in brackets) of trace metals concentrations detected in fish ($\mu g\ g^{-1}$ in dry weight). 25

Table 3.1 Mean concentrations of trace elements (µg g–1 dry weight) and standard errors (in brackets) in waters collected in the Portuguese estuaries: Douro, Mira, Sado and Tejo. 52

Table 3.2 Mean concentrations of muscle trace elements (µg g–1 dry weight) and standard errors (in brackets) in nine fish species collected in Portuguese estuaries. Estuaries: Douro, Mira, Sado and Tejo. Species: *D. labrax* (DL), *P.flesus* (PF), *D. sargus* (DS), *D. vulgaris* (DV), *H. didactylus* (HD), *S. aurata* (SA), *S. senegalensis* (SSe), *D. belotti* (DB), *S. solea* (SSo). 57

Table 3.3 Total Length and Weight means and standard errors (in brackets) of the individuals from each species and location sampled. Estuaries: Douro, Mira, Sado and Tejo. Species: *D. labrax* (DL), *P.flesus* (PF), *D. sargus* (DS), *D. vulgaris* (DV), *H. didactylus* (HD), *S. aurata* (SA), *S. senegalensis* (SSe), *D. belotti* (DB), *S. solea* (SSo). 58

Table 3.4 Mean concentrations of liver trace elements (µg g–1 dry weight) and standard errors (in brackets) in nine fish species collected in Portuguese estuaries. Estuaries: Douro, Mira, Sado and Tejo. Species: *D. labrax* (DL), *P.flesus* (PF), *D. sargus* (DS), *D. vulgaris* (DV), *H. didactylus* (HD), *S. aurata* (SA), *S. senegalensis* (SSe), *D. belotti* (DB), *S. solea* (SSo). 66-67

List of Abbreviations

CAP Canonical Analysis of Principal coordinates

TXRF Total reflection X-ray fluorescence analysis

WFD Water Framework Directive

Chapter 1

1.1 General Introduction

Estuaries constitute transitional bodies of water between rivers and the sea, where tides meet river flow and cause the mix of saltwater with freshwater (Hobbie, 2000). This junction of distinct water masses creates several different habitats that provide conditions for the development and survival of multiple organisms, providing valuable functions such as migration routes, feeding grounds, spawning areas and refuge to a high diversity of species (Elliot *et al*., 2007b ; Barbier *et al.* 2011). As such, estuaries are considered as one of the most productive, biodiverse, and valuable ecosystems in the world (Costanza *et al.* 1997). However, mankind is also a great beneficiary of estuarine services (e.g. fisheries, drinking water supply and climate regulation) and increasingly exploits coastal resources at rates proportionate to the needs of a continuously rising global population (Barbier *et al.* 2011). The incessant anthropogenic activity in coastal landscapes has led to the severe chemical contamination of the surrounding water, and currently a decrease in the capacity of estuaries to support human and aquatic health is being manifested worldwide (Gibson *et al*., 2007).

Amongst the wide range of concerning pollutants affecting estuaries, trace metals have gained notoriety for their persistence in the ecosystem and their toxicity. As inorganic chemicals, metals are not easily metabolized into less harmful compounds, thereby they persist in the ecosystem, easily accumulate in biota, and bio-magnify along the trophic chain (Förstner & Wittmann, 2012). In coastal systems, sources of these chemicals include urban effluents, industrial wastewater, river discharges, land runoff, atmospheric deposition and groundwater enrichment. Despite naturally occurring in the environment, metals are present in trace amounts, generally with low to no toxic effects (Mason, 2013). Notwithstanding, they are essential to life, such as the micronutrients (Cu, Zn, Fe, Mn, Co, Mo, Cr, and Se) and the macronutrients (Ca, Mg, Na, P, and S), which regulate important body functions through oxidation-reduction reactions (Bury *et al.*, 2003; Heinrichs *et al.*, 2020). Nevertheless, at high concentrations all metals are toxic, especially since they bioaccumulate, potentially disturbing the growth, metabolism or reproduction of the entire trophic chain, including humans (Livingstone, 2003; Fonseca *et al*., 2009; Driessnack *et al*., 2017).

Moreover, non-essential metals such as mercury, lead, and cadmium, can cause deficiency of essential elements through competition for active sites of molecules (Walker *et al.*, 2006). Considering the rapid industrialization and urbanization in coastal areas, pollution will not subside, and highly toxic species of metals will continue to be highly prevalent in estuaries and burden ecologically important species (Nriagu, 1996; Bradl, 2005; Hu *et al*., 2014).

Metal behaviour and fate are a subject difficult to address in dynamic transitional waters (Elliott & Quintino, 2007a). Contrasting with rivers, seas and lakes, the strong physicochemical gradients (e.g. oxygen, turbidity and salinity) and complex hydrogeomorphology of estuaries (e.g. sediment type, river discharge and tidal cycles), introduces a unique high degree of variability in metal cycling. Estuarine circulation, river and groundwater discharge, tidal flooding and the salinity gradient, are some of the distinct features in estuaries that affect metal partitioning differently than in other aquatic environments (de Souza Machado *et al*., 2016). Once introduced in the estuary, metals are transported and distributed through environmental compartments such as water, sediment and biota, where they may be transformed into other chemicals, and accumulate (McComb *et al*., 2014). In water, metals tend to display conservative and non-conservative behaviours. Conservative behaviour is observed when metal concentrations decrease towards the sea while non-conservative implies the opposite (Achterberg *et al*., 2003; Wang *et al*., 2009). Dissolved and suspended particulate metals in the water column are the main path of contamination to organisms (Tessier & Turner, 1995; Yamazaki *et al*., 1996). Yet, due to particle-solute interactions, flocculation and sedimentation processes, which are mediated by the physical and chemical characteristics of the environment, the majority of metals in estuaries are stored in the sediment, while only a small portion of metals actually remains in the water (de Souza Machado *et al*., 2016). Under the appropriate abiotic conditions such as high salinity, redox conditions and low pH, metals can be removed from the sediment through desorption and remobilization processes, becoming readily bioavailable to the food web once again, thereby providing the biggest reservoir of metals in the ecosystem to aquatic life (Atkinson *et al*., 2007; Kalnejais *et al*., 2007; Zhang *et al*., 2014).

Estuarine metal pollution is especially troubling for fish species because of the complex connection they have with transitional systems and their socioeconomic value to humans. Throughout their life cycles, fish species use estuaries to full-fill ontogenetic purposes in either facultative or opportunistic ways (Elliot *et al*., 2007b). For instance, anadromous and catadromous

species occupy estuaries transitionally to reach their spawning grounds, situated in rivers and at sea respectively, while estuarine species use them to complete their entire or post-larval development. As for marine/freshwater stragglers and marine migrant species, they temporarily take advantage of these productive coastal areas, notably to ensure the successful recruitment of juveniles to adult stocks offshore (Franco *et al.*, 2008; Potter *et al.*, 2015). Essentially, if the estuarine quality is compromised, the replenishment of fish populations might be severely affected both in coastal and in marine waters, inevitably upsetting the ecological integrity of all associated ecosystems. Recognizing the impact of metals on population dynamics in such productive and complex habitats is key for the effectiveness of conservation efforts and the sustainability of human estuarine resource exploitation (Vasconcelos *et al.*, 2011; Ali *et al*., 2019).

Over the last few decades, the anthropogenic degradation of ecosystems highlighted the necessity of using living organisms and their responses to stressors as indicators (i.e. bioindicators) of environmental integrity (Parmar *et al.*, 2016; Oertel & Salánki, 2003). The reasoning behind this paradigm lies in the ability of biota to reflect the contamination of the environment by incorporating pollutants in body compartments and changing their biochemistry as well as their behaviour (Sabullah *et al.*, 2015). Bioindicators offer the possibility of understanding, at multiple spatial and temporal scales, the interactions of biological communities with the environment and its harmful components (Poikane *et al.*, 2011). A suitable bioindicator usually entails organisms with a wide distribution and abundance in the ecosystem, but also with long lifespans and easy to sample (Parmar *et al.*, 2016). The use of these tools overcomes the limited biological and ecological significance of traditional approaches, which mainly consisted of physicochemical analysis of water. However, chemical approaches are not considered obsolete, nor less effective for monitoring purposes than bioindicators. In fact, they enable a more specific account on availability and distribution of pollutants in an ecosystem by directly measuring concentrations from an environmental compartment, such as water or sediment (Kotamäki *et al*., 2019; Kumar *et al*., 2019). Therefore, rather than focusing on only one approach, legislations such as the European Water Framework Directive and Habitats Directive as well as the US Clean Water Act have been promoting the development of bioindicators and their integration with physical and chemical investigations to enhance sustainable water use and the good quality of aquatic ecosystems (Hering *et al*., 2010). When both approaches are incorporated in the same monitoring program, a

comprehensive view of aquatic ecosystems is achieved, which may prompt the swift enactment of robust conservation and management strategies. This approach is particularly beneficial to address metal behaviour and fate in dynamic transitional waters, due to the complexity of biogeochemical processes controlling their temporal and spatial patterns of distribution (Borja *et al*., 2008).

Even though any organism can potentially be an indicator, fish has been a particularly prolific monitoring subject in metal research, but there are still gaps in knowledge (Souza *et al.*, 2020). Although, fish continuously uptake trace elements directly from water or sediment, via three main routes (ingestion, skin and gills), accumulation patterns in tissue have been shown to significantly vary with several characteristics of individuals (e.g. length, diet, species-specific behavior and physiology), as opposed to just proportionately reflecting environmental loads (Jezierska & Witeska, 2006). Upon entering the body, depending on their role in biological processes, chemical compounds will either be efficiently regulated and innocuously stored by organisms in cells and tissue, or trigger detoxification responses that may lead to their persistent accumulation and hazardous effects (Olsson *et al.*, 1998). Therefore, interpreting the quantification of contaminants in these species requires thoughtful experimental questions (Souza *et al*., 2013).

Fish is one of the most consumed and traded commodities worldwide. According to the yearly report on global fisheries and aquaculture from the Food and Agriculture Organization of the United Nations (FAO, 2020), fish consumption has been steadily increasing from 1961 to 2017 at a mean annual rate of 2.4%, which is almost two-fold the annual world population growth rate (16 percent) (FAO, 2020). However, the food sector has had a reasonable number of shortcomings in the monitorization and control of practices that culminated in extreme food poisoning situations (Ekino *et al.*, 2007; Leal *et al.*, 2015). In the best interest of public safety, regulatory bodies have directed their attention to setting safety standards for numerous priority pollutants in fish such as metals, mycotoxins, dioxins and PCBs. Therefore, prevention and mitigation of estuarine metal contamination demands an efficient monitoring of the ecosystem status and an accurate assessment of risks to fauna, flora and humans. In this context, advancements in environmental monitoring research have been instrumental in addressing modern ecologic goals, instituted for the betterment of human welfare and the preservation of aquatic ecosystems (Birk *et al.*, 2012). Moreover, worldwide commercialization of seafood and complex supply chains have increased the demand to pinpoint the origin and trace harvested or produced seafood (McClenachan et al 2016). Allied to growing recognition of how malpractices undermine sustainable fisheries, conservation efforts,

and human health, recent research into tools that promise advanced provenance assessments are crucial (Helyar *et al.*, 2014). Among these are chemical or biochemical natural tags, such as multi-elemental signatures, since all individuals are naturally marked by environmental, dietary or other intrinsic constraints that are essentially tamper-proof (e.g. Tanner *et al.*, 2018; Gopi *et al.*, 2019).

In this study, multi-elemental concentrations of metals in fish tissues and in water from major Portuguese estuaries were determined, and important temporal and spatial patterns of these trace elements were investigated. Portugal's long-standing relationship with the sea and Europe's commitment to the WFD directive have regularly influenced the amount of research conducted in Portuguese coastal systems. For ecotoxicological purposes, most of the analysis of elemental composition in fish and water have relied on Inductively Coupled Plasma techniques: Inductively coupled plasma-optical emission spectroscopy (ICP-OES) and ICP-mass spectrometry (ICP-MS) are the most frequently used techniques for multi-element analysis (Polak-Juszczak, 2010; Laursen *et al*., 2014). However, new technological advances have been providing cost-effective alternatives to those methods with high levels of accuracy and more simplistic methodologies (e.g. sample preparation and calibration). Therefore, in the present work we utilized Total reflection X-ray fluorescence analysis (TXRF), a versatile analytical method established in several different fields (e.g. medicine, forensics, toxicology and biomonitoring), yet still relatively overlooked due to the historic reliability on atomic absorption spectrophotometry techniques (Fernández-Ruiz, 2014; Klockenkämper & Von Bohlen, 2014; Pashkova *et al.*, 2015). A variety of studies have appraised TXRF in both water and fish elemental analysis, for its simplicity, accuracy, precision, and fast simultaneous elemental detection (Zarazúa *et al.*, 2014; Beltrán *et al.*, 2019; Vázquez *et al.*, 2020).

The work is divided in two chapters: Chapter 2, which addresses the temporal variability of trace metal signatures in one species from one estuary, and the temporal and spatial variation in waters collected throughout the entire estuarine gradient; and Chapter 3, which explores spatial differences in elemental composition of various fish species and waters from several estuaries and how the signatures change among species from those locations. The estuary selected for Chapter 2 was the Mondego estuary, a well-mixed system with very few anthropogenic pressures. The fish species selected was a marine migrant, the European flounder [*Platichthys flesus* (Linnaeus, 1758)], strictly in juvenile phases, from which muscle tissue was analysed for trace metal concentrations. Considering one fish species and limiting individuals to the same age, we aimed to account for variations in tissue metal composition induced by physiological or

metabolic differences and ensure that temporal metal patterns are strongly connected to the environment. In addition, taking into account that *P. flesus* is a marine migrant species that uses the estuary during its juvenile life stage and then spends his adulthood at sea, all individuals are assumed to have approximately the same level of exposure to environmental metals (Martinho et al., 2008; Skerritt, 2010).

In Chapter 3, four estuarine systems were considered, Douro, Tejo, Sado and Mira, as they support various fish species, including serving as important nursery grounds for several commercial fish species (Vasconcelos *et al.*, 2010), and possess contrasting hydro-morphological characteristics and anthropogenic pressures, covering a significant range of important variables in exploring environmental chemical patterns. Tejo is the largest estuary in Portugal (320 km^2), harboring large port and navigation activities, multiple industries and agriculture runoff, and its watershed is one of the most densely populated areas in the country (Lisbon region) (Vansconcelos *et al.*, 2007). Sado is the second largest estuary (212.4 km^2), followed by Douro (7.3 km^2), and both are also subjected to considerable anthropogenic activities (port and navigation activities, urbanization, agriculture), all of which can influence fish abundance and community structure (e.g. Fonseca *et al.*, 2013). The Mira estuary is the smallest system considered (4.7 km^2) and is generally considered to be under low anthropogenic pressure, albeit it has been characterized as highly vulnerable (e.g. Vasconcelos *et al.*, 2007).

Specific research on metal contamination in these coastal systems has shown that water and sediment metal contamination is high in the Tejo estuary, and moderate to low in the moderately pressured Douro (Mucha *et al.*, 2005; Ribeiro *et al.*, 2018; Iglesias *et al.*, 2020). Water surveys for the remaining estuaries, Sado and Mira are very limited, but sediment studies indicate that contamination is moderate in the sizeable, highly pressured Sado (Duarte *et al.*, 2014) and moderate to low in the small, relatively undisturbed Mira (Vasconcelos *et al.*, 2007; Chainho *et al.*, 2008; Serafim *et al.*, 2013). The fish species sampled from those estuaries encompassed various ecological guilds: seven marine migrant species, the common two banded seabream [*Diplodus vulgaris* (E. Geoffroy Saint-Hilaire, 1817)], the common sole [*Solea solea* (Linnaeus, 1758)], the senegalese sole [*Solea senegalensis* (Kaup, 1858)], the senegal seabream [*Diplodus bellottii* (Steindachner, 1882)], the white seabream [*Diplodus sargus* (Linnaeus, 1758)], the European flounder [*Platichthys flesus* (Linnaeus,1758)], and the European seabass [*Dicentrarchus labrax*); a marine straggler, the gilt-head seabream [*Sparus aurata* (Linnaeus, 1758)], and an

estuarine resident, the Lusitanian toadfish [*Halobatrachus didactylus* (Bloch & Schneider, 1801)] (Franco *et al.*, 2008; França *et al*., 2009). The two tissues analysed in the various species were muscle and liver. They are the most studied organs in metal accumulation research and present different opportunities to relate environmental contamination and fish metal burden (Sabullah *et al.*, 2015). Muscle is the main edible part of fish, making it a threat to human health, and the liver is the main site of accumulation, responsible for important transformation, storage, and distribution processes in the body (Canli & Atli, 2003; Wei et al., 2014). Accordingly, the aim of this study was to gain a deep understanding of metal concentration patterns in two metabolically distinct tissues from multiple taxa and water extracted from differently impacted locations, through time and space in some of the most important species and estuaries in Portugal. Measuring and examining metal distribution in abiotic and biotic components will inherently assess the quality of the coastal systems in question, and clarify the influence of some important factors in environmental metal partitioning and bioaccumulation, such as physiology differences, estuarine dynamics and life strategies. It will also underpin the variability of elemental fingerprints towards determination of species origin.

Disclaimer

Under the constraining conditions resultant from the COVID pandemic of 2020, it was not possible to gather and analyse the optimal data to achieve the purpose of the third chapter. During this trying period, laboratories were restricted for an extended time, delaying the progress of the thesis, particularly the biochemical and statistical analysis, results and discussion of the third chapter.

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Chapter 2: Spatial and Seasonal variations in trace metal composition of water and European Flounder (*Platichthys flesus*) tissues from a temperate estuary (Mondego).

2.1 Abstract

Trace metals are potentially toxic and ubiquitous elements, with a particularly high degree of persistence in estuaries. Consequently, they are often expressed in the chemical composition of several environmental components, potentially providing discernable elemental signals in space and time, that greatly benefit the conservation and management of ecosystems. In this study, we examine spatiotemporal trace metal concentrations between Autumn 2016 and Spring 2018, in several samples of water and *P. flesus* muscle along the Mondego estuary, using Total Reflection X-ray Fluorescence analysis. Trace metal values measured in water and fish were uniformly low and depicted the Mondego estuary as a system of reduced environmental pollution, with the exception of mercury and lead. Mercury surprisingly surpassed the values recommended in water quality and food safety guidelines whilst lead excess was noticeable in fish. Water multi-elemental signatures were not able to discriminate between seasons and sites in the well-mixed mesotidal system. However, individual metal analysis managed to highlight important spatial patterns correlated with salinity and ph. Season-specific elemental trends were uniquely determined for European flounder muscle. Tissue elemental composition data was discriminated using constrained ordination (CAP), which correctly classified a large percentage of individuals, to their respective season (76.786%). Elements were distinctly present in higher concentrations during Spring (dry season) and in lower values during Winter (wet season). Although, temporal metal patterns were most likely ascribed to variations of riverine input, size also contributed to variability with noticeable effects on Spring. Length and weight were generally negatively correlated to metal concentrations in muscle, and despite the similar proportions of all selected fish, smaller individuals were significantly observed in Spring. In conclusion, multi-elemental signatures of crucial ecological components can ultimately provide promising baselines to detect environmental

degradation in complex ecosystems and establish valuable patterns required to develop provenance studies and ensure food safety.

Keywords: estuaries, metals, juveniles, muscle, elemental composition

2.2 Introduction

Estuaries are defined as transitional areas between land and sea, commonly comprising different interconnected habitats (e.g. seagrass meadows, mudflats, mangroves) (McLusky & Elliott, 2004; Fang *et al* 2018). These are unique and highly productive aquatic ecosystems, supporting an abundant and diverse community of marine, riverine and estuarine species, which depend on the environment's ecological balance to prosper (Cabral *et al.*, 2007; Sheaves *et al.*, 2015). Many fish species that use estuaries have notably complex life cycles, and, at some point, benefit from nourishment, low predation pressure, and optimal temperature ranges commonly encountered in these dynamic ecotones (Miller, 1985; Whitfield, 2017). This is particularly important for marine migrant fish species that use estuarine areas as nursery grounds during early-life stages, prompting successful recruitment to adult coastal stocks (Beck *et al.*, 2001; Vasconcelos *et al.* 2008). However, on account of the prominent socio-economic role estuaries have in human society, many of these coastal ecosystems are unfit to sustain living organisms at full capacity (Martinho *et al.*, 2012; Islam & Tanaka, 2004).

Humans exploit complex and immensely valuable ecosystem services like water purification, fisheries, climate regulation, coastal protection, nutrient cycling and waste treatment, to supply their densely populated estuary contiguous areas around the world (Martínez *et al.*, 2007; Waltham & Connolly, 2011). Consequently, estuarine systems have attracted numerous anthropogenic activities and are currently exhibiting symptoms from a wide array of chemical contaminants (Islam & Tanaka 2004; Sun *et al.*, 2012). In this context, metals are important legacy pollutants (Förstner & Wittmann, 2012). Driven by rapid population growth and economic development, metals have been continuously introduced into the marine environment at concentrations deemed to have long-lasting effects on fauna and flora (Durrieu *et al.*, 2005; Duarte *et al.*, 2010; Couto *et al.*, 2013). Anthropogenic sources including mining, fossil fuel burning, sewage discharges, agricultural runoff and electronic waste progressively surpassed natural fluxes and insured the

overbearing presence of metals in the environment (Nieuwendijk *et al.*,1990; Halpern *et al.*, 2019). These ubiquitous compounds are otherwise associated with relatively sporadic and slow natural events such as volcanic eruptions, sea-salt sprays, rock weathering, geothermal systems, and biogenic emissions, resulting in manageable background levels (Bradl, 2005). Although several environmental policies have been established to improve water quality and ecosystem health (e.g Water framework directive, Water Quality Act, OSPAR Convention, Marine Strategy Framework Directive), metals have a high degree of persistence in aquatic systems (Smail *et al.*, 2012; Tueros *et al.*, 2009; Rouillard *et al.*, 2018)

Even at low levels of exposure, metals such as cadmium (Cd), mercury (Hg), and lead (Pb) can interact with biomolecules causing cell activity disruption or apoptosis, which in early life stages of an organism can affect growth and survival (Fonseca *et al.*, 2009; Mohammed, 2013), potentially compromising their ecological role in the ecosystem (Barbee *et al.*, 2014). Despite all metals being toxic above certain concentrations, toxicity thresholds depend on the physicochemical properties of the pollutant and on the physiological characteristics of an organism (Heinrichs *et al.*, 2020). For example, metals with no biological function (chromium, nickel, mercury, etc.), designated as not essential, have a tendency to induce neurological, carcinogenic and endocrine health problems at faster rates than metals with metabolic roles, and organisms will experience bioaccumulation rates of these substances according to their absorbance, excretion and detoxification capabilities (Morgano *et al.*, 2014; Cappello *et al.*, 2018). In contrast, elements such as copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg) and zinc (Zn), if maintained at sub-lethal levels, are classified as essential, and participate in several enzymatic reactions that catalyze the uptake and transformation processes of macronutrients, as well as participate in several oxide-reduction reactions that preserve cellular function (Bury *et al.*, 2003; Heinrichs *et al.*, 2020). This dichotomy of a seemingly unrelenting compound stresses the importance of preserving the fine balance between metal deficiency and surplus (Kennish, 1996).

Once a pollutant is bioavailable, it may bioaccumulate or even magnify in biota (e.g. Lillebø *et al.*, 2011; Fonseca et al 2019), which complicates the removal from the environment and jeopardizes the health of top predators like fish, marine mammals, birds and even humans (Sands & Peel, 2012). Therefore, assessing ecological and public health safety requires monitoring of metal presence in estuaries and the development of sustainable tools capable of managing water quality (Wilson, 2003). However, contamination status research has struggled to apply analytical tools,

developed in great part for freshwater and marine environments to the physicochemical mutable estuaries (Chapman & Wang, 2001; Wilson, 2003; Elliott & Quintino, 2007).

Estuaries are remarkable sinks of metal pollution (Kenish, 2002; Caeiro *et al.*, 2005; Fonseca *et al.*, 2019). In aquatic environments, metals can either be stored in sediments and accumulated in organisms, or be present in the water column, albeit in lesser quantities, as dissolved ions, organic complexes and colloids (Du Laing *et al.*, 2009; de Souza Machado *et al.*,2016). Storage and release of contaminants between estuarine environmental matrices is the product of hydrodynamic forces such as tides, river flow and wind, and intricate biogeochemical cycles susceptible to water parameters including temperature, salinity and pH (Morel & Price, 2003; Du Laing *et al.*, 2009; McComb *et al.*, 2014; Mohan & Walther, 2015). With yearly and seasonal shifts, the aforementioned physical and chemical processes suffer fluctuations that dictate new patterns of metal suspension and settling, both in the aqueous and solid phase. As a result, the partitioning of the polluting compounds in the environment and their bioavailability will vary in scale and intensity, temporally and spatially (Duarte & Caçador, 2012; Martínez-Soto *et al.*, 2016).

Metal composition in transitional waters has often been addressed using different teleost fish species. Overall, fish are well recognized environmental monitoring tools with several advantageous traits (Souza & Vianna, 2020). They are reliable bioindicators, due to their long lifespan, a wide distribution across all aquatic systems, their diversity of functional and trophic guilds as well as their value as a resource for man (Whitfield & Elliott 2002; Chovanec *et al.*, 2003; Pérez-Domínguez *et al.*, 2012; Herman & Nejadhashemi 2015). However, research conducted in coastal waters pertaining to the topic of metals in fish tissues, is especially extensive using flatfish species (Jensen & Cheng, 1987).

Flatfish are predominant components of estuarine fish assemblages that possess the necessary requirements to signalize ecological integrity (Martinho *et al.*, 2010; Fonseca *et al.*, 2015; Selleslagh *et al.*, 2016). Their abundance and distribution are significantly related to environmental parameters, using estuaries mainly as nurseries or temporary habitats (Beck *et al.*, 2001; Cabral *et al.*, 2007; Martinho *et al.*, 2008). In north east Europe, the Mondego estuary is a comprehensively documented ecosystem that harbors a large nursery-dependent flatfish community (Cabral *et al.*, 2007; Martinho *et al.*, 2007; Martinho *et al.*, 2008). Amidst the diversity of species, the European flounder (*Platichthys flesus*) is one of the most abundant commercially important fish in that

estuary and the only flatfish species in Europe able to occupy freshwater areas for long periods of time (Cabral *et al.*, 2007; Martinho *et al.*, 2007; Skerritt, 2010). It is a marine migrant fish, spawning in the sea in early Spring, and colonizing coastal waters from Spring to Summer with juvenile phases that last approximately two years (Martinho *et al.*, 2008; Skerritt, 2010). The species sensitivity to impacts of natural and anthropogenic stressors, coupled with its abundance and life history, are prime characteristics suitable to explore metal signatures in estuarine gradients (Vinagre *et al.*, 2004; Kerambrun *et al.*, 2013).

In this study, we analyzed trace element concentrations in estuarine waters and in European flounder muscle samples, two important components of metal cycling. Given so many underlying variables affecting metal partitioning, it is necessary to periodically measure both abiotic and biotic components along the environment, to gain a holistic view of estuarine metal pollution (Anderson & Cribble, 1998; Ellis *et al.*, 2015). This approach aims to gain a clear understanding of spatial and seasonal metal patterns in Mondego, a temperate mesotidal estuary considered to have a relative low level of pollution. The data obtained contributes to the ongoing monitoring efforts in the system and expands on the clarification of the level of degradation in a habitat with nursery functions. It will also provide further insight into the temporal variation of this species element composition, which would be a useful tool for future provenance studies.

2.3 Materials and Methods

2.3.1 Study area and sample collection

The Mondego estuary is located on the central western coast of Portugal (40º08'N, 8º50'W), at the lowest reaches of the Mondego River Basin (Pardal *et al.*, 2002). It is a well-mixed mesotidal estuary (tidal range 0.35-3 m) with a mean water flow of 79 $m^3 s^{-1}$, that fluctuates with yearly precipitation, reaching a maximum during rainy years (140 $m^3 s^{-1}$) and a minimum during dry years (27 $m^3 s^{-1}$) (Cunha & Dinis, 2002). Near the mouth of the estuary, the last 7 km are split into two contrasting arms (North and South), separated by the Murraceira island, each with a significant influence level on the local ecology. The hydrological features of the North arm support the Figueira da Foz harbor and define this waterway as the main navigation channel in the area.

According to a multimeric index model by Vasconcelos et al 2007, the Mondego estuary is very susceptible to effects derived from bank reclamation, loss of habitat and fishing mortality.

A total of 58 juvenile *P. flesus* specimens were captured using beam trawl tows in the upper portion of the Mondego estuary northern arm. The sampling station chosen was in accordance with the literature on nursery use of the European flounder and its geographical distribution in the area (Cabral *et al.*, 2007; Martinho *et al.*, 2007). Fish collection occurred over 3 seasons between Autumn 2016 and Summer 2017 whilst waters were collected in four seasons between Autumn 2016 and Spring 2018. Water sampling stations were selected throughout the salinity gradient of the Mondego. Efforts yielded 93 samples of surface water retrieved from four separate locations (Figure 1). To avoid impurities in the water, previously decontaminated high-density polyethylene (HDPE) plastic bottles and flasks were used to capture and store samples, respectively (Liang et al 2020). Additionally, at every station, when either water or a fish was collected, abiotic data such as temperature, salinity, pH, and dissolved oxygen were measured with a probe. Upon collection, both fish and waters were transported in refrigerated crates to the laboratory. *P. flesus* were individually weighed (g) and total length determined (cm), and a portion of muscle tissue was then collected, weighed, and placed in labelled vials to be stored at -20 ºC until further processing.

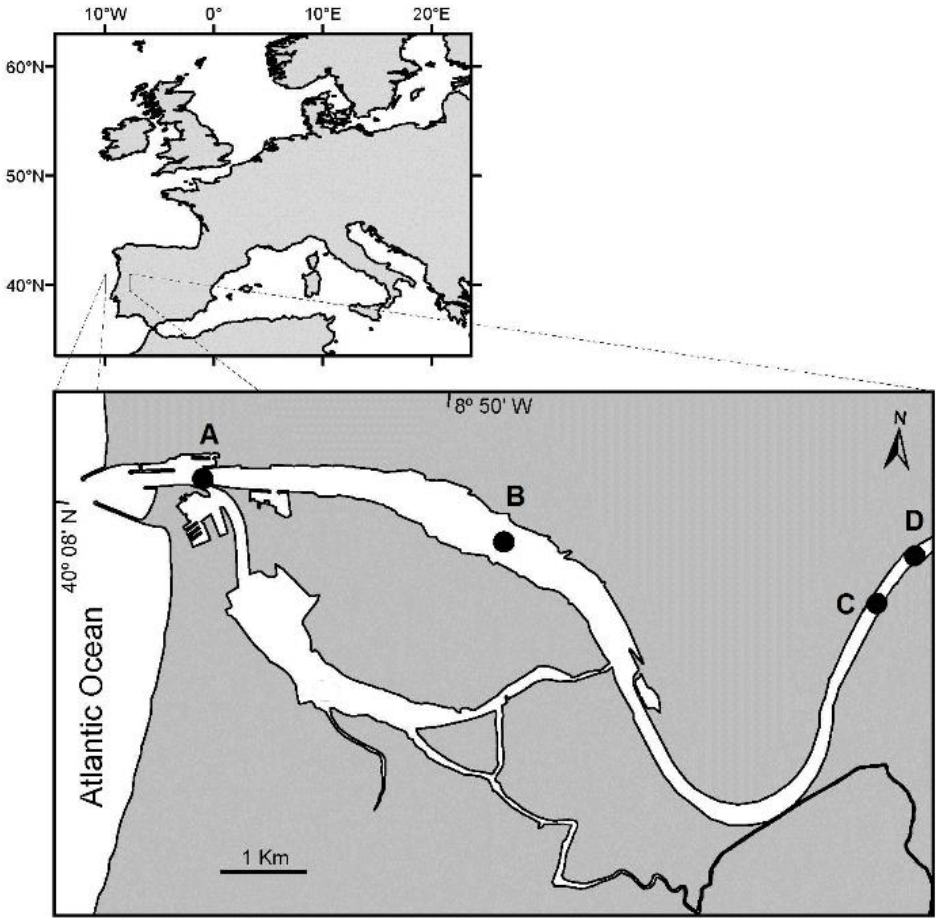

Figure 2.1 Map showing sampling sites within the Portuguese estuary, Mondego. Water sampling was conducted in sites A (Lower estuary), B (Middle estuary), C and D (both Upper estuary) and fish were gathered from sites C and D.

2.3.2 Sample preparation and elemental analysis

Sample preparation for elemental analysis, generally followed protocols from Duarte *et al.* (2014) and Caçador *et al.* (2012). All labware used for trace metal determination was previously decontaminated by soaking in 10% HCl for 24 h, and then rinsed in deionized water (Reverse Osmosis, Elga Purelab Prima) to avoid cross-contamination. Also, all chemicals used were trace metal free (concentrations below 0.001%). Briefly, water samples were first filtered with an acid clean GF/C Whatman filter and immediately acidified with HNO_3 (pH < 1). Fish muscle samples

were first freeze-dried with a Laboratory Freeze Dryer Cryodos-50, TELSTAR for 48–72 h at -50 °C, and their dry weight determined. The acid digestion procedure for liquefaction of muscle samples consisted in digesting ca. 100 mg of the sample with a 2 mL of HNO_3:$HClO_3$ (7:1) mixture during 3 h in a Teflon reactor at 110 ºC (Caçador *et al.*, 2012).

Elemental concentrations in samples were determined by Total X-ray Fluorescence spectroscopy (TXRF S2 PICOFOX, Brucker). Instrumental recalibration and analytical blanks were used for quality control. Elemental concentrations were determined in water and muscle samples by comparison with an internal standard (Gallium). The accuracy and precision of the analytical methodology for elemental determinations were assessed by replicate analysis of certified reference material TORT-2 (animals). Trace metal concentrations in the reference material determined by TXRF were consistently within the range of certified values (Student's t tests $p < 0.05$).

2.2.3 Statistical analyses

Preliminary tests on raw data were conducted to verify if normality and homogeneity assumptions were met, based on Shapiro Wilks and Levene's tests, respectively. Multivariate analysis methods were applied to normalized muscle and water chemical composition data in order to elucidate spatial and temporal chemical patterns.

Canonical Analysis of Principal coordinates (CAP) were used to obtain a visual representation of how well a priori groups (Season and Location) were discriminated. Subsequently, PERMANOVA tests were applied to validate if similarities among groups (factors) could be confirmed by an experimental design and to ascertain the significance of the CAP distribution. Pair-wise comparison tests were used to test similarities among all pairs of factors considered. All multivariate tests were done using the PERMANOVA+ package in Primer v6.

Individual factors and their potential associations with variables of interest were explored using univariate methods. Whenever data did not meet the necessary requirements for univariate parametric tests, seasonal and spatial differences were assessed with a Kruskal Wallis test (KW), followed by a Dunns pairwise test if the null hypothesis was rejected. Simple metrics were applied to address seasonal estuarine contamination by considering flounder metal concentrations as a

proxy for environmental pollution. Individuals were grouped according to the season of capture and had their relative condition factor (Kn) calculated using morphometric data to briefly evaluate seasonal fish fitness in the presence of toxic elements (Le Cren, 1951; Jisr *et al.*, 2018). The equation is shown below:

$$Kn=Wt/\ aLt^{b}$$

where Wt is total weight (g), Lt is total length (cm) and a and b represent respectively the regression coefficients, allometric growth and weight length relationship interception.

To compare total metal concentrations among flounder individuals, the Metal Pollution index was computed in accordance with Usero *et al.*, (1996). The MPI equation is the following:

$$MPI=(Cf_1Cf_2Cf_n)^{1/n}$$

where Cf is the concentration of the metal n in the sample.

Metal ratios were calculated for solely, non-essential elements by simply dividing sample concentrations by food standards stipulated in health organizations guidelines.

Additionally, correlation tests Spearman for non-parametric models, were employed to consider a relationship between the MPI and Kn, as well as to highlight other relevant interactions between abiotic components, morphologic factors and individual metals with the chemical components detected in both water and fish. All univariate tests were done using R Statistics software.

2.4 Results

2.4.1 Water metal concentrations

The abiotic factors analysed, displayed a significant spatial variation along the estuarine gradient (Table 2.1). Lower (A), mid (B) and upper (C and D) sections of the estuary were distinguished from each other by salinity (Kruskal Wallis, H=48.1, $p< 0.001$), and pH (H= 26.78, $p< 0.001$). Salinity and pH were shown to be positively correlated (Spearman, rho=0.32, p=0.005). Water parameters in B and C stations were not statistically different. Seasonality, on the other hand, was only evident in water temperature (H=47.45 $p< 0.001$) and dissolved oxygen. These two

parameters were negatively correlated (rho=0.57, p< 0.001), with lower dissolved oxygen in Summer (H=30.04, p< 0.001).

Table 2.1- Means and standard errors (in brackets) of the abiotic factors: Temperature, Salinity, pH and Dissolved Oxygen. Measured in all sampling sites across the estuarine gradient of the Mondego.

Season	Site	Temp (C°)	Salinity (‰)	pH	DO (mg/l)
Spring	A	14.3 (0.5)	35 (0.0)	8.1 (0.0)	8.4 (0.0)
	B	16 (0.0)	4.1 (0.0)	7.8 (0.0)	8.5 (0.0)
	D	18.3 (0.0)	0.0 (0.0)	7.4 (0.0)	8.9 (0.0)
Summer	A	17.7 (0.6)	35 (0.0)	7.9 (0.1)	8.2 (0.2)
	B	19.3 (1.1)	23 (1.9)	7.5 (0.2)	7.9 (0.3)
	C	21.9 (0.8)	7.3 (2.4)	7.7 (0.0)	7.5 (0.3)
	D	18.2 (0.6)	0.0 (0.0)	7.6 (0.1)	7.4 (0.3)
Autumn	A	15.9 (0.0)	32.9 (1.2)	7.9 (0.1)	9 (0.4)
	B	12.5 (0.6)	21.2 (3.6)	7.6 (0.0)	8.7 (0.2)
	C	11.1 (0.3)	4.9 (1.5)	7.7 (0.0)	9.4 (0.5)
	D	12.7 (0.0)	0.1 (0.0)	7.4 (0.0)	8.4 (0.2)
Winter	A	15 (0.0)	35 (0.0)	8.1 (0.0)	9.5 (0.0)
	B	13.7 (0.0)	1.7 (0.0)	8.1 (0.0)	9.3 (0.0)
	C	14.2 (0.2)	0.0 (0.0)	7.9 (0.1)	9 (0.2)
	D	13.8 (0.0)	0.0 (0.0)	7.4 (0.0)	8.7 (0.0)

Water elemental analysis resulted in the detection of 16 trace metals in 93 samples. Concentrations ranged between 123 and 0.0001 μgL^{-1} (Na, and As and Ni, respectively, Table 2.2). There was no significant spatial and temporal elemental water signature detected by PERMANOVA analysis. Accordingly, CAP visual representation did not discern among different sampling sites and seasons (Figure 2.2). Despite the lack of clear differences following the multivariate approach, the use of a univariate model evidenced significant spatial differences along the estuarine gradient, for a few metals. Na (Kruskal Wallis, H=18.68, p< 0.001), Cr (H=1.646, p=0.028), Mn (H=24.53, p< 0.001), Sr (H=26.15, p< 0.001), Cd (H=12.36, p=0,001) and Hg (H=24.91, p< 0.001), all had significantly different concentrations in each sampling site (Dunn, p<0.039), except between site C and D, the two most upstream sites. Higher Na and Hg concentrations were consistently present near the mouth of the estuary (A and B), whereas Mn and Cd concentrations were higher in the site with the highest riverine influence (site D).

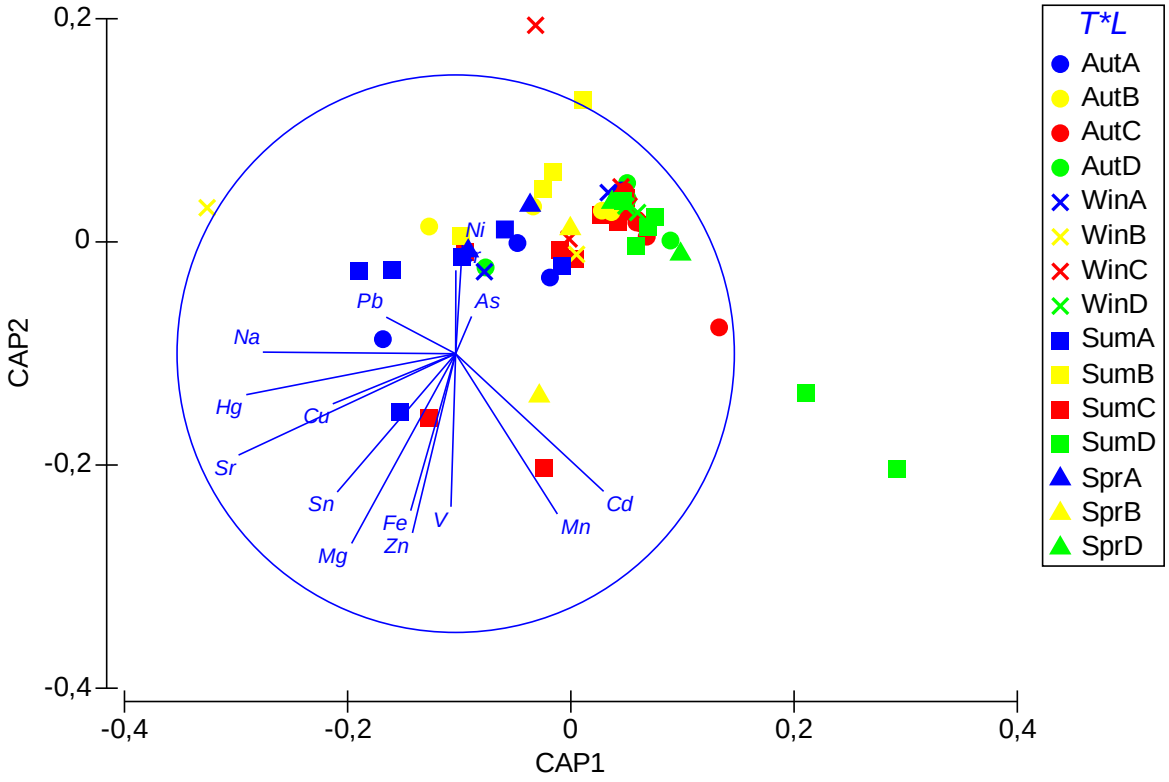

Figure 2.2 Diagram of the Canonical Analysis of Principal coordinates (CAP), showing the distribution of chemical elements found in water considering time and location. Locations: A, B, C and D. Time: Spring, Summer, Autumn and Winter. Lines represent individual metal contribution to the distribution, aiming to their direction of influence and with lengths proportional to their relative importance for distribution.

Table 2.2 Means and standard errors (in brackets) of trace metals concentrations detected in water (μgL^{-1} in dry weight).

Season	Site	N	Na	Mg	V	Mn	Fe	Sr	Sn
Spring	A	2	53.911 (3.516)	4.801 (9.601)	0.003 (0.006)	—	0.047 (0.043)	0.622 (0.104)	0.713 (0.385)
	B	2	37.568 (22.392)	7.415 (5.69)	0.078 (0.156)	—	0.14 (0.236)	0.246 (0.159)	3.591 (6.584)
	D	2	17.354 (23.404)	1.494 (2.987)	0.012 (0.004)	0.004 (0.008)	0.067 (0.083)	0.027 (0.021)	0.736 (0.19)
Summer	A	6	114.101 (253.578)	7.328 (21.028)	0.048 (0.144)	—	0.19 (0.71)	1.179 (1.685)	3.134 (6.545)
	B	4	73.431 (58.976)	3.235 (5.763)	0.004 (0.015)	—	0.047 (0.08)	0.532 (0.729)	0.963 (0.581)
	C	10	40.921 (135.413)	4.882 (16.745)	0.026 (0.128)	0.015 (0.109)	0.155 (0.827)	0.453 (1.515)	1.821 (8.621)
	D	6	5.215 (10.984)	2.72 (7.582)	0.035 (0.128)	0.045 (0.154)	0.109 (0.427)	0.052 (0.161)	0.941 (2.864)
Autumn	A	4	49.061 (66.561)	5.458 (14.173)	0.014 (0.032)	0.006 (0.024)	0.102 (0.222)	0.729 (1.45)	0.931 (1.861)
	B	4	33.172 (78.98)	2.062 (4.97)	0.008 (0.015)	0 (0.001)	0.034 (0.058)	0.406 (0.829)	0.842 (1.315)
	C	8	5.62 (15.388)	1.556 (4.983)	0.013 (0.028)	0.004 (0.013)	0.051 (0.269)	0.042 (0.122)	0.422 (1.18)
	D	4	22.965 (64.301)	3.085 (10.769)	0.006 (0.009)	0.003 (0.006)	0.035 (0.043)	0.145 (0.535)	0.428 (0.763)
Winter	A	2	36.047 (49.142)	5.986 (10.142)	0.006 (0.011)	—	0.031 (0.057)	0.382 (0.499)	0.799 (1.564)
	B	2	122.939 (197.107)	—	0.03 (0.059)	—	0.078 (0.034)	0.803 (0.858)	2.307 (2.268)
	C	4	83.201 (288.339)	1.756 (6.517)	0.014 (0.035)	0.002 (0.003)	0.082 (0.229)	0.203 (0.458)	1.44 (4.05)
	D	2	1.238 (1.433)	0.623 (0.309)	0.018 (0.013)	0.003 (0.001)	0.022 (0.016)	0.009 (0.005)	0.326 (0.048)

Table 2.2, Continuation- Means and standard errors (in brackets) of trace metals concentrations detected in water (μgL^{-1}).

Season	Site	N	Cr	Ni	Cu	Zn	As	Cd	Hg	Pb
Spring	A	2	—	—	0.004 (0.004)	0.043 (0.015)	—	—	1.453 (0.701)	0.014 (0.027)
	B	2	—	—	0.008 (0.009)	0.248 (0.381)	—	0.011 (0.021)	0.939 (0.843)	0.001 (0.001)
	D	2	—	0.001 (0.001)	0.002 (0.000)	0.119 (0.045)	0.001 (0.001)	0.054 (0.108)	0.071 (0.094)	0.078 (0.153)
Summer	A	6	—	0.001 (0.003)	0.011 (0.039)	0.203 (0.458)	0.001 (0.004)	0.018 (0.109)	1.336 (1.756)	0.158 (0.925)
	B	4	0.007 (0.012)	0.003 (0.012)	0.004 (0.002)	0.034 (0.042)	—	—	0.516 (0.089)	0.004 (0.009)
	C	10	—	—	0.005 (0.017)	0.092 (0.303)	—	0.013 (0.074)	0.522 (1.718)	0.005 (0.021)
	D	6	—	0.001 (0.004)	0.004 (0.01)	0.137 (0.327)	0.001 (0.004)	0.104 (0.377)	0.138 (0.361)	0.003 (0.005)
Autumn	A	4	—	0 (0.001)	0.005 (0.008)	0.163 (0.237)	—	—	0.929 (1.338)	—
	B	4	—	—	0.002 (0.003)	0.019 (0.039)	—	—	1.089 (2.801)	0.011 (0.039)
	C	8	—	—	0.001 (0.002)	0.039 (0.082)	—	0.05 (0.214)	0.064 (0.191)	0.003 (0.01)
	D	4	—	—	0.002 (0.005)	0.063 (0.099)	0 (0.001)	0.023 (0.089)	0.331 (1.248)	0.029 (0.092)
Winter	A	2	—	0.001 (0.001)	0.004 (0.007)	0.06 (0.104)	—	—	0.581 (0.929)	0.002 (0.000)
	B	2	—	—	0.01 (0.000)	0.107 (0.214)	—	—	4.185 (6.955)	—
	C	4	0.001 (0.002)	0.007 (0.027)	0.008 (0.025)	0.084 (0.235)	0.002 (0.008)	0.005 (0.019)	0.437 (1.289)	0.117 (0.432)
	D	2	—	0.001 (0.001)	0.002 (0.003)	0.041 (0.028)	—	0.007 (0.013)	0.016 (0.007)	0.001 (0.001)

Spearman correlation tests revealed several monotonic correlations among abiotic components (temperature, pH, salinity, dissolved oxygen) and metals (Na, Mn, Sr, Cd, Hg). Notably, salinity and pH had positive relationships with Na, Sr, and Hg ($p< 0.001$), but negative relationships were observed with Mn ($p< 0.001$) and Cd ($p= 0,004$). Moreover, temperature was positively correlated with Na ($p= 0.019$) and Sr ($p= 0.003$).

Trace elements, Na, Sr, and Hg had strong positive correlations when compared to each other (Spearman, $p< 0.001$) and weak negative correlations when compared to Cd ($p<0,01$), and Mn ($p< 0.002$). Cd and Mn were positively correlated to each other (rho= 0.52, $p< 0.001$).

2.4.2 Flounder tissue concentrations

The *P. flesus* specimens collected throughout the study period were selected to be roughly the same size and weight (Table 2.3). Yet, morphometric factors differed in Spring (Kruskal Wallis analysis; Length= 7.406 cm, H=33.38, $p< 0.001$; Weight= 4.827g, H= 33.07, $p< 0.001$) and individuals were smaller comparatively to the rest of the seasons. Nevertheless, the morphometric derived condition index (Kn), did not significantly vary among seasons (H = 0.306, p=0.858).

Table 2.3 Length. Weight. Relative condition factor (Kn) and Metal pollution Index means of fish in each studied season (standard errors in brackets).

Season	N	Length (cm)	Weight (g)	MPI	Kn
Autumn	30	11.35 (0.2)	15.17 (0.9)	7.25 (0.4)	1.02 (0.0)
Spring	16	7.40 (0.2)	4.83 (0.4)	7.54 (0.6)	1.00 (0.0)
Winter	10	11.76 (0.7)	17.50 (3.1)	9.43 (1.5)	1.02 (0.0)

Elements in fish muscle varied between 3569.3 (Na) and 0.004 (Co) µg g-1 from a list of 19 metals detected in 58 samples. Elements composition in juvenile flounder muscles differed seasonally (Table 2.4) (Pseudo F=3.314, p value=0.001) and amongst all seasons ($t<2.16$, $p<0.013$). Consequently, a high percentage of the samples (76.786%) were correctly assigned to the a priori Season groups (Autumn, Winter, Spring), as observed in Figure 2.3. This seasonal pattern can be attributed to the succeeding relevant metals: K (H=17.11, $p< 0.001$). Cr (H=14.78, $p< 0.001$). Mn

(H=17.52, p=0.0001568) and As (H=29.71, p< 0.001). In spring, fish tissues had higher concentrations of K, Cr and Mn, while in winter the trace element As was particularly more abundant (Table 2.4). Positive monotonic correlations were detected between K and Cr as well as K and Mn (Spearman, p< 0.001), whereas negative correlations were only recorded between As and K (rho= -0.293, p=0.028). When testing the previous metals against the physical parameters, length and weight, K, Cr and Mn exhibited negative relations (p<0.001), whilst As presented a positive relation (p<0.001).

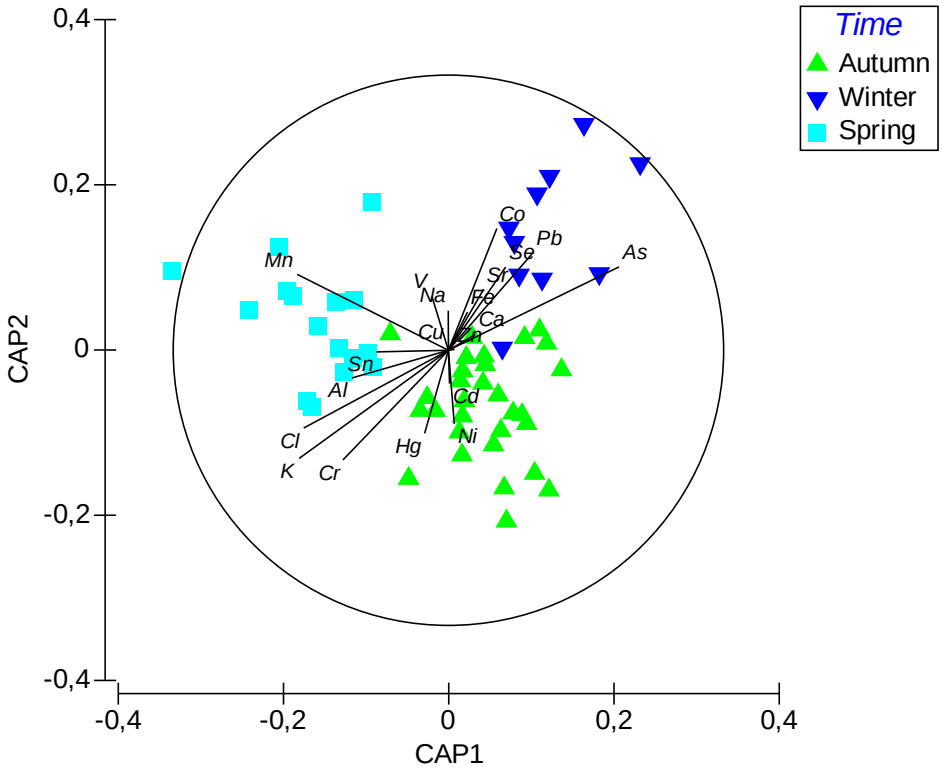

Figure 2.3 Diagram of the Canonical Analysis of Principal coordinates (CAP), showing the distribution of chemical elements found in fish from different seasons. Time: Spring, Summer, Autumn and Winter. Lines represent individual metal contribution to the distribution, aiming to their direction of influence and with lengths proportional to their relative importance for distribution.

Fish contamination, assessed broadly via MPI and fractionally by calculating metal ratios, did not vary temporally, except for the Cr ratio (Kruskal Wallis. H=14.86. p< 0.001), which was lower in Winter (Figure 2.4 Table 2.4). Despite the absence of any seasonal variation in most ratios, two elements exceeded the recommended threshold concentrations. Pb and Hg, surpassed values of 1

in Winter, when compared to food safety standards. Furthermore, the Hg ratio also had a small number of samples that exceeded 1 in Spring and Autumn. All other ratios were below 1 throughout the study period (Figure 2.4).

Table 2.4 Means and standard errors (in brackets) of trace metals concentrations detected in fish ($\mu g\ g^{-1}$ in dry weight).

Season	N	MPI	Na	Al	K	Ca
Spring	16	7.541 (0.568)	3384.901 (217.291)	41.254 (4.858)	790.538 (64.087)	141.022 (9.189)
Autumn	30	7.252 (0.429)	3213.358 (170.397)	31.571 (3.392)	633.092 (44.124)	156.486 (30.449)
Winter	10	9.432 (1.534)	3569.267 (703.594)	23.294 (5.301)	305.015 (62.736)	181.447 (51.286)

(Continued)

Season	N	V	Mn	Fe	Co	Se
Spring	16	0.292 (0.135)	0.661 (0.055)	4.794 (0.344)	0.004 (0.004)	0.274 (0.036)
Autumn	30	0.157 (0.049)	0.368 (0.031)	4.733 (0.575)	—	0.284 (0.012)
Winter	10	0.322 (0.232)	0.417 (0.091)	6.011 (2.398)	0.031 (0.021)	0.404 (0.097)

(Continued)

Season	N	Sr	Sn	Cr	Ni	Cu
Spring	16	1.699 (0.177)	32.492 (4.406)	0.187 (0.032)	0.025 (0.01)	0.41 (0.036)
Autumn	30	1.706 (0.182)	25.013 (2.437)	0.151 (0.024)	0.038(0.009)	0.407 (0.044)
Winter	10	2.428 (0.822)	21.953 (7.448)	0.012 (0.008)	0.015 (0.01)	0.411 (0.07)

(Continued)

Season	N	Zn	As	Cd	Hg	Pb
Spring	16	12.864 (1.134)	0.675 (0.096)	0.051 (0.051)	1.954 (0.123)	0.694 (0.259)
Autumn	30	13.065 (1.073)	1.84 (0.158)	0.104 (0.104)	2.054 (0.139)	1.018 (0.338)
Winter	10	13.183 (2.224)	3.377 (0.796)	—	1.589 (0.233)	2.64 (0.595)

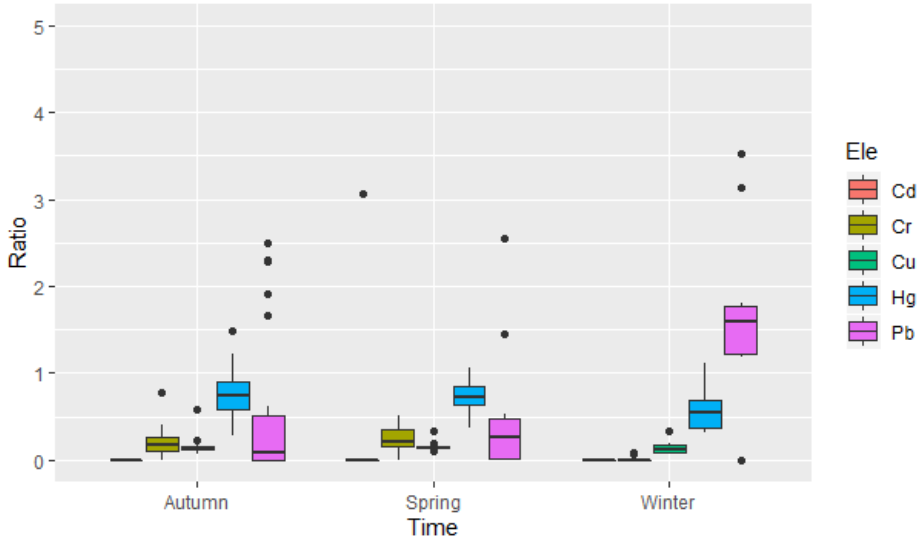

Figure 2.4 Ratios of toxic metals in fish based on European guidelines established for food safety displayed.

2.5 Discussion

The low values of harmful metals measured in this study and their reduced spatiotemporal variability, portray the Mondego estuary as a well-mixed system of low environmental pollution. Metal distribution patterns observed were in great part concomitant with the inherent environmental salinity balance and to a lesser degree, linked to anthropogenic influences. Elements observed in highest concentrations in water and fish tissue, such as Na and Mg, were essential, naturally occurring metals attributed to geochemical processes, whereas less available elements such as known pollutants Cd, Hg and Pb are introduced anthropogenically and were mostly available below harmful limits. The Mondego estuary extends across a warm temperate region for 26 km in length, effectively connecting the Mondego mountain river to the Atlantic Ocean. Both river and ocean fundamentally generate the estuarine water circulation responsible for metal mobility by means of river flow and tidal cycles respectively and contribute with chemically distinct waters to the elemental composition of transitional systems as sources of abundant and rare crustal metals (Mason, 2013).

Our abiotic factor analysis denoted a weak temporal discrimination power of trace element quantities in water but managed to highlight important spatial patterns governed by saltwater and

freshwater inputs. Usually, trace metals are more abundant in oceanic waters than in inland freshwaters. Thus, metals which have traversed past the mixing zone in estuaries and did not decrease linearly with salinity are considered to behave non conservatively (Boyle *et al.*, 1974). The elements Na, Sr, Hg were shown to vary non-conservatively seawards, while Mn and Cd varied conservatively. Unsurprisingly, the elements Na, a quintessential ion of saline water, and Sr, another principal constituent of salt-water, were positively correlated with salinity and pH, reflecting the transition of ocean to river along the estuary (He & Xu, 2015).

Salinity is a decisive component of metal mobility (Turner, 1996). Increased salinity promotes metal desorption from particulate matter and facilitates its dissolution consequently raising total trace metal content in the water (Valenta *et al.*, 1986, Turner, 2003). The area monitored in the estuary is a deep channel, with a 5–10 m depth during high tide, which causes the upstream propagation of marine tidal water to be fast and its daily salinity variations to be high (Costa *et al*., 2013), indicating that hydrodynamic forces are feasibly another factor to recognize in metal partitioning. Even though the salting out mechanism can bear some responsibility in enhancing metal levels in water, the distribution of highly reactive elements like mercury is readily understood as a strong expression of pollution, especially since its cycle is still a subject of debate for its complexity. (Canela & Jardim, 1997; Baeyens *et al.*, 1998; Black *et al.*, 2012). Ultimately, the most credible hypothesis for high concentrations of the toxicant near the mouth of the estuary, is excessive anthropogenic inputs from urban areas and harbor-related activities (Horvat, 1997; Rodrigues *et al.*, 2006). Mercury concentrations were persistently higher in the stations A and B, which corresponded to the sites closest to the harbor and areas with highest human density.

In contrast to elements that behaved non-conservatively, concentrations of Mn and Cd were higher in areas strongly subjected to riverine discharge and steadily decreased towards the ocean. As reported by Duinker *et al.* (1979) in the Scheldt and Rie estuaries, dissolved manganese can be prevalent in the upper estuary due to the more reductive conditions present in the freshwater saturated area. Lower salinity, pH and dissolved oxygen create a less chemically stable environment that drives the dissolution of this compound from suspended particles and its diffusion from the sediment (Morris *et al.*, 1982). In contrast, under oxidizing conditions, manganese can sequester metal ions from solution by reducing its reactivity in the Mn oxide form and co precipitating with other metals, including Cr, Cd, Cu, Pb and Zn, which could partly explain reduced dissolved Cd in the lower estuary (Zwolsman *et al.*, 1993). Like manganese, the

remobilization of cadmium from particles and sediment is heavily related to its internal cycling, however, anthropogenic inputs have a greater impact on how this metal is partitioned in the environment (Yang & Sañudo-Wilhelmy, 1998; Zwolsman *et al.*, 1998). The most affected area by Cd contamination in the Mondego estuary was adjacent to several long stretches of land used for agriculture (rice production) (Teixeira *et al.*, 2008). Despite the regulations imposed in Europe to reduce the environmental accumulation of Cd, in many countries its use in fertilizers is still widely popular and poorly regulated (Ulrich, 2019). It is likely that agricultural runoff is enriching the water with this metal, and biogeochemical processes are encouraging its dissolution in the upper estuarine zone. Besides the anthropogenic processes directly affecting the estuary, the river that pours into the system is comprised of a large drainage basin (6670 Km2) permeated by polluting, industrial, agricultural and urban areas (Pinto *et al.*, 2013), collecting various chemical inputs along its course and transporting them into the estuary via fluvial regimes (Pardal *et al.*, 2002).

Overall, the harmful metal concentrations in Mondego estuarine water were lower than in other estuaries with higher anthropogenic pressures. In the context of Portuguese estuaries, this can be evidenced by the much higher values reported of Zn, Ni, Cd and Pb in Tagus (Duarte *et al.*, 2014), Guadiana (González-Ortegón *et al.*, 2019) and Douro (Ribeiro *et al.*, 2018). Compared to other European waters of differently polluted estuaries, our Mondego results were consistently lower, than the values reported for the pollutants Cd, Pb, Zn and Cu in several studies (Turner *et al.*, 1992; Dassenakis *et al.*, 1997; Zwolsman *et al.*, 1997). Mercury was the only toxicant in Mondego water that seemed uncharacteristically available in high concentrations (0.016-4.185 μgL^{-1}) and according to the European Union's guidelines, 42 out of the 93 water samples surpassed the mercury limit imposed for surface waters in Directive 2000/60/EC (maximum allowable concentration, 0.07 μgL^{-1}) (European Commission, 2006). Understanding trace element spatial availability and acknowledging sources of pollution, provides the important groundwork capable of explaining seasonal trends of metals in estuarine ecosystems (Pereira *et al.*, 2005).

Fish tissue analysis yielded 5 metals out of the 20 studied with distinct elemental concentrations each season. All fish were strictly gathered from the same section in the upper estuary, where individuals were subjected to the same environmental conditions. Seasonal metal concentrations in fish are expected to be strongly driven by riverine discharge since Mondego is a river dominated estuary (Pereira *et al.*, 2005). The few metals that varied seasonally were generally found in higher

concentrations stored in muscle during the dry season, when warmer climate increases evaporation and lowers river inputs. Even though a low river flow is known to contribute to less metals being flushed into the estuary (Waeles *et al.*, 2005), the corresponding weaker flush of organic matter may favor metal accumulation in the water through reduced formation of soluble inorganic complexes, and cause an increase in their bioavailability during warm seasons (Yang & Sañudo-Wilhelmy, 1998; Zwolsman *et al.*, 1998; Remoundaki *et al.*, 2007). Variation in riverine input directly affects the quantity of contaminants flushed into the estuary and simultaneously alters the abiotic factors responsible for metal speciation, namely salinity and organic matter (Zhang & Wang, 2007a). The partitioning of metals in water as previously stated will be conditioned by salinity and inevitably affect fish metal uptake, not only as a function of environmental concentrations, but also by affecting fish metabolism (Zhang & Wang, 2007b; Dutton & Fisher, 2011).

For instance, K is an efficiently regulated element by fish as an important component of enzymes, and it plays an essential role in maintaining osmotic balance, therefore this metal varies with seasonal salinity shifts (MacLeod *et al.*, 1958; Epstein *et al.*, 1969). Potassium levels were higher during Spring, when salinity increased, requiring the cell membrane potential to be kept through the pumping of this element into tissue. In addition, research on metal contamination in aquatic systems notes that higher temperatures would increase fish metabolism, leading to a higher metal uptake by organisms (Somero *et al.*, 1977; Siscar *et al.*, 2014).

Although fish tissue contamination is generally a function of their environment, the age, size, physiology, and behavior of an organism can substantially impact bioaccumulation (Zhang & Wang, 2007; Jakimska *et al.*, 2011). Specimens in our study were picked as juveniles and selected to be roughly the same size and weight to minimize discrepancies in metal bioaccumulation. Yet, it was ascertained that Spring individuals were slightly smaller. This could account for the temporal variation in tissue elements, especially for Mn and Cr, which changed concentrations significantly only in that season. Metals with seasonal variability were present in muscle at quantities inversely related to flatfish size, which was something not conveyed by the relative condition factor and metal pollution index. This inverse relation between body mass and metal levels is corroborated in the work of De Wet *et al.* (1994) for the metals Fe, Mn, Zn, Cu, Ni and Pb. Taking into consideration that age is not a strong factor in our situation, the reasoning behind this effect in our data, mainly rests on the fact that small organisms have higher metabolic rates

than their larger counterparts, and to meet energy requirements they must ingest higher amounts of food, which increases the probability of exposing tissues to diet-borne metal contamination (Henry *et al*., 2004; Zhang and Wang, 2007a). Further insight on the results is best provided by comparing the muscle metal burden in Mondego *P. flesus* with other European investigations using individuals from the same species and age group.

In a study by Vasconcelos *et al.* (2011), the essential metals Cr and Zn were analyzed in the muscles of juvenile *P. flesus* in three estuaries (Douro, Ria de Aveiro and Mondego) differentiated by anthropogenic levels of pollution and hydrology. Average concentrations of Cr and Zn in all estuaries surpassed the maximum values obtained in our work. It was ascertained by the authors that metals assessed were present in low quantities, regardless of the level of habitat contamination, and no differences could be detected among estuaries. Accordingly, it was suggested that the time *P. flesus* spends in estuaries (approximately three months) might account for the low contamination of muscle. Other research, assessing strictly juvenile *P. flesus* muscle concentrations in Europe, is limited to the work of Henry *et al.*, (2012). The study in question, covered a large range of metals (e.g. Cr, Ni, Cu, As) from three French estuaries of low anthropogenic impact (Canche, Authie and Somme) and one highly polluted (Seine), and managed to reveal that muscle metal composition in *P. flesus* from anthropogenically distinct systems can differ despite the low residence time of juveniles. Metal concentrations measured in fish, reflected the different anthropogenic levels of each habitat, and were found to be generally above our reported results, apart from lead and cadmium which were altogether below. On par with water quality, the levels of hazardous elements in Mondego fish, were predominantly low and, as illustrated in our metal ratio calculations using the standards established by the European Union, under the hazardous limit for health. However, quantities of Hg and Pb in some fish exceeded the guidelines stated in the COMMISSION REGULATION (EC) No 1881/2006. These metals were not proportionally available in water, which suggests that sediment may account largely for tissue concentrations.

Given that metals are legacy pollutants, it is conceivable that a benthic species like *P. flesus,* might be gradually exposed to these toxicants from a major metal sink like sediments, that was originally enriched during the Mondego estuary heavy pollution years (Kerambrun *et al.*, 2013; Maulvault *et al.*, 2015). During the 90s, the ecosystem registered a severe decline in water quality and an increase in eutrophication events that dramatically altered the structure of the species community

(Martins *et al.*, 2001). From that period on, environmental pressures have been limited to relatively low inputs from agriculture, fish farming, activities associated with the commercial harbour and extreme seasonal climatic events (Martins *et al.*, 2001; Baptista *et al.*, 2010; Verdelhos, 2010; Nyitrai *et al.*, 2013; Pinto *et al.*, 2013). Currently, the estuary has mostly recovered from years of anthropogenic pressures and is placed amongst the least polluted coastal bodies of water in Portugal (Vasconcelos et al 2007a; Pinto *et al.*, 2013), with several protection programs (IBA, RAMSAR, National Agricultural Reserve and National Ecological Reserve) (Costa *et al.*, 2013) aiding in its ongoing maintenance and controlling the sources responsible for ecosystem impoverishment (Lillebø *et al.*, 2005; Verdelhos, 2010). Still, it is not possible to rule out historic contamination as a reason for metal bioavailability. Remobilization of metals from particulate to dissolved phases is easily achieved in a dynamic water body like an estuary due to the previously stated redox conditions of the environment, interactions with organic matter, bioturbation and the hydrological regime of the system (e.g. tidal current, water level oscillation, river flow). For instance, metals and other pollutants from past anthropogenic events were reported to endure in the water of the Mondego river and its associated tributaries in other investigations mainly because of the sediment's storage capacity. The European flounder is a benthic species, directly exposed to substrate pollution either as a result of constant proximity to the bottom or through the ingestion of sediment particles (Moles *et al.*, 1994). Furthermore, since *P. flesus* occupies a high trophic level (carnivorous) in estuaries, contaminated prey consumption can bio-magnify metal concentrations in the body (Fonseca *et al.*, 2019).

Muscle metal analysis has been firmly established in pollution monitoring strategies worldwide, ensuring species health as well as the good quality of the associated ecosystem. Among the different tissues commonly analyzed for metal contamination in fish (notably, liver, gills and kidneys), muscle generally displays the lowest affinity for these compounds (Durrieu *et al.*, 2005), however, it is the main organ targeted for consumption by humans, and contaminants stored in this tissue pose a great health risk to man. Periodically measuring muscle elemental composition diminishes this seafood safety threat by progressively monitoring contamination status of commercially important species. Data gathered in this study contributes to the present assessment of *P. flesus* metal contamination and establishes appropriate baselines to eventually inform future research using this bioindicator as part of environmental degradation models.

The successful discrimination of fish captured in different seasons using muscle also highlights the potential development of natural tags of habitat use based on metal patterns in tissue. The metal in muscle belonging to a marine migrant fish species, as is the case with *P. flesus*, can be purposed as a fingerprint tied to the spatial and temporal variations of water chemistry and used to trace its origins (Swearer *et al.*, 2003; Forrester, 2005; Ricardo *et al.*, 2017). Recognizing the provenance of an organism is particularly important to evaluate stock replenishment for marine migrant fish species that use the estuary to complete their life cycle (Gillanders *et al.*, 2003; Vasconcelos *et al.*, 2007b; Selleslagh *et al.*, 2016; Campana *et al.*, 2000). However, previous studies have mainly focused on trace elemental signatures in the hard tissue of otoliths, greatly dismissing muscle due to its low degree of accumulation and reduced sensitivity to short-term environmental changes (Vasconcelos *et al.*, 2007b; Martinho *et al.*, 2020). Therefore, otoliths have been widely and sufficiently studied to the point of becoming perhaps the most reliable tissue in identifying nursery use, migratory patterns, and population structure (Campana *et al.*, 2000; Vasconcelos *et al.*, 2008). Nevertheless, the scarce research on trace element composition in muscle has demonstrated promising results in discriminating fish origin worldwide, either as a standalone technique or combined with other approaches. For example, in a study by Guoet *et al.* (2013) conducted in East China, muscle elemental profiling of four commercial marine species allowed their discrimination to the respective habitats with an accuracy of 97.92% and 100%, depending on the statistical method. Furthermore, in Gopi *et al.* (2019) a model using muscle elemental analysis to discern the geographic origin of wild caught and farmed bass had an accuracy of 72%, but when combined with stable isotopic analysis, the new model increased in accuracy to 81%.

Multi-elemental signatures of water and fish, if consistent, may in fact prove to be a resourceful baseline to detect abnormal metal values and inform on mitigation measures, ensuring species health as well as the good quality of the associated ecosystem. In view of the economic importance of the European flounder as a resource to humans and its ecological role as a nursery-dependent fish, a risk assessment related to the high mercury levels in muscle tissue should be pursued in tandem with a more comprehensive and updated exploration of the harmful element in water and sediment. As far as we are aware, any attempts to characterize metal distribution along the full extent of the Mondego estuary by means of surface water sampling has been unprecedented in literature and should be encouraged.

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Chapter 3: Multi elemental signatures in soft tissue of several fish species from Portuguese estuaries

3.1 Abstract

Excessive anthropogenic activity in estuaries is introducing a myriad of chemicals in estuaries such as metals with toxic properties to organisms and deleterious repercussions to the ecology of these systems. During their complex life cycles, fish species greatly benefit from the ecosystem services provided by the variety of habitats in coastal waters and thereby are expected to be greatly impacted by estuarine pollution. This study aimed to assess estuarine metal contamination and predict the provenance of fish by characterizing the elemental signatures in water and soft tissues (liver and muscle) of nine fish species (*Diplodus vulgaris, D. bellottii, D. sargus, Platichthys flesus, Solea senegalensis, S. solea, Dicentrarchus labrax, Sparus aurata and Halobatrachus didactylus*) from four major Portuguese estuaries (Douro, Tejo, Sado, and Mira), using Total X-ray Fluorescence spectroscopy analysis. Canonical ordination methods showed that multi-elemental signatures in liver allowed for a better discrimination of fish estuary and species (accuracy range: 39.47%-77.27%) than muscle (30.44%-71.43%). However, the elemental composition of both tissues was noticeably more suitable in discriminating fish from different estuaries. Water CAP analysis indicated some similarity among the chemical composition of water in the various estuary groups. Although, among the systems analysed, Mira and Douro had significantly higher levels of hazardous metals, water samples from all estuaries generally surpassed the Hg limit imposed for surface waters by the European Union. According to the metal ratios in muscle calculated using guidelines for food safety, fish from Sado and Tejo were the most metal polluted and species such as *H. didactylus* and *D. labrax* were the most affected. Overall, elemental signatures in fish liver and muscle tissue might be efficient tools to determine fish provenance. Moreover, elemental analysis of abiotic and biotic components of estuaries provide crucial information about the state of the ecosystem and prevents the deterioration of biota and human health.

Keywords: estuaries, metals, muscle, elemental signatures

3.2 Introduction

Estuaries characteristically provide a mosaic of interconnected habitats with different ecological functions to different species (Jenkins *et al*., 1997; Phil *et al*., 2002). They are key environments in the recruitment and survival of many commercially and ecologically important fish populations (Beck *et al*., 2001). While estuarine resident and diadromous fish species have been known to depend entirely on the estuary to reach full maturity and ultimately thrive, for opportunistic species like some marine migrant fish, these ecotones may not be necessary to their development, but rather highly beneficial (Franco *et al.*, 2008; Potter *et al.*, 2015). They essentially function as nurseries that provide nutrient rich and sheltered environments to larva and subadult stages, until their recruitment to adult stocks offshore (Potter *et al.*, 2015). Given fish are usually highly mobile top predators of estuarine food chains, they drive ecologically important exchanges of matter and energy among segregated and interconnected ecosystems (Able, 2005). These organisms greatly contribute to the complex biogeochemical processes in estuaries necessary to sustain and maintain productivity at various trophic levels (Disa et al., 2017). Therefore, the growing anthropogenic pressures currently affecting estuaries and fish species, such as overexploitation, pollution and habitat degradation, can drive decreases in abundance and biodiversity of aquatic fauna and flora with serious repercussions to the quality of the local ecosystem, potentially extending the negative impacts to any environment ecologically linked to coastal water bodies (Dobson *et al.*, 2006). Additionally, these disruptions inevitably place fish stocks under pressure and since they are a valuable economic and nutritional resource worldwide, the food safety and welfare of man will be threatened (Steinberg *et al.*, 1994; Zeitoun & Mehana, 2014; Bosch *et al.*, 2016).

Taking into consideration the sensitivity of estuarine ecosystems, metal contamination is one of the leading concerns in these environments (Tomlinson *et al*., 1980). Metal pollution is a global challenge in estuarine systems perpetually decreasing water quality and potentially endangering all life forms in contact with the environment (Suedel et al., 1994). Metals are common waterborne elements, minimally and naturally supplied to estuaries by lithogenic sources and excessively by anthropogenic sources (e.g. fossil fuel combustion, sewage wastewater and mining) (Halpern *et*

al., 2019). The rising and widespread application of metals for industrial, domestic, agricultural, and technological purposes has been dramatically propelling the exposure of estuarine biota to large quantities of these toxicants (Bradl, 2005; Barletta *et al.*, 2019). When metals are deposited in the ecosystem, they can insidiously accumulate in organisms and along the trophic chain, inflicting various degrees of toxicity according to their biological role (Tchounwou *et al.*,2012). For instance, essential metals such as copper (Cu), chromium (Cr), iron (Fe), manganese (Mn) and nickel (Ni) are important catalysts in biological macromolecules for oxidation-reduction reactions and if not adequately supplied, organisms may suffer deficiency diseases (Bury *et al*., 2003; Sfakianakis *et al*., 2015). On the other hand, when in high concentrations these beneficial compounds can became harmful and cause tissue and cellular damage. Comparatively, non-essential metals such as mercury (Hg), lead (Pb) and cadmium (Cd), have no known physiological or biochemical functions, generally induce toxicity at low concentrations and easily accumulate in organisms to a much larger extent (Fonseca *et al*., 2009; Mohammed, 2013). Therefore, assessing the danger these pollutants pose to estuarine aquatic life is a priority of modern society and several monitoring programs and strategies have been proposed to address this endeavour (Birk *et al*., 2012; Zhang *et al.,* 2017).

In the late 60s, the pervasive and pronounced burden of pollutants on the environment led to the wide use of fish as bioindicators and it became commonplace in estuarine metal contamination research to analyse the multi elemental composition of their soft tissues (Oertel & Salánki, 2003; Burger, 2006). Measuring the concentrations of various elements in organs, such as gills, liver, kidneys and muscle, relays important information about the bioavailability of pollutants in the environment, what abiotic conditions influence their uptake and accumulation in the body and how they affect the metabolism of organisms (Clearwater *et al*., 2002; Vicente-Martorell *et al*., 2009). Answering these questions is crucial to define the health of ecosystems and safeguard human exploitation of estuarine services. Muscle and liver are the most studied organs in metal accumulation studies and present different opportunities to relate environmental contamination and fish metal burden (Sabullah *et al.*, 2015). Muscle is the main edible part of fish hence it has a great importance for human health, whereas liver is an important agent in the sequestration of metals and detoxification (Canli & Atli, 2003; Wei *et al.,* 2014). Muscle is less metabolically active than liver, therefore it will accumulate metals at a slower rate, but the elemental signatures will most likely vary less over long periods of time (Saygı & Atasagun, 2012). Liver tissue usually

presents higher metal loads, which could be a proxy of local environmental influence, particularly during early life-stages and young adults (Zeitoun & Mehana, 2014; Yancheva *et al*., 2016). These biochemical patterns in fish tissues can be extremely telling of the habitat use during the lifetime of an organism hence their designation as natural tags in biological monitoring (Gillanders, 2009; Vasconcelos *et al.*, 2008).

On account of the ecologic value of fish and its notorious role as a profitable and healthy product to man, natural tags have been in constant demand to avoid food fraud, public health issues and environmental deterioration (Ranaldi & Gagnon, 2010 ;El Sheikha & Xu, 2017). Natural tags, such as otolith chemical composition and tissue stable isotope ratios, have been increasingly effective in the reconstruction of fish life history, and currently, they are the most prominent methods in assessing relationships between the environment and biological contamination (Elsdon et al., 2008; Reis-Santos et al., 2015). The utility of these tags stems from their capacity to provide spatiotemporal information on fish habitat use, without the necessity of physically tagging individuals, which often involves procedures not viable for organisms in larval life stages and small sized juveniles (Thorrold et al., 2002; Tanner *et al*., 2016). Benchmark techniques rely upon inferring on the organism's whereabouts and contamination through the measurement of biochemical signatures in tissues, caused by the incremental accumulation of several trace elements from environmental exposure or diet (Elsdon *et al*., 2008; Zeigler *et al*., 2011). To unravel the origin of fish, these multi-elemental approaches are typically conducted on calcified structures (mainly otoliths), as the preferred tissue of analysis due to their metabolically inert nature and time keeping capabilities, while soft tissues are largely reserved for the study of specific isotopic forms of a few elements (e.g. carbon, oxygen and sulphur) with known environmental variations (Campana et al., 2000; Trembaczowski, 2011; Trueman et al., 2012). However, multi elemental trials using soft tissues have shown promising results in discerning geographical origin and production method of fish (wild or farmed), which is an indication they might be eligible as a reliable tool to study areas with ecological importance to fish (Chaguri *et al.*, 2015; Li *et al.*, 2016). This hypothesis could be substantiated in a way similar to how elemental fingerprints from the whole otolith can be statistically analysed and subsequently matched with previously defined groups to uncover their origin (Vasconcelos *et al*., 2008). Yet, since this is a relatively new concept, the fundamentals of tracking fish origin with soft tissue elemental composition need to be comprehensively understood and that entails testing habitat discrimination in a multi-taxa

approach, by proactively considering the effect of several factors responsible for elemental patterns (e.g. species physiology, metabolic functions of organs, and water physiochemistry) (Jezierska & Witeska, 2006;).

The present study aimed to use multi-elemental analysis to investigate the elemental signatures in liver and muscle of nine fish species (*Diplodus vulgaris*, *D. bellottii*, *D. sargus*, *Platichthys flesus*, *Solea senegalensis*, *S. solea*, *Dicentrarchus labrax*, *Sparus aurata* and *Halobatrachus didactylus*) exposed to the environmental conditions of four distinctly pressured Portuguese estuaries (Douro, Tejo, Sado and Mira) and to differentiate among the chemical composition of the estuarine waters. If a consistency is obtained in fish tissue biochemistry in each estuary, and species differences in metal uptake prove to be sufficiently neglectable, it would be possible to develop reliable traceability tools, using the analysed organs to ascertain the provenance of fish, which are highly valuable economic resource to humans, but also potentially hazardous if contaminated with some trace elements. Moreover, multi-elemental analysis of environmental compartments has been relying on the same techniques for years, despite the existence of potentially more suitable tools for this purpose (Klockenkämper & Von Bohlen, 2014). In this work, Total reflection X-ray fluorescence analysis (TXRF) technique was chosen for its less labor-intensive features and high efficiency in simultaneous elemental reading compared to conventional methods (Vázquez et al., 2020).

3.3 Materials and Methods

3.3.1 Study site and sample collection

Along the Portuguese coast, five fish species were caught between March and June 2019, in four major estuaries (Douro, Tejo, Sado and Mira) (Figure 3.1). These systems support various fish species, including important nursery grounds for several commercial fish species (Vasconcelos *et al.*, 2010), and possess contrasting hydro-morphological characteristics and anthropogenic activity, which covers a range of important variables when exploring environmental chemical patterns. Tejo is the largest estuary in Portugal (320 km^2), with one of the most populated areas in the country (Lisbon region). Sado is the second largest estuary (212.4 km^2), followed by Douro (7.3 km^2) and then Mira (4.7 km^2). In a study conducted by Vasconcelos *et al.* (2007), which

compares the anthropogenic pressures among Portuguese coastal systems, Tejo, Sado and Douro were rated as the most pressured estuaries, but the least vulnerable, whereas Mira was under low anthropogenic activity and was considered highly vulnerable.

Fish sampling was carried out by beam trawl tows with the support of professional fishermen, following previous research into the selected systems on species geographical distribution and population abundance (Vasconcelos *et al.*, 2010; França *et al.*, 2011). Accordingly, the collection time spanned from Spring to Summer, when higher fish biodiversity and abundances have been reported (Vasconcelos *et al.*, 2010). After collection, fish specimens were transported in refrigerated crates to the laboratory where their weight (g) and total length (cm) were measured. Subsequently, a portion of muscle and liver tissue was collected, weighed, and stored in a labelled vial at -20ºC for later processing. In every site where fish were collected, water samples were also collected using decontaminated high-density polyethylene (HDPE) plastic bottles for capture, and flasks for storage (Liang *et al*., 2020).

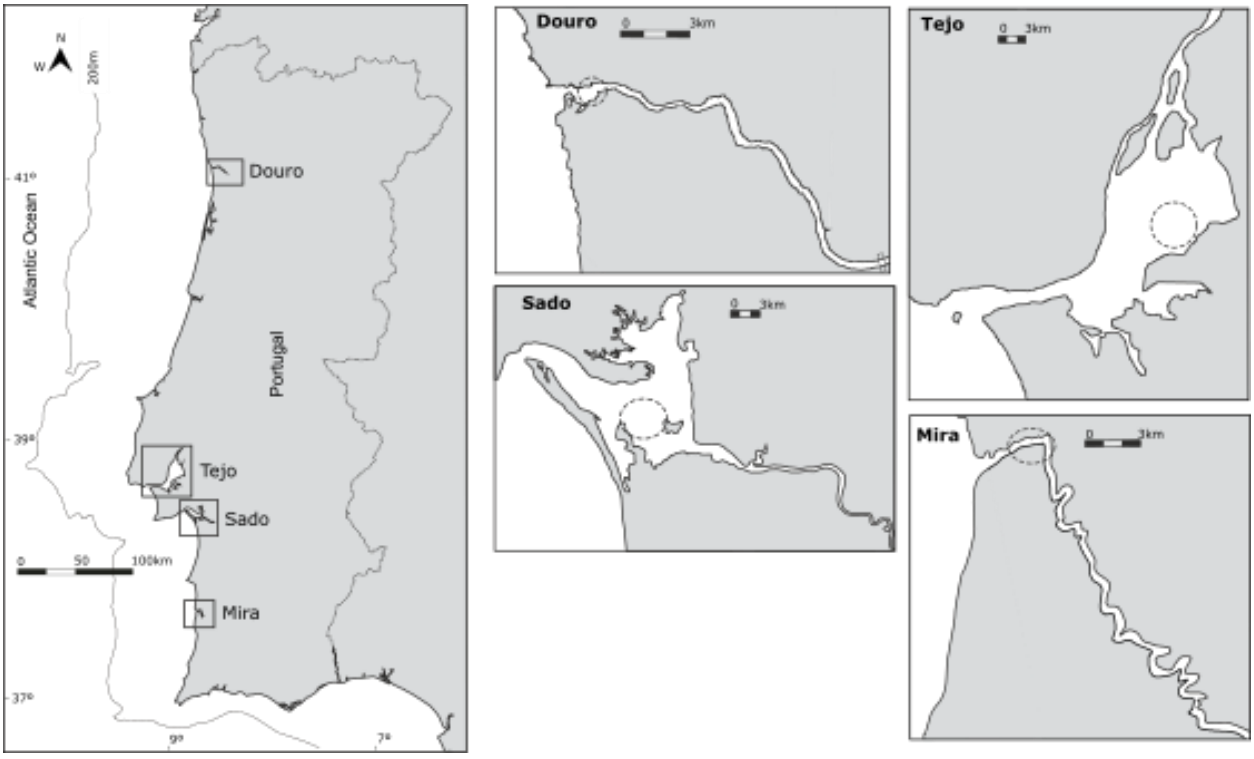

Figure 3.1 Map of Portugal showing the location of each studied estuary (Douro, Tejo, Sado and Mira estuaries).

3.3.2 Sample preparation and elemental analysis

Water and fish sample preparation followed adapted protocols from Duarte *et al.*, (2014) and Caçador *et al.*, (2012). Prior to the elemental analysis, all necessary lab equipment was immersed in 10% HCl for 24h and afterwards washed with deionized water (Reverse Osmosis, Elga Purelab Prima) to avoid contamination. Chemicals employed in the process were all trace metal free. Water processing consisted in filtering samples using GF/C Whatman filters, previously cleaned with acid and acidifying the remaining solution with HNO_3 (pH < 1). In the end samples were placed in Eppendorfs and stored at 4°C until analysis. As for fish samples, muscle and liver samples were freeze-dried with a Laboratory Freeze Dryer Cryodos-50, TELSTAR for 48–72 h at -50 °C and then their dry weight was determined. Dried tissues (ca. 100 mg) were liquefied in an acid digestion with 2 mL of a HNO_3:$HClO_3$ (7:1) mixture, during 3 h, in a Teflon reactor at 110 ºC (Caçador *et al.*, 2012).

Sample multi-elemental composition was determined by Total X-ray Fluorescence spectroscopy (TXRF S2 PICOFOX, Brucker). Trace element quantification in water and tissue was accomplished via the internal standard method by applying Gallium to analytes. The quality of the analytical methodology was ensured by instrumental recalibration and blanks. Additionally, replicate TXRF analysis of certified reference material TORT-2 (animals) was performed to assess the accuracy and precision of the analytical procedure, and consistently returned elemental concentrations within the range of certified values (Student's t tests $p < 0.05$).

3.3.3 Statistical analyses

The data produced by the TXRF readings was organized in separate sets corresponding to the three types of samples analyzed, namely water, liver, and muscle samples. All raw data was checked for parametric assumptions, verified with Shapiro Wilks and Levene's tests. Elemental profiles of muscle and liver were independently analysed, first with location and species integrated in the same multivariate models, and then both factors were assessed individually. The number of species was not equal in each estuary, therefore, subsets of data were created with the intent that, any possible statistical biases due to skewness were avoided and to ensure that all pertinent research hypothesis were tested. As a result, data was pooled from the following factor levels: Mira, Tejo, *H. didactylus* and *D. labrax*, to explore species and estuarine specific signatures. Water had a

single independent variable (location) so multivariate analysis was conducted accordingly. All multivariate datasets were normalized.

The discriminatory power of elemental fingerprints in distinguishing amongst a priori groups (location and species) was quantified and visually represented, using Canonical Analysis of Principal Coordinates (CAP). When high percentages of correctly classified groups were achieved in canonical ordinations (above 60%), an Analysis of Similarity (ANOSIM) was applied to test the statistical significance of the distinct elemental concentrations. Additionally, under the same circumstances a similarity percentage analysis (SIMPER) was used in order to determine which elements were responsible, to a large extent, in the discrimination of species and estuaries. Multivariate analysis was strictly done with PERMANOVA+ package in Primer v6.

Differences in individual element and morphometric parameter, between estuaries and species, were explored by univariate methods using non-parametric Kruskal Wallis tests (KW) and subsequent Dunn's pairwise tests. Interactions among variables were examined using non-parametric Spearman Correlation tests. Univariate analysis employed R Statistics software.

Values of priority pollutants, such as the potentially hazardous metals Cu, Zn and the highly toxic Cd, Pb and Hg were also compared with food safety standards to evaluate the level of fish contamination from different estuaries. Ratios of these elements in fish muscle were calculated to evaluate the contamination level of fish from each estuary.

3.4 Results

3.4.1 Water elemental analysis

Elemental concentrations in estuarine waters ranged from <0.001 to 393,81 μgl^{-1} (Cn and As, respectively) (Table 3.1). Univariate analysis evidenced significant variations in water elemental concentrations among estuaries. The elements that evidenced differences among estuaries, following Kruskal Wallis tests were: Na ($H=30.55$, $p<0.001$), Mg ($H= 7.749$, $p= 0.049$) Mn ($H= 4.564$, $p= 0.013$) Cu ($H=8.765$, $p= 0.032$), V ($H= 10.04$, $p=0.007$), Sr ($H= 9.515$, $p= 0,021$), and Pb ($H= 8.168$, $p= 0.038$). Noteworthy, Douro had the highest concentration of Cd and Hg, whilst Mira had the highest concentrations of Cu and Pb.

Table 3.1 Mean concentrations of trace elements (µg g–1 dry weight) and standard errors (in brackets) in waters collected in the Portuguese estuaries: Douro, Mira, Sado and Tejo.

Loc	N	Na	Mg	V	Cr	Mn
Douro	18	88.171 (13.484)	4.278 (0.68)	3.278 (0.836)	3 (0.97)	1.836 (1.004)
Mira	14	293.214 (32.983)	11.699 (2.1)	14.715 (5.105)	12 (3.508)	0.857 (0.592)
Sado	15	223.2 (32.685)	6.945 (1.88)	0.6 (0.412)	7.667 (2.284)	0.8 (0.439)
Tejo	24	175.188 (17.52)	7.648 (1.26)	7.293 (2.615)	4.626 (1.637)	—

(continued)

Loc	N	Fe	Co	Ni	Cu	Zn
Douro	18	74.507 (20.408)	—	0.667 (0.302)	4.612 (1.158)	61.521 (10.679)
Mira	14	203.643 (53.275)	—	0.143 (0.097)	11.929 (2.254)	77.5 (17.125)
Sado	15	152.284 (42.185)	—	0.867 (0.601)	7.869 (1.819)	108.681 (28.673)
Tejo	24	110.465 (22.483)	0.292 (0.292)	0.958 (0.63)	6.334 (1.16)	79.762 (27.43)

(continued)

Loc	N	As	Sr	Cd	Sn	Hg	Pb
Douro	18	—	376.944 (74.396)	2.056 (1.479)	393.809 (61.177)	165.699 (44.132)	4.78 (1.966)
Mira	14	0.001 (0.001)	216.269 (95.754)	—	93.214 (61.041)	78.142 (65.849)	60.874 (32.845)
Sado	15	—	96.031 (53.746)	—	264.995 (91.142)	47.569 (35.125)	21.016 (13.981)
Tejo	24	—	300.6 (71.019)	—	236.833 (69.948)	27.005 (17.774)	30.543 (14.783)

Water canonical ordination analysis using the elemental signatures managed to correctly assign 47,887% of water samples to the respective estuaries (Figure 3.1).

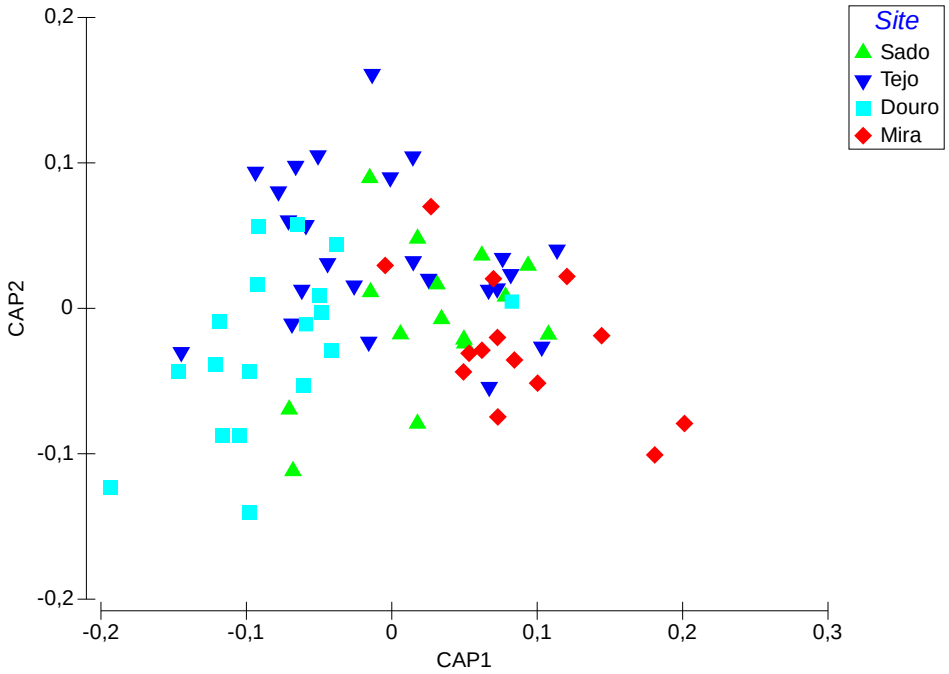

Figure 3.2 Canonical Analysis of Principal coordinates (CAP), showing the distribution of chemical elements found in water from different estuaries. Sites: Sado, Tejo, Douro and Mira.

3.4.2 Muscle elemental analyses

The Canonical ordination methods exhibited different arrangements of samples according to the similarities among elemental profiles in fish muscle samples. The CAP comprising the entire range of estuaries and species, separated only 35.417% of samples correctly, considering their a priori groups (Figure 3.3). CAPs assessing *H. didactylus* and *D. labrax* distributed samples between the estuaries Mira, Tejo and Douro with an accuracy of 68% (Figure 3.4) and 71.429% (Figure 3.5), respectively. In the Tejo, 51.282% of fish were correctly assigned to their species group (Figure 3.6), while in the Mira estuary the accuracy was of 30.435% (Figure 3.7).

For the highest performing CAP, which used data pooled from *D. labrax*, ANOISM did not detect any differences among the concentrations of that species in Douro, Tejo and Mira estuaries.

The dissimilarities among groups revealed by SIMPER analysis were generally low. The dissimilarity percentage between Tejo and Mira was 37.61%, between Mira and Douro 19.97%, and between Tejo and Douro 34.47%. A combination of several elements was required to

differentiate groups. The elements Zn, Cd and As were among the highest contributors to variations in elemental signatures.

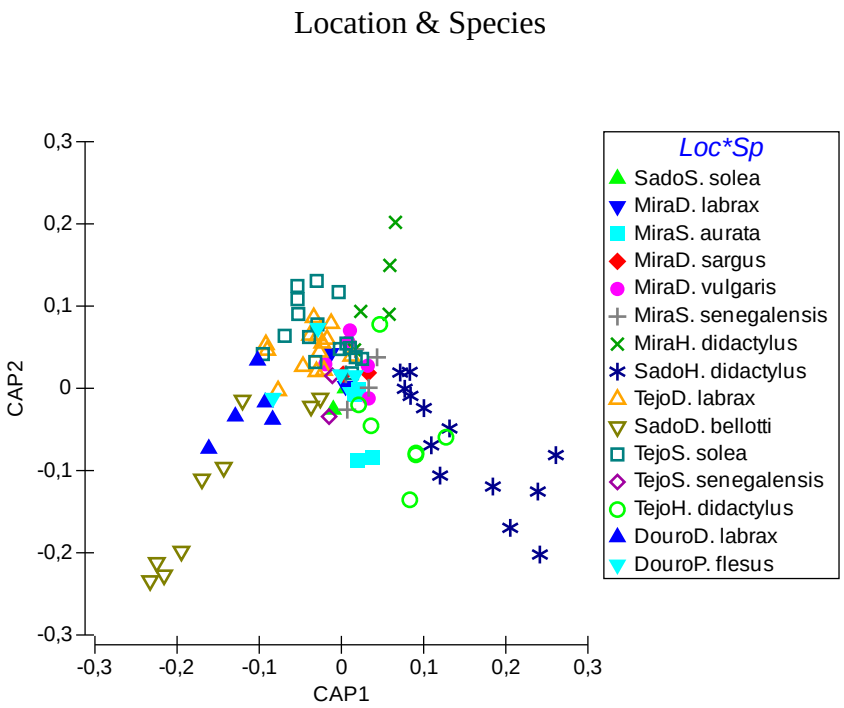

Figure 3.3 Canonical Analysis of Principal coordinates (CAP), showing the grouping of species and estuaries based on chemical elements found in fish muscle samples from different estuaries. Sites: Sado, Tejo, Douro and Mira, including the species: *Diplodus vulgaris*, *D. bellottii*, *D. sargus*, *Platichthys flesus*, *Solea senegalensis*, *S. solea*, *Dicentrarchus labrax*, *Sparus aurata* and *Halobatrachus didactylus*

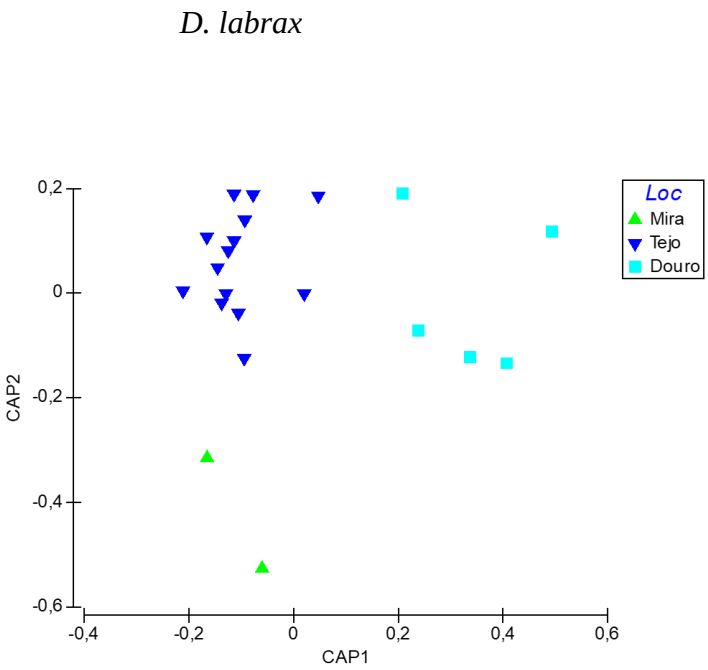

Figure 3.4 Canonical Analysis of Principal coordinates (CAP), showing the grouping of *Dicentrarchus labrax* from different estuaries based on chemical elements found in fish muscle samples. Sites: Tejo, Douro and Mira.

H. didactylus

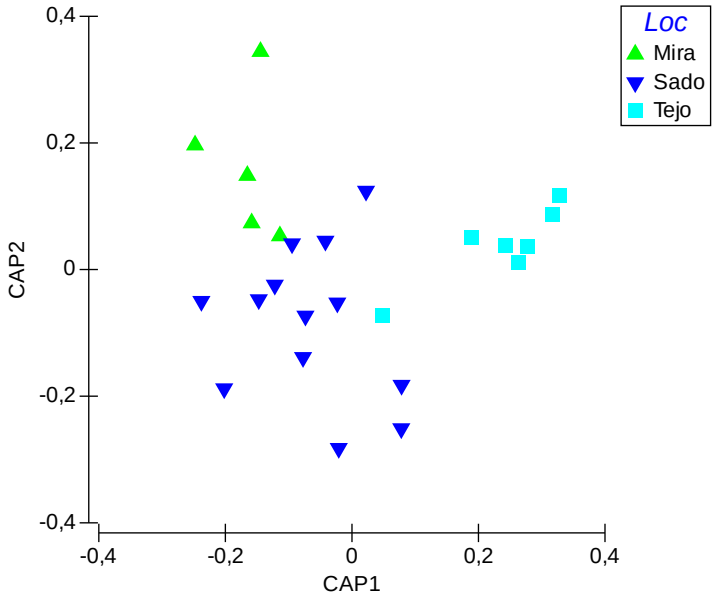

Figure 3.5 Canonical Analysis of Principal coordinates (CAP), showing the grouping of *Halobatrachus didactylus* from different estuaries based on chemical elements found in fish muscle samples. Sites: Tejo, Sado and Mira.

Tejo

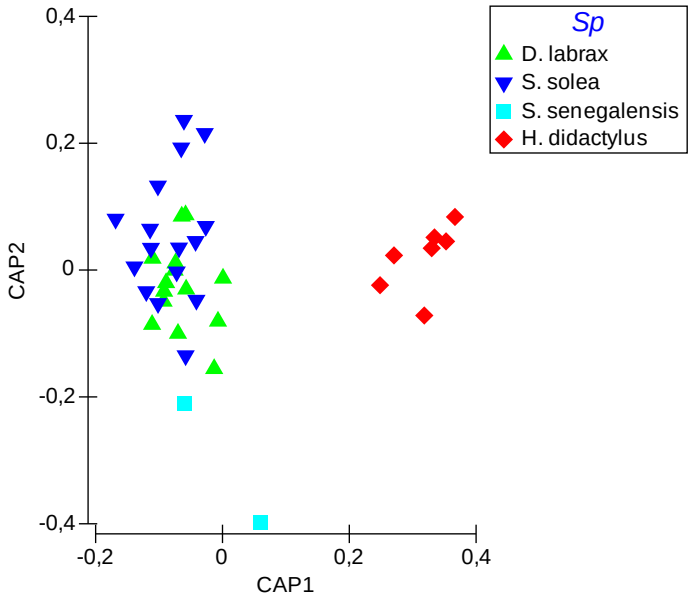

Figure 3.6 Canonical Analysis of Principal coordinates (CAP), showing the muscle grouping of the species: *Solea senegalensis, Solea. solea, Dicentrarchus labrax, Sparus aurata* and *Halobatrachus didactylus* from Tejo based on chemical elements found in fish muscle samples

Mira

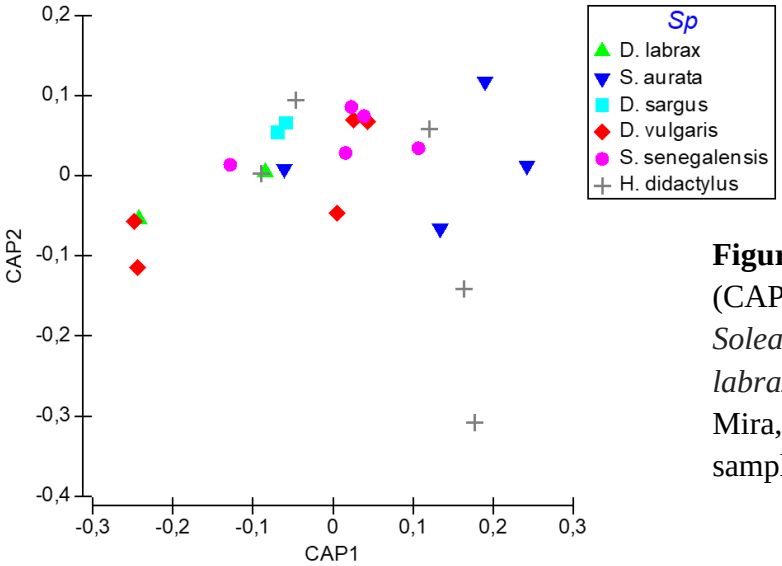

Figure 3.7 Canonical Analysis of Principal coordinates (CAP), showing the muscle grouping of the species: *Solea senegalensis, Diplodus vulgaris, Dicentrarchus labrax* and *Halobatrachus D. sargus, didactylus* from Mira, based on chemical elements found in fish muscle samples

Muscle elemental composition of the various fish species analyzed is presented in Table 3.2. Concentrations differed between estuaries and species. Inter-estuarine variations were ascribed to the elements Al (Kruskal Wallis, H=11.49, p=0.009), K (H=10.38, p=0.016), Mn (H=15.36, p=0.002), Cu (H=17.35, p<0.001), Zn (H=18.75, p<0.001), As (H=22.86, p<0.001), Se (H=8.721, p=0.03), Sr (H=12.3, p=0.006), Hg (H=21.52, p<0.001), and inter-species variations to Cr (H=15.02, p=0.046), Mn (H=22.87, p<0.01), Co (H=4.825, p=0.028), Cu (H=19.7, p=0.012), Zn (H=21.53, p=0.006), As (H=39.27, p<0.001), Sr (H=15.97, p=0.043), Sn (H=16.79, p=0.032) and Hg (H= 22,85, p= 0.039).

The elemental concentrations of K (H=5.053, p=0.03), As (H=5.053, p=0.03) and Se (H=3.868, p=0.0492) in *S. solea*, differed between Tejo and Sado (p=0.03), and in *H. didactylus* concentrations were generally different in the Tejo estuary (p<0.025), namely for Al (H=6.593, p=0.037), Mn (H=7.452, p=0.024), Fe (H=7.634, p=0.022), Cu (H=9.965,p=0.007), Zn (H=7.145, p=0.028), and only As (H=8.431, p=0.015) statistically differentiated Mira from Sado fish elemental composition. The metal Zn (H=6.02, p=0,049) varied between Mira and Douro (p=0.024) in *D. labrax* muscle samples.

Additionally, within each estuary there were also differences. In the Tejo estuary, *H. didacylus* had significantly higher concentrations of As (H=13.47, p=0.004), Cu (H=13.01, p=0.005), Co (H=2.934, p=0,01423), Mn (H=12.93, p=0.005) than the other species (p<0.043) and within Douro, elemental compositions of *D. labrax* and *P. flesus* were distinguished by variations in Cl (H=4.811, p= 0.028), K (H=6.818, p=0.009), Ca (H=5,771, p=0,016), Sr (H=3.938, p=0.047) and Sn (H=6.818, p=0.009). *H. didactylus* and *D. bellotti* (p=o.oo1) from Sado had differences ascribed to the elements Zn (H=7.705, p=0.021) and As (H=10.78, p=0.005).

Table 3.2, Mean concentrations of muscle trace elements (µg g–1 dry weight) and standard errors (in brackets) in nine fish species collected in Portuguese estuaries. Estuaries: Douro, Mira, Sado and Tejo. Species: *D. labrax* (DL), *P.flesus* (PF), *D. sargus* (DS), *D. vulgaris* (DV), *H. didactylus* (HD), *S. aurata* (SA), *S. senegalensis* (SSe), *D. belotti* (DB), *S. solea* (SSo).

Loc	Sp	N	Na	Al	Cl	K	Ca	V	Cr
Douro	DL	5	4179.52 (850.07)	47.15 (10.05)	5070.74 (447.41)	811.89 (129.8)	122.99 (16.67)	0.13 (0.07)	0.04 (0.03)
	PF	5	2317.79 (654.84)	30.65 (8.02)	3127.27 (584.07)	404.83 (49.64)	51.22 (13.75)	0.03 (0.02)	0.03 (0.02)
Mira	DL	2	2954.14 (1192.81)	25.12 (25.12)	4718.72 (132.4)	656.55 (306.23)	19.77 (13.17)	0.21 (0.05)	0.05 (0.05)
	DS	2	1245.98 (28)	29.92 (4.06)	2803.88 (200.98)	479.28 (65.17)	23.95 (1.18)	0.06 (0.06)	0.09 (0)
	DV	5	2868.23 (410.48)	33.86 (16.01)	4721.72 (605.38)	639.46 (120.71)	105.58 (35.9)	0.08 (0.05)	0.05 (0.03)
	HD	5	3759.43 (1448.22)	22.04 (12.17)	5420.56 (1824.81)	577.23 (129.09)	345.43 (175.22)	0.02 (0.01)	0.37 (0.16)
	SA	4	3530.38 (1030.55)	36.91 (3.78)	5204.78 (1268.9)	610.56 (155.63)	137.86 (56.5)	0.03 (0.03)	0.24 (0.09)
	SSe	5	2640.58 (830.5)	28.86 (9.09)	3974.61 (876.39)	332.25 (57.55)	20.84 (7.29)	0.04 (0.04)	0.05 (0.03)
Sado	DB	9	4298.52 (655.33)	59.63 (12.59)	5996.01 (808.83)	881.27 (150.44)	524.15 (239.72)	0.08 (0.03)	0.19 (0.1)
	HD	13	2903.53 (337.41)	45.03 (9.44)	5041.13 (701.69)	668.5 (96.68)	188.58 (42.32)	0.12 (0.02)	0.21 (0.05)
	SSo	2	2657.1 (129.24)	38.07 (4.82)	4197.46 (9.39)	1098.77 (39.46)	74.36 (7.22)	0.14 (0.05)	0.05 (0.05)
Tejo	DL	14	3964.56 (606.75)	72.04 (14.87)	5366.4 (775.4)	817.86 (140.24)	224.87 (48.58)	0.13 (0.05)	0.12 (0.04)
	HD	7	3972.06 (579.98)	101.2 (40.32)	6140.28 (984.43)	1044.95 (205.49)	112.54 (67.68)	0.39 (0.27)	0.2 (0.08)
	SSe	2	3071.79 (49.68)	34.85 (34.85)	5612.66 (62.53)	936.64 (184.08)	320.29 (165.14)	0.24 (0.06)	0.12 (0.02)
	SSo	16	3938.91 (398.17)	66.02 (17.7)	5378.76 (312.95)	721.98 (40.68)	174.14 (46.37)	0.21 (0.1)	0.13 (0.03)

(continued)

Loc	Sp	N	Mn	Fe	Co	Ni	Cu	Zn	As
Douro	DL	5	0.22 (0.02)	5.69 (1.93)	—	—	0.53 (0.15)	15.32 (3.15)	1.58 (0.44)
	PF	5	0.37 (0.17)	43.17 (29.48)	0.02 (0.02)	0.01 (0.01)	15.3 (12.39)	13.21 (4.77)	1.59 (0.41)
Mira	DL	2	0.09 (0.09)	4.1 (0.57)	—	—	0.15 (0.05)	4.49 (1.43)	0.48 (0.06)
	DS	2	0.09 (0.02)	1.21 (0.26)	—	0.01 (0.01)	0.06 (<0.001)	1.99 (0.24)	0.92 (0.68)
	DV	5	0.21 (0.07)	4.91 (1.07)	—	—	0.16 (0.02)	4.38 (0.57)	1.48 (0.56)
	HD	5	1.41 (0.64)	4.21 (1.81)	—	0.02 (0.02)	0.37 (0.15)	6.73 (2.38)	2.55 (0.91)
	SA	4	0.21 (0.08)	2.77 (0.89)	—	0.1 (0.06)	0.2 (0.06)	6.98 (1.85)	4.83 (1.64)
	SSe	5	0.12 (0.04)	3.42 (0.62)	—	0.02 (0.01)	0.41 (0.29)	4.18 (1.04)	1.43 (0.43)
Sado	DB	9	0.41 (0.12)	26.61 (18.54)	0.06 (0.03)	0.02 (0.01)	4.97 (3.82)	17.47 (3.34)	3.3 (0.88)
	HD	13	0.39 (0.06)	4.29 (0.73)	—	0.01 (0.01)	0.3 (0.05)	5.86 (0.75)	12.04 (2.01)
	SSo	2	0.28 (0.01)	4.37 (1.14)	—	—	0.22 (0.02)	6.2 (0.18)	4.28 (0.26)
Tejo	DL	14	0.34 (0.05)	15.22 (7.95)	0.01 (0.01)	0.04 (0.03)	8.21 (5.87)	8.45 (1.45)	1.11 (0.22)
	HD	7	1.89 (1.03)	63.65 (42.86)	0.11 (0.08)	0.71 (0.71)	2.93 (1.67)	14.35 (4.11)	7.71 (1.75)
	SSe	2	0.47 (<0.01)	4.31 (0.31)	—	—	0.26 (0.09)	7.37 (1.81)	2.66 (0.56)
	SSo	16	0.46 (0.09)	4.41 (0.37)	—	0.02 (0.01)	0.31 (0.04)	7.94 (0.86)	1.15 (0.11)

Table 3.2, (continued) Mean concentrations of muscle trace elements (µg g–1 dry weight) and standard errors (in brackets) in nine fish species collected in Portuguese estuaries. Estuaries: Douro, Mira, Sado and Tejo. Species: *D. labrax* (DL), *P.flesus* (PF), *D. sargus* (DS), *D. vulgaris* (DV), *H. didactylus* (HD), *S. aurata* (SA), *S. senegalensis* (SSe), *D. belotti* (DB), *S. solea* (SSo).

Loc	Sp	N	Se	Sr	Cd	Sn	Hg	Pb
Douro	DL	5	0.37 (0.09)	1.28 (0.26)	—	34.79 (7.42)	2.68 (0.58)	0.86 (0.61)
	PF	5	1.66 (0.91)	0.57 (0.2)	—	14.23 (1.44)	2.41 (1.28)	0.32 (0.13)
Mira	DL	2	0.19 (0.04)	0.4 (0.21)	0.33 (0.33)	32.41 (2.67)	0.86 (0.01)	0.81 (0.16)
	DS	2	0.06 (0.03)	0.19 (0.08)	0.21 (0.21)	12.61 (0.41)	0.42 (0.08)	0.28 (0.28)
	DV	5	0.21 (0.05)	0.86 (0.09)	0.29 (0.29)	27.04 (6.18)	1.76 (0.59)	0.73 (0.63)
	HD	5	0.46 (0.17)	4.81 (3.18)	0.17 (0.12)	27.36 (11.69)	4.3 (1.66)	1.1 (0.81)
	SA	4	0.46 (0.13)	1.18 (0.54)	—	16.98 (5.23)	1.06 (0.39)	0.28 (0.16)
	SSe	5	0.15 (0.03)	0.27 (0.07)	0.09 (0.09)	20.38 (3.88)	0.71 (0.11)	0.44 (0.18)
Sado	DB	9	0.78 (0.49)	8.19 (3.58)	0.1 (0.1)	39.19 (12.06)	2.82 (0.55)	1.64 (0.75)
	HD	13	0.22 (0.03)	2.32 (0.61)	0.08 (0.08)	31.05 (4.35)	2.62 (0.46)	0.7 (0.28)
	SSo	2	0.52 (0.11)	0.82 (0.18)	—	37.45 (6.65)	1.73 (0.17)	0.66 (0.03)
Tejo	DL	14	1.12 (0.55)	2.39 (0.59)	—	38.32 (5.76)	2.47 (0.38)	1.59 (0.34)
	HD	7	0.72 (0.3)	1.68 (0.76)	—	41.01 (9.81)	3.79 (0.83)	1.46 (1.23)
	SSe	2	0.31 (0.07)	3.02 (1.52)	—	30.94 (1.38)	1.65(<0.001)	0.11 (0.11)
	SSo	16	0.31 (0.03)	1.87 (0.43)	—	33.16 (6.18)	3.07 (0.32)	1.6 (0.41)

The size of fish was not correlated with the concentrations of statistically important elements in the muscle. However, sizes varied among species and estuary (Table 3.3).

Table 3.3 Total length and weight means and standard errors (in brackets) of the individuals from each species and location sampled. Estuaries: Douro, Mira, Sado and Tejo. Species: *D. labrax* (DL), *P.flesus* (PF), *D. sargus* (DS), *D. vulgaris* (DV), *H. didactylus* (HD), *S. aurata* (SA), *S. senegalensis* (SSe), *D. belotti* (DB), *S. solea* (SSo).

		Muscle	Liver		
Loc	Sp	N	N	Lt (cm)	Wt (g)
Douro	DL	5	5	28.96 (0.71)	256.19 (10.62)
	PF	5	5	28.32 (0.466)	277.35 (15.33)
Mira	DL	2	2	30.95 (1.65)	324.92 (51.11)
	DS	2	2	21.15 (0.35)	185.19 (1.94)
	DV	5	5	13.78 (0.244)	41.14 (2.5)
	HD	5	5	12.48 (1.821)	44.35 (20.97)
	SA	4	4	22.775 (1.001)	174.45 (26.02)
	SSe	5	3	15.04 (1.211)	36.81 (10.33)
Sado	DB	10	10	16.28 (0.494)	79.57 (8.16)
	HD	13	11	15.277 (1.624)	94.65 (26.37)
	SSo	2	2	24.4 (2.6)	130.47 (34.96)
Tejo	DL	14	14	24.293 (2.632)	200.08 (46.85)
	HD	7	6	23.129 (2.464)	284.09 (64.43)
	SSo	2	2	10.35 (2.25)	12.04 (7.59)
	SSe	16		10.813 (0.959)	17.72 (6.23)

Ratios calculated using food safety guidelines assessed fish contamination in different estuaries and species for Cu, Zn, Cd, Hg and Pb. The value 1 implies the guideline matches our concentrations in a given metal. Above this mark, the presence of elements is considered dangerous to fish and human life. Metal ratios above 1 were observed for Hg in *D. bellottii, D. labrax, H. didactylus* and *S. solea*, Pb in *D. bellottii, D. labrax* and *S. solea*, and Cd in *D. sargus* (Figure 3.8). Fish from Tejo surpassed thresholds of Hg and Pb, whereas in Sado only the metal Hg was elevated (Figure 3.9). The elements Cu and Zn were consistently below harmful levels in all estuaries and species (Figure 3.10 and 3.11).

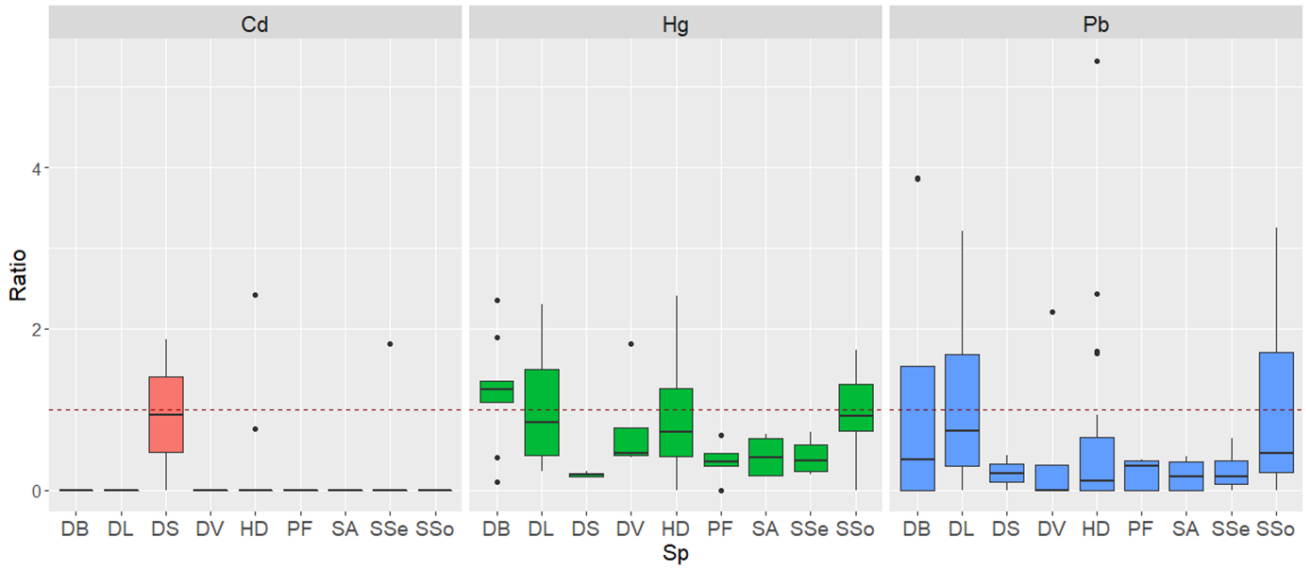

Figure 3.8 Boxplots depicting metal ratios in muscle of all species analyzed, calculated using European Union food safety standards from the COMMISSION REGULATION (EC) No 1881/2006. Dotted line indicates a ratio of 1. Estuaries: Douro, Mira, Sado and Tejo. Species: *D. labrax* (DL), *P.flesus* (PF), *D. sargus* (DS), *D. vulgaris* (DV), *H. didactylus* (HD), *S. aurata* (SA), *S. senegalensis* (SSe), *D. belotti* (DB), *S. solea* (SSo).

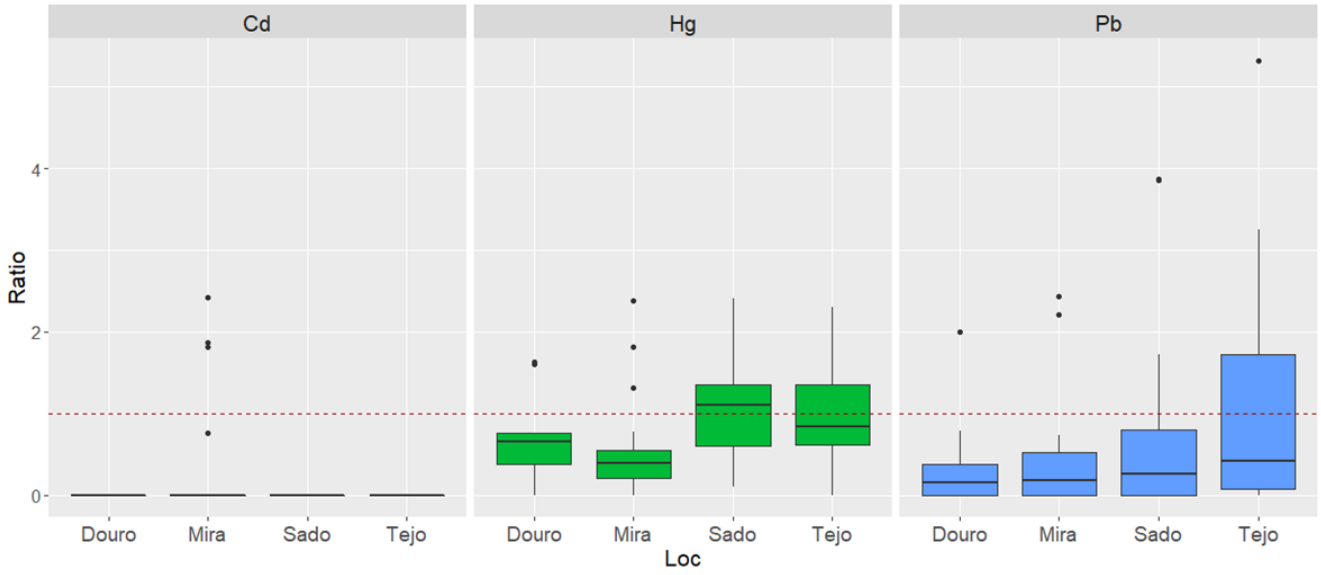

Figure 3.9 Boxplots depicting metal ratios in muscle of fish from all estuaries analyzed, calculated using European Union food safety standards from the COMMISSION REGULATION (EC) No 1881/2006 (European Commission, 2006b). Dotted line indicates a ratio of 1. Estuaries: Douro, Mira, Sado and Tejo. Species: *D. labrax* (DL), *P.flesus* (PF), *D. sargus* (DS), *D. vulgaris* (DV), *H. didactylus* (HD), *S. aurata* (SA), *S. senegalensis* (SSe), *D. belotti* (DB), *S. solea* (SSo).

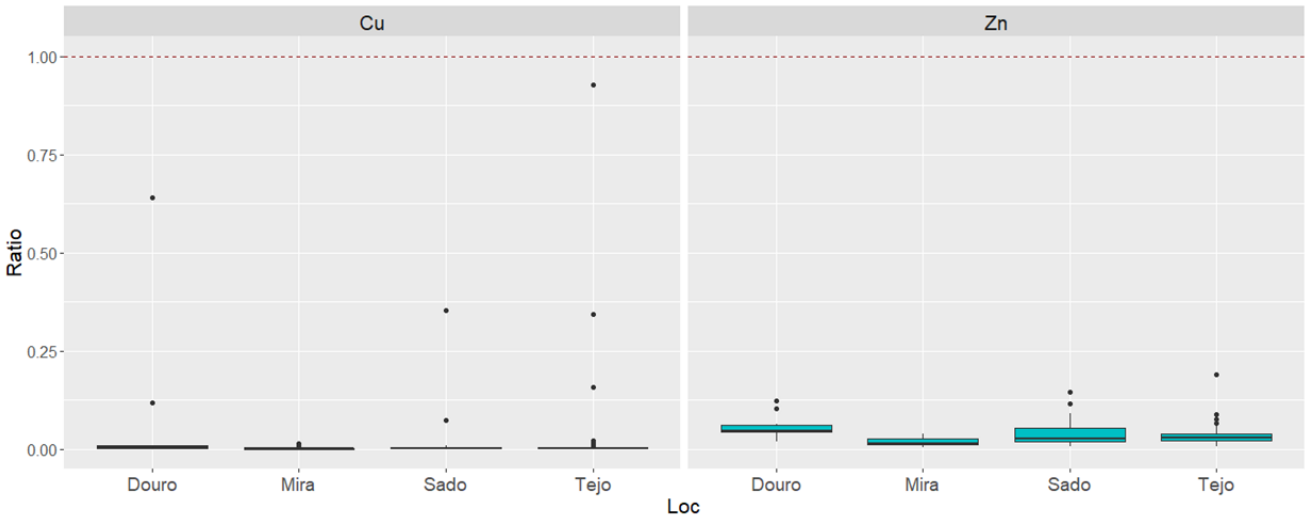

Figure 3.10 Boxplots depicting metal ratios in muscle of fish from all estuaries analyzed, calculated using guidelines from the United Kingdom Ministry of Agriculture, Fisheries and Food (MAFF, 1995). Dotted line indicates a ratio of 1. Estuaries: Douro, Mira, Sado and Tejo. Species: *D. labrax* (DL), *P.flesus* (PF), *D. sargus* (DS), *D. vulgaris* (DV), *H. didactylus* (HD), *S. aurata* (SA), *S. senegalensis* (SSe), *D. belotti* (DB), *S. solea* (SSo).

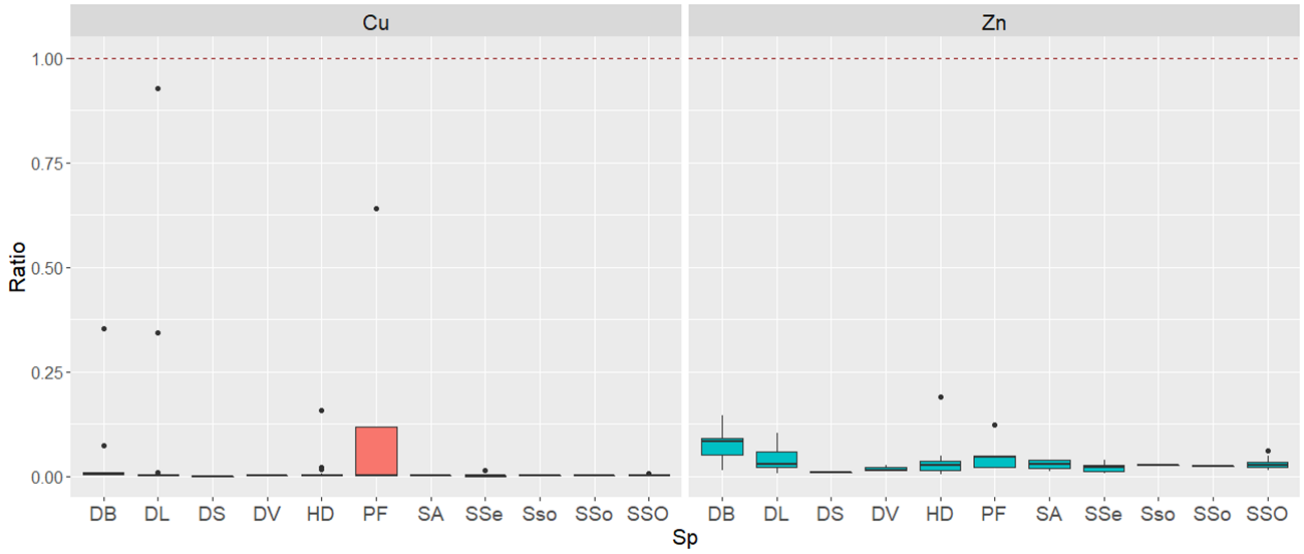

Figure 3.11 Boxplots depicting metal ratios in muscle of all fish species analyzed, calculated using guidelines from the United Kingdom Ministry of Agriculture, Fisheries and Food (MAFF, 1995). Dotted line indicates a ratio of 1. Estuaries: Douro, Mira, Sado and Tejo. Species: *D. labrax* (DL), *P.flesus* (PF), *D. sargus* (DS), *D. vulgaris* (DV), *H. didactylus* (HD), *S. aurata* (SA), *S. senegalensis* (SSe), *D. belotti* (DB), *S. solea* (SSo).

3.4.3 Liver elemental analyses

Liver chemical composition generally evidenced the most perceptible elemental differences among estuary and species groups. CAP model with both species and estuaries displayed an accuracy of 39.474% in the assignment of samples to a priori groups (Figure 3.12). Data pooled from *H. didactylus* was distributed by estuary of origin with a 77.273% accuracy (Figure 3.13), whereas *D. labrax* data was discerned with 57.143% accuracy (Figure 3.14). Liver elemental composition separated species within the Mira estuary with 52.381% accuracy (Figure 3.15), and Tejo species with a 72.727% accuracy (Figure 3.16).

The multivariate assessment of similarities in CAPs with an accuracy above 65%, was performed for *H. didactylus* and Tejo data. There were no significant differences detected using ANOSIM. SIMPER results also showed low dissimilarity values. Dissimilarity percentages observed between *H. didactylus* captured in different estuaries were the following: 40.03% for Sado and Tejo, 40.48% for Tejo and Mira, and 40% for Mira and Sado. Regarding Tejo data, percentages were: 37.12% for *D. labrax* and *S. solea*, 40.15% for *D. labrax* and *H. didactylus*, and 33.80% for *S. solea* and *H. didactylus*. Individual elements contributed very little to the differentiation of groups. At least eight elements out of the sixteen, were usually required to reach a contribution of 50% to the dissimilarity among groups. The elements frequently shown to contribute the most for differences among groups in *H. didactylus* data were Pb, Al, V, Mn and Fe, and for Tejo data, Sn, As, Al and Mn.

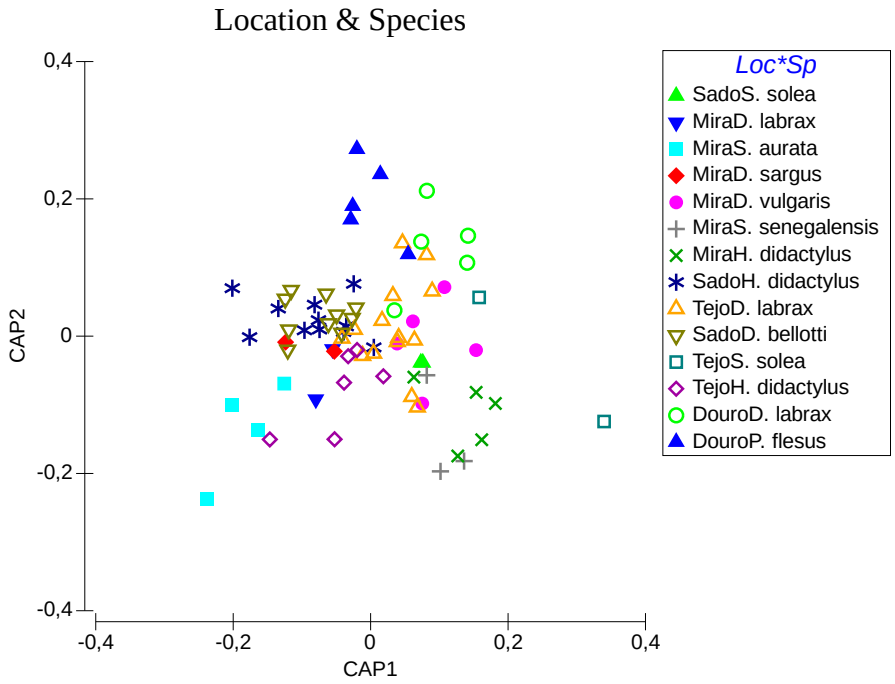

Figure 3.12 Canonical Analysis of Principal coordinates (CAP), showing the grouping of species and estuaries based on chemical elements found in fish liver samples from different estuaries. Sites: Sado, Tejo, Douro and Mira, including the species: *Diplodus vulgaris*, *D. bellottii*, *D. sargus*, *Platichthys flesus*, *Solea senegalensis*, *S. solea*, *Dicentrarchus labrax*, *Sparus aurata* and *Halobatrachus didactylus*

H. didactylus

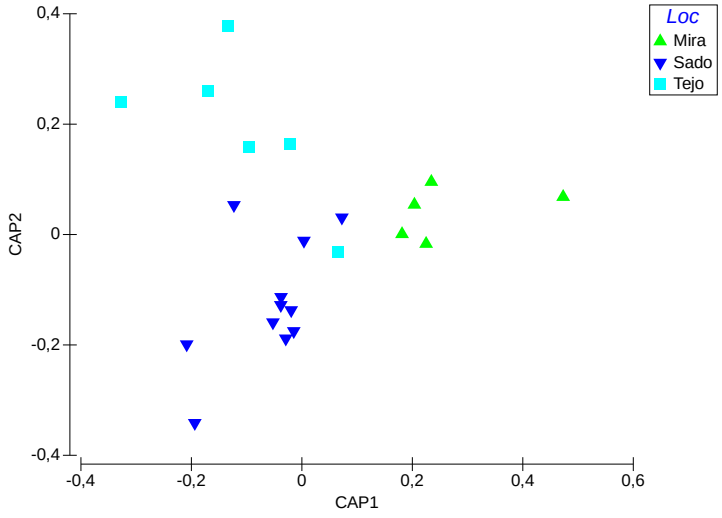

Figure 3.13 Canonical Analysis of Principal coordinates (CAP), showing the grouping of *Halobatrachus didactylus* from different estuaries based on chemical elements found in fish liver samples. Sites: Tejo, Sado and Mira.

D. Labrax

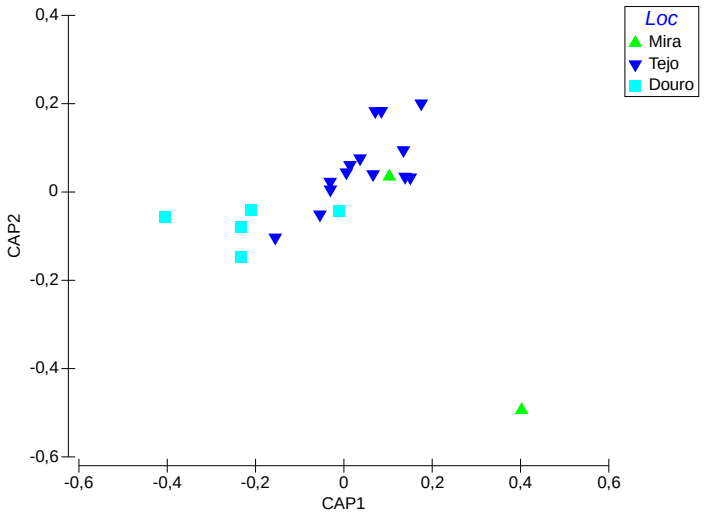

Figure 3.14 Canonical Analysis of Principal coordinates (CAP), showing the grouping of *Dicentrarchus labrax* from different estuaries based on chemical elements found in fish liver

Mira

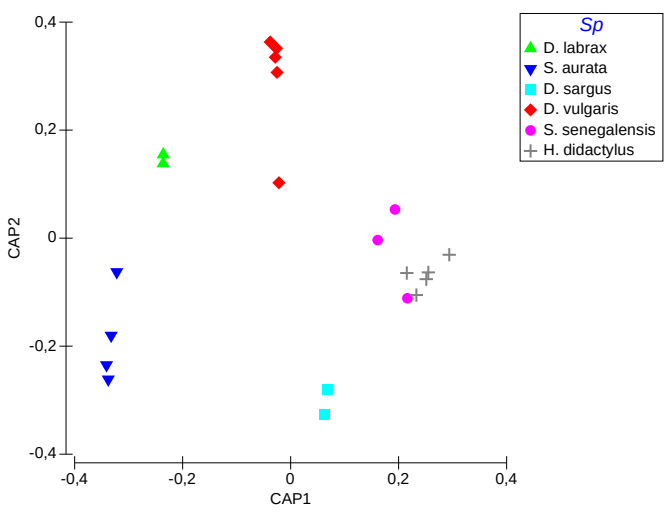

Figure 3.15 Canonical Analysis of Principal coordinates (CAP), showing the muscle grouping of the species: *Solea senegalensis, Diplodus vulgaris, Dicentrarchus labrax* and *Halobatrachus didactylus and D. sargus* from Mira, based on chemical elements found in fish liver samples

Tejo

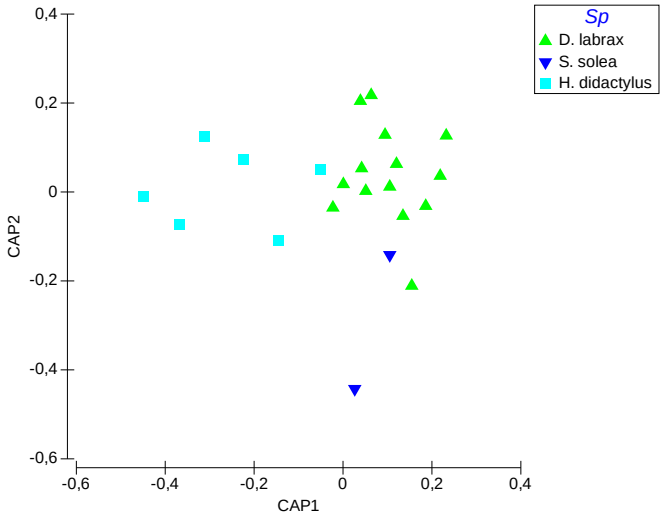

Figure 3.16 Canonical Analysis of Principal coordinates (CAP), showing the muscle grouping of the species *Solea. solea, Dicentrarchus labrax,* and *Halobatrachus didactylus* from Tejo based on chemical elements found in fish liver samples

Elements responsible for the variation between estuary groups were further explored using Kruskal Wallis tests. Elemental composition of fish liver samples is presented in Table 3.4. Overall, the elements responsible for the variations among all species were V (H=17.2, p=0.022), Mn (H=20.67, p=0.008) and As (H=29.23, p<0.001) whereas only As (H=21.72, p<0.001) and Hg (H=9.887, p=0.019) values varied between the entirety of estuaries.

Differences among estuaries were observed for two species: *D. labrax* and *H. didactylus*. The metal As was responsible for the differences in *D. labrax* elemental signatures (H=6.02, p=0.049) between Mira and Douro (p=0.024). Fluctuations in *H. didactylus* liver composition between Sado, Tejo and Mira were attributed to the elements Mn (H=7.199, p=0.027), Fe (H=7.038, p=0.029), As (H=7.773, p=0.021) and Pb (H=6.162, p=0.045). The highest concentrations of the previously mentioned metals were recorded in the *H. dicatylus* from Tejo (p<0.031).

Within the same estuarine system, variations among species were also confirmed. Ca (H=3.938, p=0.047) and Mn (H=3.938, p=0.047) concentrations varied between *P. flesus* and *D.labrax* in Douro. Levels of Mn (H=7.998, p=0.018) and As (H=8.497, p=0.014) differentiated *H. didactylus* elemental signatures from other species in Tejo. In Mira concentrations of Fe (H=15.51, p=0.008) in *H. didactylus* were low compared to the other fish.

Table 3.4, Mean concentrations of liver trace elements (µg g–1 dry weight) and standard errors (in brackets) in nine fish species collected in Portuguese estuaries. Estuaries: Douro, Mira, Sado and Tejo. Species: *D. labrax* (DL), *P.flesus* (PF), *D. sargus* (DS), *D. vulgaris* (DV), *H. didactylus* (HD), *S. aurata* (SA), *S. senegalensis* (SSe), *D. belotti* (DB), *S. solea* (SSo).

Loc	Sp	N	Na	Al	Cl	K	Ca	V	Cr
Douro	DL	5	3277.201 (533.901)	36.55 (3.342)	4694.089 (883.031)	690.062 (68.356)	65.905 (14.219)	0.172 (0.088)	0.087(0.07)
	PF	5	5536.082 (1033.666)	43.102 (22.821)	6620.194 (642.868)	621.086 (119.229)	26.092 (8.641)	0.05 (0.05)	0.034(0.034)
Mira	DL	2	2125.572 (440.219)	54.16 (29.84)	4302.427 (169.424)	656.148 (166.688)	16.07 (1.025)	0.186 (0.037)	—
	DS	2	3764.187 (362.853)	47.262 (47.262)	5565.677 (971.355)	899.738 (574.241)	93.66 (89.455)	0.099 (0.099)	—
	DV	5	3484.606 (1012.073)	131.765 (51.078)	5888.066 (1676.995)	901.474 (142.152)	44.273 (13.082)	0.283 (0.074)	0.204(0.19)
	HD	5	4551.147 (1560.678)	212.815 (163.726)	5609.664 (720.327)	539.605 (160.583)	12.495 (4.342)	0.088 (0.038)	0.069(0.03)
	SA	4	3662.979 (848.877)	95.33 (58.07)	5050.904 (974.193)	946.008 (186.658)	17.485 (1.855)	0.087 (0.053)	0.088(0.068)
	SS	3	3556.557 (366.045)	66.37 (13.861)	8024.594 (538.936)	882.699 (121.886)	48.513 (10.096)	0.027 (0.027)	0.155(0.079)
Sado	DB	10	5368.907 (1281.027)	103.732 (61.763)	5319.27 (954.071)	538.34 (84.42)	50.364 (24.059)	0.058 (0.031)	—
	HD	11	3380.775 (597.204)	81.138 (23.292)	4454.801 (646.554)	656.27 (163.559)	76.537 (33.161)	0.132 (0.04)	0.097(0.062)
	SSo	2	4130.099 (2325.406)	70.683 (34.841)	6179.135 (2423.255)	867.369 (294.728)	30.408 (14.757)	0.196 (0.094)	0.19(0.19)
Tejo	DL	14	3443.78 (964.878)	28.516 (8.11)	4865.008 (1071.57)	623.256 (78.985)	58.962 (24.44)	0.215 (0.067)	0.074(0.037)
	HD	6	3519.006 (1140.192)	62.279 (36.352)	4869.595 (1078.295)	692.786 (130.094)	36.895 (12.871)	0.481 (0.267)	0.04(0.04)
	SSo	2	5303.717 (835.838)	100.125 (43.83)	6570.45 (671.43)	922.191 (340.642)	52.509 (43.034)	0.589 (0.205)	0.154(0.154)

(continued)

Loc	Sp	N	Mn	Fe	Co	Ni	Cu	Zn	As
Douro	DL	5	1.13 (0.35)	29.812 (10.743)	0.102 (0.042)	0.04 (0.03)	46.198 (20.593)	33.172 (10.47)	2.193 (0.599)
	PF	5	0.307 (0.102)	59.871 (17.576)	0.431 (0.122)	0.011 (0.011)	12.414 (3.263)	38.834 (8.013)	1.336 (0.111)
Mira	DL	2	0.571 (0.026)	77.246 (16.321)	—	0.224 (0.224)	9.018 (2.116)	17.719 (1.564)	0.437 (0.041)
	DS	2	2.315 (1.942)	149.651 (90.531)	0.261 (0.137)	—	29.253 (21.996)	45.009 (21.677)	3.005 (1.479)
	DV	5	1.629 (0.314)	62.225 (14.06)	0.093 (0.072)	0.036 (0.025)	8.99 (4.277)	25.267 (6.417)	1.465 (0.386)
	HD	5	1.028 (0.25)	13.458 (1.49)	—	—	3.822 (0.799)	14.711 (1.036)	1.351 (0.304)
	SA	4	1.208 (0.249)	191.156 (35.706)	0.085 (0.085)	0.005 (0.005)	5.811 (1.12)	29.243 (5.825)	2.452 (0.692)
	SS	3	1.759 (0.199)	62.078 (16.627)	—	0.042 (0.024)	10.192 (7.566)	16.631 (3.916)	0.865 (0.146)
Sado	DB	10	0.957 (0.155)	93.582 (17.227)	0.251 (0.099)	0.018 (0.017)	6.947 (1.532)	33.576 (4.575)	4.412 (0.861)
	HD	11	0.901 (0.199)	55.269 (9.247)	0.101 (0.042)	0.047 (0.029)	11.088 (2.356)	25.416 (3.693)	6.279 (1.419)
	SSo	2	1.518 (0.511)	69.651 (25.224)	0.127 (0.127)	0.045 (0.045)	20.362 (8.5)	20.206 (8.207)	2.48 (0.978)
Tejo	DL	14	0.756 (0.153)	91.086 (19.813)	0.141 (0.048)	0.024 (0.007)	40.011 (14.429)	22.698 (3.161)	1.255 (0.389)
	HD	6	1.924 (0.292)	85.429 (28.181)	0.101 (0.047)	—	8.581 (2.412)	21.556 (5.528)	7.968 (2.303)
	SSo	2	1.94 (1.287)	44.928 (3.125)	0.173 (0.173)	—	43.709 (13.284)	22.459 (3.412)	0.689 (0.131)

Table 3.4 (continued) Mean concentrations of liver trace elements (µg g–1 dry weight) and standard errors (in brackets) in nine fish species collected in Portuguese estuaries. Estuaries: Douro, Mira, Sado and Tejo. Species: *D. labrax* (DL), *P.flesus* (PF), *D. sargus* (DS), *D. vulgaris* (DV), *H. didactylus* (HD), *S. aurata* (SA), *S. senegalensis* (SSe), *D. belotti* (DB), *S. solea* (SSo).

Loc	Sp	N	Se	Sr	Cd	Sn	Hg	Pb
Douro	DL	5	3.535 (1.286)	0.846 (0.194)	—	26.346 (8.224)	4.766 (1.135)	1.529 (0.632)
	PF	5	0.81 (0.177)	0.963 (0.231)	—	28.078 (2.248)	5.191 (1.254)	2.339 (0.788)
Mira	DL	2	1.378 (0.562)	0.325 (0.152)	—	29.404 (7.163)	2.511 (0.086)	0.507 (0.507)
	DS	2	1.107 (0.539)	1.898 (0.94)	—	33.945 (10.578)	4.052 (0.716)	0.5 (0.387)
	DV	5	1.147 (0.278)	0.784 (0.206)	—	40.174 (8.421)	4.612 (1.244)	3.13 (1.047)
	HD	5	1.297 (0.354)	0.738 (0.223)	0.402 (0.402)	32.582 (5.663)	3.68 (0.206)	4.698 (1.528)
	SA	4	1.563 (0.298)	0.555 (0.079)	—	35.937 (7.984)	4.57 (0.921)	1.616 (1.616)
	SS	3	0.723 (0.239)	0.535 (0.151)	—	37.443 (4.75)	4.316 (1.125)	2.001 (1.21)
Sado	DB	10	1.597 (0.323)	1.362 (0.311)	—	22.833 (3.907)	8.613 (2.367)	1.304 (0.665)
	HD	11	0.988 (0.154)	1.245 (0.365)	0.333 (0.333)	31.73 (5.178)	5.229 (0.639)	1.56 (0.401)
	SSo	2	1.662 (0.04)	0.6 (0.396)	—	19.181 (4.144)	6.484 (2.353)	0.649 (0.649)
Tejo	DL	14	3.445 (0.945)	1.199 (0.429)	—	26.899 (6.034)	3.452 (0.456)	0.767 (0.221)
	HD	6	0.84 (0.188)	1.101 (0.298)	—	30.857 (10.026)	4.671 (0.999)	0.894 (0.514)
	SSo	2	1.893 (0.413)	1.592 (0.458)	—	68.287 (35.94)	3.846 (0.252)	0.125 (0.125)

3.5 Discussion

Multi-elemental signatures in fish soft tissues generally allowed the discrimination of their estuary of origin and among species, albeit with different degrees of efficiency. Still, an intense overlap between elemental signatures was observed, when classifying fish with ordination models considering both species and estuaries. However, assessing the two factors independently yielded clear discrimination results, which revealed that fish samples were assigned to a priori estuary groups with a noticeable higher accuracy (range: 57%-77%) than to species groups (34%-72%). This was evidenced in both muscle and liver samples, which might suggest that elemental signatures are mainly driven by the environment, and secondarily by inter-species differences. However, water metal concentrations didn't reflect fish metal burden, likely due to the dynamic nature of estuaries and to the fact that water sampling was a single event in time and may vary greatly throughout fishes' life.

Metal distribution and bioavailability in water is controlled by several environmental factors, among them anthropogenic, hydrological, physicochemical, and geomorphological features. According to previous studies in our assessed Portuguese estuaries, all the selected systems have been found to potentially contain metal concentrations denoting concerning environmental

pollution in various ecologically important areas: Tejo (Duarte *et al.*, 2014), Sado (Serafim *et al.*, 2013), Douro (Ribeiro *et al.*, 2018) and Mira (Chainho *et al.*, 2008). However, it is comprehensively understood that Tejo and Sado are the most anthropogenically pressured and metal polluted systems, whereas Mira and Douro are the least pressured and polluted (Vasconcelos *et al.*, 2007; Serafim *et al.*, 2013). In the present study, water metal content in Tejo and Douro was below or within the ranges reported by other studies in the same estuaries (Duarte *et al.*, 2014; Ribeiro *et al.*, 2018). Yet, there is an overall lack of studies analysing metal in water, thereby no references suitable for comparison were found for Mira and Sado estuarine systems.

Contradictory patterns were observed in our results, and harmful metals, usually sourced by human activity, were more abundant in waters of the previously mentioned least polluted estuaries, namely Douro (highest concentrations of Cd and Hg) and Mira (highest concentrations of Cu and Pb). According to European Union guidelines, Hg levels in the water of all estuaries was above the limit imposed for surface waters in Directive 2000/60/EC (maximum allowable concentration, 0.07 $\mu g l^{-1}$) (European Commission, 2006). In our study, waters were indiscriminately sampled along the estuarine gradient. As such, water analysis displays a generalized representation of the elemental content in the several characteristically mutable estuarine environments, and signatures are prone to have poorly defined patterns. Supporting this argument is our CAP analysis of water multi-elemental concentration, which showed a considerable overlap of elemental signatures, indicating a lack of distinguishable differences (or high variability within each estuary) among the chemical composition of the various estuary systems. Due to high water exchange rates in estuaries (e.g. tidal flow, wave action, freshwater influx) and spatiotemporal fluctuations in physicochemical parameters (e.g. organic matter, salinity and temperature), metal concentrations in water are constantly changing and in contrast to biota metal burden, may not provide an accurate account of estuary pollution (Adams & Tremblay, 2003; de Souza Machado *et al.*, 2016). Biological indicators such as fish, continuously store pollutants proportionally to their availability in the environment, but unlike water retain them for long periods of time integrating past events of pollution in estuaries (Whitfield & Harrison, 2014). However, it is necessary to explore biological factors influencing fish metal uptake from the environment to justify variability in tissue composition (Wood, 2011).

It is evident in our study, and confirmed in several other investigations, that various factors such as physiological, behavioral and life strategy differences among fish species have critical effects in tissue metal composition (Durrieu *et al.*, 2005; França *et al.*, 2005; Pedro *et al.*, 2015). The ordination of the liver subset data pooled from Tejo showed that the species *D. labrax*, *S. solea* and *H. didactylus* had very distinct metal content in their tissue and could be assigned to their respective species groups with a high precision of 72,727%. In this particular case, the success in separation can either be strongly attributed to the inherent accumulation differences of physiological distinct organisms or a consequence of life strategies. Estuarine resident species such as *H. didactylus* spend their entire life cycle in estuaries, where they perpetually accumulate metal loads under very restrictive conditions, whereas marine migrant species like *S. solea* and *D. labrax*, temporarily reside in estuaries, mostly during juvenile phases, and are exposed to different contaminated waters throughout their life (Vasconcelos *et al.*, 2010). In some studies, it is evidenced that species that use estuaries temporarily, possess lower contamination levels than long-term residents (Usero *et al.*, 2003; Henry *et al.*, 2004; Durrieu *et al.*, 2005). As such it is fair to assume that estuarine residents accumulate concentrations of pollutants that reflect more accurately estuarine metal content than marine migrants. In fact, this phenomenon might justify the results between the liver CAP assessing the data pooled from *H. didactylus* and *D. labrax*. The species *H. didactylus* was distributed between the estuaries Mira, Tejo and Douro with a higher accuracy of 77,273% than *D. labrax*, which correctly classified 57,143% samples.

Moreover, the size of the estuary could be another factor influencing fish metal content. The elemental signatures in fish from Tejo probably allowed for such a high degree of distinction among species because this estuary is a large system and organisms can have a broader selection of habitats with very distinct physicochemical conditions (França *et al.*, 2010). Comparatively, ordinations including muscle and liver from fish inhabiting the relatively small Mira, assessed a wider range of species, namely *D. labrax*, *H. didactylus*, *S. aurata*, *D. sargus*, *D. vulgaris* and *S. senegalensis*, and produced separations based on species groups with lower percentages of correctly classified samples (52.381%). However, it is possible that the low number of species groups in Tejo or the reduced number of individuals sampled in Mira interfered with the precision of CAPS and produced misleading results. A smaller variety of species entails less variability allowing for a higher likelihood that individuals are assorted correctly, and low sample replicates introduces biased elemental concentrations.

Despite the fact that fish strongly manifest elemental patterns according to their constant exposure to the environment and biological features throughout their lifetime, metal levels are expressed differently in several tissues (Zhang & Wang, 2007). Unlike the metabolically inert calcified tissues (e.g. otolith and scales) in organisms, soft tissues (e.g. liver, muscle and gonads) are directly involved in biochemical processes that affect the rate of elemental accumulation from the environment and ultimately drive their capability of representing the identity of species and estuaries accurately (MacNeil *et al.*, 2006; Tzadik *et al.*, 2017). The elemental storage, bio-transformative and excretion properties of liver seem to confer this tissue a unique selective accumulation of elements that produces better defined signatures than muscle in our study. Liver samples generally expressed the differences in elemental composition among estuaries for a given species, and among fish species, better than muscle samples, and correctly matched a superior number of individuals with their respective identities and origins, highlighting the differential suitability of the two tissue signatures in the identification of fish provenance and in recording taxa specific biochemical patterns. Previous research has only concretely shown that it is possible to characterize groups of environments using the elemental variation in muscle with both highly metabolically regulated elements and non-essential elements (Chaguri *et al.*, 2015; Li *et al.*, 2016). The applicability of liver to accomplish the same ends is largely unexplored. However, several ecotoxicological studies have concluded that liver, due to its crucial role in detoxification, accumulates greater concentrations of trace elements than muscle, especially non-essential metals that pose a great toxicity risk to aquatic life, generally reflecting environmental metal pollution more accurately (Fernandes *et al.*, 2007; Mohammadnabizadeh *et al.*, 2014). Similar patterns emerged in our results, and liver concentrations of essential and non-essential metals such as the highly toxic Pb and Hg were observed to be generally far greater than in muscle.

Differences in liver tissue composition among fish species and individuals from different estuaries relied on a very small number of non-essential elements namely V, As, Hg and Pb, with the only exceptions being Mn and Fe which are essential. Conversely, a generally wider range and larger number of elements in muscle contributed to the differentiation of both estuaries and species. Specifically, the essential chemical compounds Fe, Se, Cl, K, Zn, Mn, Cu and Co, and the non-essential As, Sr, Sn, Al. This is probably another outcome of the metabolic differences in tissues given that liver can reduce its elemental burden through detoxification processes, but also accumulate more specifically certain compounds. Noteworthy, non-essential elements in the liver

with a great degree of toxicity (Hg and Pb), and therefore highly accumulated in this tissue, appeared to play a significant larger role in differentiating among the elemental composition of fish species and individuals from distinct estuaries. Although the elemental signatures used to separate groups were very variable, there was some consistency in the importance of some chemical compounds in predictive models. The discrimination of liver signatures seemed to stem from the differences in Mn and Al content, while in muscle it was from Zn, Cd and As variations.

As proposed for otolith natural tags, muscle and liver elemental fingerprints must meet certain criteria to be viable natural tags. 1) Elemental signatures must have distinguishable and highly reproducible traits 2) able to persist over long periods of time without suffering major changes and 3) attain the characterization of all subjects considered in a study (Campana et al., 2000). Although our results can vouch for the first two assumptions, soft tissue temporal stability is weak. Elemental composition in muscle has been shown to have seasonal variability (Chapter 2), but it remains to be properly assessed to what extent it affects discrimination of estuarine provenance, i.e. if seasonal variability is significantly lower than spatial variability. In a provenance study by Kim *et al.* (2010), small yearly and seasonal differences in muscle elemental composition were detected among wild and farmed salmon from the Pacific Ocean, sampled between 2006 and 2008. These temporal fluctuations were beyond the scope of the authors objectives and so they were only briefly addressed. In a work conducted by Chaguri *et al.* (2015) seasonality seemed to have a strong effect on the separation of marine wild Whitemouth Croaker individuals from different origins in Brazil. Additionally, this species life strategies in estuaries is similar to marine migrant species, thereby the majority of fish in our study such as *D. labrax*, *S. solea* and *D. sargus* might have had their elemental composition altered between seasons. Liver elemental composition may also display seasonal patterns, in addition to strong shifts due to ontogenic development, such as the onset of maturation and the reproduction cycle. For instance, the male and female individuals belonging to the species *H. didactylus* from Tagus estuary in Pedro *et al.* (2015) were observed to accumulate metal in tissues differently among each other and during their reproductive period. Therefore, understanding the seasonal and yearly patterns can attenuate the precision dampening effects of variability on predictive models using elemental profiles (Reis-Santos *et al.*, 2012). Due to the lack of attempts to study time as a variable in the discrimination power of soft tissue elemental composition, it is advisable to explore this variable at multiple levels in future research, especially in liver.

The present results seem to confirm that the level of pollution in estuaries can be more efficiently reflected in fish metal burden than in water content, by displaying the highest concentrations of the statistically significant hazardous elements As and Hg in Sado and Al in Tejo, which are the most pressured and metal polluted environments amidst our selected estuaries (Vasconcelos *et al.*, 2007). The ratios calculated with the levels of Cd, Hg and Pb to assess muscle contamination were above the legal limits proposed by the European Commission in some estuaries and species (European Commission, 2006). They provide strong evidence of the effects life strategies and estuarine pressures have on muscle contamination. The most anthropogenically affected estuaries Sado and Tejo (mainly by industrial and urban wastewater) had the highest amounts of Hg and Pb (Vansconcelos *et al.*, 2007). The fish that accumulated the highest concentrations of Pb and Hg were from species that are strongly exposed to the sediment and usually feed strictly on invertebrates (Reis-Santos *et al.*, 2008; França *et al.*, 2009) namely *D. labrax*, *S. solea* and *H. didactylus*, which highlights the relevance this environmental compartment as a potential source of metals (França *et al.*, 2009; Vasconcelos *et al.*, 2011; Pedro *et al.*, 2015; Polak-Juszczak, 2017). Although still not fully understood, fish dietary metal uptake from contaminated prey is a relevant source of metal in muscle (Barwick & Maher, 2003). The transference of metal through the food chain subsequently leads to the biomagnification of these pollutants in top predators such as fish. In our selected estuaries, notably Douro, Tejo and Mira concentrations of metals in benthic invertebrates are known to change among habitats and species as well as seasonally, potentially introducing another important degree of variability in fish metal composition (França *et al.*, 2004; Mucha *et al.*, 2005; Rodrigues *et al.*, 2014). Furthermore, the zinc and copper ratios calculated with the guidelines established by the United Kingdom Ministry of Agriculture, Fisheries and Food, were all below the danger threshold for human health, regardless of estuarine origin or species (MAFF, 1995).

In conclusion, fish tissue (muscle and liver) multi-elemental analysis using TXRF provides an effective method to determine metal pollution levels in estuarine systems. Elemental signatures gathered from such approaches can effectively characterize the environment individuals inhabited for the majority of their life possibly allowing for a serviceable traceability tool for aquatic organisms in dynamic ecosystems like estuaries. However, the unpredictability of the water chemical content, the pronounced differences among species elemental composition from the same estuary and the different organ affinity to chemical compounds observed in this study underline

the potential limitations of liver and muscle elemental profiling in predicting the provenance of organisms. Biogeochemical processes are bound to alter over time and spatially the signatures in the ecosystem, therefore, future endeavors assessing the quality of provenance tools derived from muscle and liver should attempt to measure the effect of abiotic and biotic variables in the discrimination power of elemental signatures.

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