Multi-element fingerprint to discriminate the geographical origin of *Ruditapes philippinarum* (Adams & Reeve, 1850) of the Tagus and Sado estuaries.

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Resumo

As técnicas de rastreamento podem ser uma ferramenta analítica importante na determinação da origem geográfica dos bivalves. O objetivo deste estudo foi distinguir as populações de *Ruditapes philippinarum* entre os estuários do Tejo e do Sado, através da determinação do perfil químico de origem. Para avaliar a variabilidade espacial do padrão químico da amêijoa, foram escolhidos em cada estuário três locais de amostragem com caraterísticas hidrológicas e geomorfológicas diferentes. A técnica ICP-MS permitiu determinar a concentração de 16 elementos traço (Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Cd, Ce e Eu) nos tecidos moles da amêijoa. A PCA foi utilizada para a ordenação de dados e as hipóteses foram testadas com PERMANOVA, complementado com o método Monte Carlo. O modelo utilizado foi, dois fatores: fator "Estuário" (2 níveis fixos); fator "Local" (6 níveis fixos aninhados a "Estuário"), $p < 0.05$. A ordenação mostrou a separação entre as amostras dos estuários Tejo e Sado, sendo Zn, Mn, Cr, Co, Cu, Se e Ce os elementos que melhor explicam a separação. Detetaram-se diferenças significativas entre estuários e entre os locais Gâmbia e Herdade do Pinheiro. O sedimento dos dois estuários apresentaram padrões de distribuição espacial semelhantes, onde as amostras a montante registaram concentrações elementares mais elevadas dos que as amostras a jusante. Foi possível obter um "*fingerprint"* de origem geográfica entre os estuários do Tejo e do Sado. O *"fingerprint"* químico que permite identificar as ameijoas do estuário Tejo caracteriza-se pela dominância de Zn, Co, Se e Ce e o *"fingerprint"* químico que permite identificar as do estuário do Sado caracteriza-se pela dominância de Cr e Cu.

É essencial avaliar a estabilidade dos perfis químicos para além da influência dos fatores intrínsecos e extrínsecos de modo a garantir a reprodutibilidade da ferramenta de rastreabilidade.

Palavras-chave: Rastreabilidade; Perfil químico de origem; *Ruditapes philippinarum*; Estuário do Tejo; Estuário do Sado.

Abstract

Traceability techniques could be an important analytical tool to determine bivalve's geographical origin. The aim of this study was determination of a chemical fingerprint to discriminate the *Ruditapes philippinarum* of the Tagus and Sado estuaries. The spatial variability patterns of clam´s chemical profile were assessed, collecting samples of three sites with different hydrological and geomorphological characteristics in each estuary. The contents of 16 trace-elements (Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Cd, Ce and Eu) in clam's soft tissue were determined by inductively coupled plasma mass spectrometry. Principal component analysis was used in data ordination, and the hypothesis were tested with permutational multivariate analysis of variance, pairwise PERMANOVA and Monte Carlo test to complement of permutation level, following the model two-factor with "Estuary" (2 levels fixed), "Sites" (6 level fixed and nested in Estuary) ($p < 0.05$). The ordination demonstrated that collected clams in Tagus estuary were separated from those of Sado estuary by the elements Zn, Mn, Cr, Co, Cu, Se and Ce. Although the significant differences were only obtained between estuaries and Gâmbia and Herdade do Pinheiro sites. The sediment from both estuaries showed similar spatial distribution patterns the upstream sites presented high element concentrations compared to downstream sites. Was possible to obtain a chemical profile of the geographical origin "fingerprint" between Tagus and Sado estuaries. However, no significant differences were detected between all sites in each estuary. The clam´s geographic origin in Tagus estuary is characterised by the dominance of the elements Zn, Co, Se and Ce and clam´s geographical origin in Sado estuary is characterised by the dominance of Cu and Cr.

To develop the precision, accuracy and sensibility of the traceability tool is essential to assess the elemental stability through time, beyond intrinsic and extrinsic factors that influence the presence of the elements in clams.

Keywords: Traceability; Fingerprinters; *Ruditapes philipinarum*; Tagus estuary; Sado estuary.

Resumo alargado

A apanha de bivalves no estuário do Tejo, tem vindo aumentar exponencialmente nos últimos anos. A amêijoa japonesa, espécie exótica *Ruditapes philippinarum*, é das mais procuradas pelos mariscadores, devido à sua abundância, rápido crescimento e resiliência. A falta legislação eficaz conduziu ao falseamento da origem destes bivalves, as ameijoas são apanhadas no estuário do Tejo e comercializadas como sendo do estuário do Sado.

Recursos marinhos de elevado interesse económico têm sido alvo de monitorização recorrendo a técnicas moleculares ou químicas como métodos de rastreabilidade. Estas técnicas têm permitido determinar a origem destes recursos na cadeia alimentar e comercial. A sua utilização para o rastreamento da origem de organismos através da identificação dos elementos químicos tem sido bem-sucedida, apresentando eficácia e reprodutibilidade.

Com a finalidade de distinguir as populações de *R.philippinarum* através da determinação do perfil geoquímico de origem, recorreu-se a um método de rastreamento analítico para a identificação do perfil químico dos tecidos moles da amêijoa japonesa. A técnica ICP-MS ("Inductively Coupled Plasma Mass Spectrometry") permitiu determinar a concentração de múltiplos elementos químicos em simultâneo no tecido mole da amêijoa, com precisão, exatidão e sensibilidade.

No seguimento de travar a comercialização falseada e reduzir o risco saúde pública, surgiu a necessidade de investigar quais as diferenças dos perfis químicos da espécie *R. philippinarum* dentro e entre os estuários do Tejo e do Sado para determinar a origem geográfica. Foram testadas as seguintes hipóteses: i) Existem diferenças significativas no perfil químico das amêijoas entre os estuários do Tejo e do Sado; ii) Existem diferenças significativas no perfil químico das amêijoas entre locais dentro de cada estuário; iii) Existe relação entre o perfil químico do sedimento e das amêijoas amostradas. Para identificar o padrão químico de distribuição da amêijoa ao longo dos estuários, utilizou-se como parâmetro de variação a escala espacial.

Foram selecionados três locais de amostragem em cada estuário, tendo em conta os seguintes critérios: ocorrência da amêijoa-japonesa, características hidrológicas (influência de sub-bacias) e características geomorfológicas (tipos de sedimentos) potencialmente diferentes. No estuário do Tejo os locais escolhidos para efetuar a amostragem foram Alcochete, a Baia do Montijo e a Baia do Seixal. No estuário do Sado foram selecionados como locais de amostragem a Gâmbia, a Herdade do Pinheiro e o Cabeço do Ratão.

Para melhor identificar e relacionar os elementos químicos que determinam o perfil químico de origem da amêijoa foi também analisado o perfil geoquímico do sedimento. O sedimento é considerado um parâmetro de georreferência da origem de muitos organismos bentónicos.

As amostras da amêijoa e do sedimento foram previamente submetidas à digestão ácida na placa quente e posteriormente analisada a concentração de 16 elementos traço (Sc, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sr, Cd, Cs, Ce, Eu e Th), escolhidos com base na ocorrência frequente em bivalves e em ambientes estuarinos.

A frequência dos elementos químicos nos bivalves está particularmente dependente da sua biodisponibilidade, toxicidade (especiação) e da capacidade de bioacumulação dos espécimes. Estando estes fatores associados a variáveis bióticas e abióticas, foi possível identificar padrões de distribuição espacial nos perfis químicos da amêijoa ao longo dos estuários Tejo e do Sado.

A ordenação PCA (principal component analysis) mostraram a separação entre as amostras do estuário Tejo e do estuário do Sado, sendo Zn, Mn, Cr, Co, Cu, Se e Ce os elementos que melhor explicam a separação entre estuários. Com a análise multivariada PERMANOVA (permutational multivariate analysis of variance) a dois fatores “Estuário” (fixos com dois níveis) e “Locais” (fixos e aninhados em Estuário com seis níveis), detetaram-se diferenças significativas entre locais e estuários ($p < 0.05$). O teste “pairwaise” PERMANOVA e o método Monte Carlo realizados para os locais de amostragem permitiram identificar diferenças significativas entre locais Gâmbia e Herdade do Pinheiro. Os perfis químicos do sedimento amostrado apresentaram padrões de distribuição espacial similares nos dois estuários, mas foram detetadas diferenças significativas entre locais e estuários. A determinação da concentração dos elementos permitiram diferenciar o perfil químico das ameijoas entre locais e estuários, identificando o padrão de distribuição espacial.

As influências litológicas, fluviais e antropogénicas parecem ser relevantes para explicar as diferenças dos padrões químicos identificados nas amêijoas analisadas, como por exemplo a ocorrência Zn, Fe e Mn nos dois estuários. A disponibilidade significativa de metais como o Cu, Cr, Ni e Co nos estuários do Sul de Portugal deve-se à influência da Faixa Piritosa Ibérica. As elevadas concentrações de Mn, Ni, Co, e Se nas amêijoas amostradas em Alcochete podem ser relacionadas com a influência litológica, fluvial do Rio Tejo e da presença das pradarias marinhas na Reserva Natural do estuário do Tejo.

Atualmente, a presença de pesticidas têm provocado um grande impacto no estuário do Tejo chegando a ultrapassar os níveis aceitáveis pela Diretiva 2013/39/EU. O arsénio e Se são constituintes de pesticidas, herbicidas e inseticidas que explicam as elevadas concentrações destes elementos químicos nas ameijoas de Alcochete e da Baía do Montijo.

As baias do Montijo e do Seixal caracterizam-se por um elevado nível de poluição, vasa arenosa com altos conteúdos de matéria orgânica, baixo hidrodinamismo e elevada influência de marés. Estas condições ambientais promovem o aumento do tempo de residência e especiação dos metais, que por sua vez favorece a crescente acumulação dos vários metais nas amêijoas amostradas. Com as pradarias marinhas de Corroios a baía do

Seixal beneficia da retenção de uma grande quantidade de elementos químicos como o Cu, sendo este o elemento com maior concentração nas amêijoas amostradas neste local.

O estuário do Sado é fortemente poluído devido às atividades industriais, antigas minas, agricultura e aquacultura. Estas influências antropogénicas e litológicas definem o perfil químico das amêijoas amostradas neste estuário. Os locais Gâmbia e Herdade do Pinheiro apresentam características de hidrodinamismo reduzidas com grandes quantidades de matéria orgânica proveniente dos canais de Alcácer e Águas de Moura. As amêijoas amostradas na Gâmbia apresentaram as concentrações mais altas de Zn e Ni. Para além de terem funções metabólicas essências para os organismos, estão relacionados com a influência litológica e fluvial do local. As ameijoas amostradas na Herdade do Pinheiro foram as que obtiveram a maior concentração para maioria dos elementos Fe, As, Mn, Se, Co, Cd, and Eu. Os valores elevados As e Se identificados nas amêijoas é devido à utilização de fertilizantes e pesticidas na agricultura principalmente na produção dos Arrozais. As amêijoas do Cabeço do Ratão evidenciaram-se pela elevada concentração de Cu sendo um local influenciado pelas marés.

O estudo atual mostrou que é possível obter um perfil químico de origem geográfica *"fingerprint"* entre os estuários do Tejo e do Sado. No entanto não foi possível detetar diferenças significativas entre locais em cada estuário. O *"fingerprint"* químico que permite identificar as ameijoas do estuário Tejo caracteriza-se pela dominância de Zn, Co, Se e Ce e o *"fingerprint"* químico que permite identificar as ameijoas do estuário do Sado caracteriza-se pela dominância de Cr e Cu.

Ao identificar os elementos químicos que mais se diferenciam entre locais e estuários foi possível definir elementos de rastreabilidade que definem a origem geográfica das amêijoas. A variação temporal dos perfis químicos para avaliar a estabilidade dos elementos é essencial para determinar a precisão e exatidão da ferramenta de rastreabilidade, visto que existem fatores intrínsecos e extrínsecos que influenciam a presença de determinados elementos químicos nas amêijoas.

Palavras-chave: Rastreabilidade; Perfil químico de origem; *Ruditapes philippinarum*; Estuário do Tejo; Estuário do Sado.

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Abbreviators list

Chemical elements					
Ag	Silver	Cs	Cesium	Na	Sodium
Al	Aluminum	Cu	Copper	Ni	Nickel
Ar	Argon	Eu	Europium	O	Oxygen
As	Arsenic	Fe	Iron	P	Phosphorus
Ba	Barium	H	Hydrogen	Pb	Lead
C	Carbon	He	Helium	Rb	Rubidium
Ca	Calcium	Hg	Mercury	Rh	Rhodium
Cd	Cadmium	Ir	Iridium	Sc	Scandium
Ce	Cerium	K	Potassium	Se	Selenium
Cl	Chlorine	Mg	Magnesium	Sr	Strontium
Co	Cobalt	Mn	Manganese	Th	Thorium
Cr	Chromium	N	Nitrogen	Zn	Zinc
OH	Hydroxide	NH3	Ammonium	U	Uranium
Units					
Ppt	Part per trillion	ppb	Part per billion	ppm	Part per million
NIST	National Institute of Standards and Technology	CRM	Certified reference materials	SE	Standard Error

Chapter I: Introduction

1. Introduction

The invasive bivalve *Ruditapes philippinarum* ecosystems have been the primary concern in Tagus and Sado estuaries. Using a traceability tool will provide the impact assessment of illegal fishery activities and reduce the consumption risk and guarantee marine ecosystem sustainability. Tracking and controlling the spread of invasive species, the intensive harvesting and mislabelling origin are the main goals of this project.

The developed traceability tool will distinguish the chemical profile of near populations and allowing to know their geographical origin.

1.1 *Ruditapes philippinarum* (Adams & Reeve, 1850)

The Japanese carpet shell also known as the small-neck or Manila clam, scientifically named *Ruditapes philippinarum* (Adams & Reeve, 1850), is an edible bivalve species from Veneridae family with an increasing ecological and economic importance. This bivalve is also recognised as a good bioindicator of metal pollution and a sentinel species of environmental quality in aquatic systems (Ji et al., 2006; Chainho et al., 2013). Their wide distribution is due to a long life cycle with a high tolerance to heavy metals, salinity and temperature variations.

1.1.1 Biology and ecology

a) Habitat and ecological distribution

Ruditapes philippinarum is a benthic bivalve that lives buried up 10-20 cm depth, surrounded by sediment, muddy sand and among buried cobbles (Sousa *et al*., 2009; H. Zhao & Zhang, 2016b). Considered as a filter feeder with a high food conversion efficiency and a broad feeding spectrum, they can accumulate a large number of metals aggregated in suspended particles, reveals a higher predisposition to uptake heavy metals than other bivalve's species (Ji *et al*., 2006; Moschino *et al*., 2012). This species has a privileged occurrence in bays and estuaries at middle and low intertidal zones (Sousa *et al.*, (2009), *cit. in* Gosling, (2003)), tolerating high salinities ranges (16 and 36) and temperatures (FAO, 2018).

Most bivalves play an essential role in many coastal ecosystems with the ability to modify, maintain and or create habitats, by removing particles from the water column and depositing undigested material into sediment surface as faeces and pseudofaeces (Newell, 2004; Costa *et al*., 2017). The metabolic engineering activities can significantly alter ecosystem structure and functioning. The high filtration rate of the invasive *R. philippinarum* developed an important role in the benthic inorganic carbon cycle and nutrient fluxes (Nizzoli *et al*., 2011). They are responsible for increasing the grazing pressure in aquatic systems promoting several processes such as sediment organic matter enrichment, ammonium release from the

sediment, modification of nitrate and nitrite fluxes stimulating nitrification and nitrate reduction (Welsh *et al*., 2015) and promoting the phosphorous recycling (Nizzoli *et al*., 2006). The bivalve's influence in those mechanisms provides oxygen distribution by the sediment mixing (*i.e*. bioturbation) influencing the pathway of the organic matter mineralisation. These processes enhance the reduction of turbidity in the water column increasing the light penetration through sediment surface and stimulate microphytobenthos production (Sousa *et al.*, 2015; Newell, 2004; Welsh *et al*., 2015). In several cases, mostly in water lakes and other freshwater bodies can occur a fast phytoplankton growth leading to a bloom-forming that change the water composition due to the toxin-produce. In Migné *et al.*, (2017) and Costa *et al.*, (2017) studies it was suggested that invasive clam *R. philippinarum* metabolic activities modify water quality increasing inorganic carbon and ammonium followed by the high sedimentation, being more effective than native species. Nevertheless, the invasive species effectiveness is due to the high amount of colonised nitrifying and denitrifying bacteria (Welsh *et al*., 2015). This invasive behaviour can lead to several impacts in marine ecosystem promoting several habitat changes, altering nutrient dynamics and phytoplankton composition by the selective grazing of phytoplankton species that induce the dominance of toxic groups, changing biotic interactions and promoting biofouling enhancement (Costa *et al*., 2017).

b) Ecologic interactions

Majority manila clam reveals a strong invasive behaviour that highly interacts with native species leading to ecosystem modifications. *Ruditapes philippinarum* and endemic species *Ruditapes decussatus* have similar habitat and ecological niche trending to an intense interspecific competition for the same resources and several environment changes (Chainho *et al.*, 2015; Velez *et al*. 2015a; Chiesa *et al*. 2018). At some environments the *R. philippinarum* establishment can cause starvation, interfere with the respiration, reproduction and growth of native species, promoting their decline (Sousa et al. 2009; Chainho *et al.* 2015; Ramajal, 2016).

Nevertheless some researches supported the concept of species coexistence, regarding the higher density of the native species detected on several environments, suggesting that the introduced clam has not yet supplanted the native species by occupying the native clam ecological niche entirely, like it was studied at Óbidos lagoon in Velez *et al*., (2015b) and Ria of Aveiro in Rodrigues, (2017). The spatial distribution of these species are not only due to metal contamination levels, they were also observed at low and high contaminated areas in Velez *et al.*, (2015a). The metal tolerance is not the primary mechanism responsible for the replacement of native species (Velez *et al.*, 2015c), the different growth parameters can be limiting factors such as the quality and quantity of food supply, recruitment

rate and environmental changes, resilience may be the leading causes for the native species replacement and community structures modification (Moura *et al*., 2017).

The accumulation of empty shells of invasive clams can change the sediment geomorphology, influencing the estuarine hydrodynamics, in some cases increasing the microhabitat complexity and heterogeneity bounding free spaces for native fixation after spawning (Sousa *et al.* 2009).

c) Reproductive adaptations

Manila clam (*R. philippinarum*) is a gonochoric species the gonads are characterised by a diffused tissue closely linked to the digestive system and the muscular foot, expanding into a visceral mass with the gametogenesis progression (Drummond *et al*., 2006; FAO, 2018). The reproductive cycle is longer than native species revealing a higher fit through environmental variations. Dang *et al.* (2010) detected the presence of spatial and temporal variability in spawning events at the kilometre scale. The optimal temperature for gonadal development can vary between 20 °C and 22 °C (FAO, 2018), the lower temperature limit reported for gonadal activity was 8^0C, the minimum for the gonadal ripening is 12 °C and 14 °C for spawning which is a broader range comparing with native's gonadal activities (Drummond *et al.*, 2006; Delgado & Peres-Camacho, 2007; FAO, 2018). The main influence to enhance the gametogenesis and spawning is the temperature which is closely associated to geographical locations beyond these variations have been reported annual oscillations at the timing, duration and number of spawnings per year regarding food availability, which strongly influences the number of gametes produced(Drummond *et al*., 2006).

The *R. philippinarum* as an invasive species revealed adaptive advantages over the native species with the higher gonadal development rate and the higher capacity for gonadal regeneration increasing the reproduction activity for longer (Delgado & Peres-Camacho, 2007).

1.1.2 Distribution and commercialisation

a) Origin and distribution

The bivalve *R. philippinarum* is native from Indo-Pacific region and it was introduced for culture purposes nearly 70's in European temperate waters such as Atlantic (Portugal, France, Spain, Ireland, England) and Mediterranean coastal waters (France and Italy) (Velez *et al.,* 2015a; FAO, 2018; Chiesa *et al*., 2018). The native population was detected in Philippines, South and East China, Yellow Sea, Japan, Okhotsk and around Southern Kuril Islands (FAO, 2018). The principal purpose for the species spread, and the establishment is

the socio-economic enhancement that competitively led to the world commercial cultivation (Figueira & Freitas, 2013; Velez *et al*., 2015a).

b) World production and economic importance resource

The Japanese carpet shell has a considerable commercial value for world fisheries and aquaculture, it is one of the most commonly consumed bivalve species and has been extensively cultivated. Their culture was initiated in wild seeds by the Asian traditional fishing activities, regarding the increasing importance in the food chain by the high protein nourishment with minerals and less fat (H. Zhao &Zhang, 2016b; Chiesa *et al*., 2018).

The massive aquaculture production and natural reproduction resulted in further geographical expansion and population growth, becoming a target of intensive harvesting and fishing activities, with the significant contribution to the clam landings in Europe (FAO, 2018).

c) Occurrence and distribution in Portugal

In Portugal, *R. philippinarum* was introduced nearly 80's in Ria Formosa (Algarve in 1984), appearing for the first time in FAO statistics in 2009. The introduced population rapidly adapted to a new environment revealing a high resistance with fast immune response to bacteria contamination (Moreira *et al.*, 2012), with a long and high reproduction rate (Delgado & Peres-Camacho, 2007). This leaded to an geographical expansion through other aquatic systems such as estuarine, lagoon and coastal systems (e.g. Tagus estuary in 2000, Ria Aveiro in 2006; Albufeira lagoon in 2010, Sado estuary in 2010, Óbidos Lagoon in 2014 and Mondego estuary in 2016) (Figueira & Freitas, 2013; Velez *et al.*, 2015a; Chiesa *et al.*, 2018; NipoGES, 2018). Otherwise is hard to relate the leading cause of this phenomena because the native and non-native populations temporal evolutions are unknown (Chainho *et al.*, 2015).

1.2 Seafood traceability

Seafood is an essential global protein source with increasing growth in world production and trade over the past 60 years (Hellberg & Morrissey, 2010), becoming the most traded food product in the world (Leal *et al*., 2015). Currently, with improved levels of foreign trade and seafood processing, combined with demands for specific seafood types increased fraudulent actions. Fraudulent actions can occur in any part of the food supply chain, ranging from large-scale multinational importers with an economic impact to individual restaurants or retail outlets (Hellberg & Morrissey, 2010; Leal *et al*., 2015).

Traceability is a process developed to avoid fraudulent actions, determining food product origin by tracking their path. The main goal of this tools is to have a broad scale application in order to describe a unique profile. To achieve it the developed method must be

rapid, cost-effectiveness, reliable with a high potential for automation skill to determine seafood origin, regardless the stage or level processing and distribution (Hellberg & Morrissey, 2010).

The traceability tool enforcement has been solving several illegal seafood trade problems. H. Zhao & Zhang (2016a) at Jiaozhou Bay in China, developed a traceability method to recognise traded products origin in order to avoid illegal captures and improve better resource management. Traceability main purpose is to enrich the market product by the enhancement of consumer confidence, regarding the food safety and quality certification (*e.g*. PDO – Protected Designation of Origin) (Leal *et al*., 2015; Zhao & Zhang, 2016a).

The lack of raw materials labelling by food industries and the difficulty of solving public health issues, led to the development of analytic and scientific tools in order to attest food products origin, quality insurance and food safety (Jacquet & Pauly, 2008; Figueira *et al*., 2013; Leal *et al*. 2015).

1.2.1 Biotechnological tools for seafood traceability

Biotechnological methods have been developed to identify species through several food types and processing levels. Some molecular techniques use referenced markers with discriminatory criteria to identify geographic origin and being stored in databases (*e.g.* DNA barcoding). The geographic origin characterisation can result from a set of analytical techniques such as data processing and analysis supported by mathematical and statistical methods (Peres *et al*., 2007).

Molecular techniques have been used to determine the geographic origin they are based on protein or deoxyribonucleic acid (DNA) they can determine several profiles from different species. They are mostly reliable for fresh or lightly processed seafood. Otherwise, it becomes unusable in the heavily processed foods because proteins degrade (Rasmussen & Morrissey, 2008). In contrast, the DNA-based methods have several advantages over protein-based methods, including higher information content, higher resistance to degradation, increased specificity and sensitivity, with a presence in all cell types (Galimberti *et al*., 2013; Leal *et al*., 2015). Other molecular techniques such as enzyme-linked immunosorbent assays technique (ELISA) for heat-treated seafood products (Carrera *et al*., 1999; Asensio *et al*., 2003), is a high precision method that requires species-specific antibodies development and doesn't work well in species closely related (Hellberg & Morrissey, 2010).

The main steps to identify a unique DNA origin profile are DNA isolation, polymerase chain reaction (PCR) amplification and detection of species building a database. Recent researches revealed DNA markers and PCR-based methods as useful traceability tool, with high sensitivity applied in plant cultivars, animal breeds and seafood tracking raw materials in food industry processes (Hellberg & Morrissey, 2010; Galimberti *et al*., 2013). PCR-denaturing

gradient gel electrophoresis (PCR-DGGE) has been mainly developed to food traceability and safety in order to characterise living bacterial communities found in seafood products for geographic origin determination and yeasts in fermented products technologies (Nguyen *et al*., 2008; Galimberti *et al*., 2013; Leal *et al*.,2015).

DNA barcoding as a new identification system was developed to analyse the variability by using a standard region of the genome called “DNA barcode”. DNA barcoding figuratively resembles an infrared scanner which applies a high resolution to identify different taxa, based on interspecific differences storing in specific databases (Galimberti *et al*., 2013).

In molecular tools, development is important to regard the time and costs processing, reproducibility, reliability, the range of target species, and deal with the difficulty to recover and identify DNA from processed products, complex food matrices and mixed- species samples (Hellberg & Morrissey, 2010; Galimberti *et al*., 2013)

Lipid analysis has important aim in biology and chemistry they can be used for traceability appliance, but several lipids are affected by biotic (*e.g*. age, sex, reproductive cycle, and phylogeny) and abiotic (*e.g.* diet, temperature, depth, and salinity) factors. Otherwise, the fatty acids are an essential energy source and unleash a cellular maintenance role which is used as biomarkers of biochemical and ecological environment conditions. Grahl-Nielsen (2010) in order to minimise the effect of seasonality associated with diet, by using the bivalve´s adductor muscle, they are rich in polar lipids with less seasonal variation. This method is relatively low cost and fast but as traceability tool can be less reproducibility when is consider complex food matrices.

1.2.2 Trace elemental fingerprint (TEF)

a) Hard structures success

Analytic techniques have been essential to trace seafood products, the chemical profiles can be composed by mineral compounds describing the organism geographic origin, can distinguish populations and stocks through the hard structure analysis (*e.g.* mollusc shells, invertebrate statoliths or fish otoliths) (Carson *et al.*, 2013; Sorte *et al*., 2013; Ricardo *et al*., 2015). The most common trace elements found in marine species are Al, Ba, Ca, Co, Cr, Cu, Mg, Mn, Pb, Zn, Sr and U. The elements Ba, Mn, Mg, Pb and Sr, can play a discriminatory role in several habitats (Carson *et al*., 2013; Ricardo *et al*., 2015). Mineral elements are more stable than organic compounds, regardless of being influenced by the environmental variations and recorded in hard structures (Zhao *et al*., 2011; Leal *et al*., 2015; H. Zhao & Zhang, 2016a). The multi-element quantification in bivalve’s hard structure has been improved with the development of inductively coupled plasma spectrometry (ICP) techniques. Examples of different techniques applications: Elemental analysis in mussel shell with Laser Ablation

Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) in Becker *et al*. (2005); Inductively Coupled Plasma Mass Spectrometry (ICP-MS) to determine manila clam origin with multi-element fingerprinting in H. Zhao & Zhang, (2016a); In Ricardo *et al*. (2015) the Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES) technique to quantify elements in *C. edule* shells and Carson *et al*. (2013) Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) technique for elemental analysis in bivalve's shells and fish otoliths.

The environment influence in bivalve`s shell chemistry has mainly focused the researches on their appliance as an environmental recorder for better know the populations dynamic and origin as it was developed in Becker *et al*. (2005) among others as Sorte *et al*. (2013), Cathey *et al*. (2014) and more recently Norrie *et al.* (2016). The elements incorporation is influenced by the combination of several biological and environmental factors such as growth rate, temperature, salinity and the availability in surrounding environments (Norrie *et al*., 2016). To avoid the seasonal influence Sorte *et al*., (2013) determine a stable geochemical signature between juvenile and adult mussels analysing the most recently formed shells (growth rings) with the less temporal differences caused by variations through age. This method started to be used as a tool to understand the organism's connectivity at recruitment (Becker *et al*., 2005, Cathey *et al*., 2014).

The method revealed different accuracy according to local physical parameters. Sorte *et al*. (2013) observed that the open coasts geochemical signatures can vary on a large scale compared with bays or estuaries where the water is less exchanged. However, Carson *et al*. (2013) assessed several species different sets of elements as best discriminant among and between open-coast, bays and lagoons sites maintaining a distinct chemical signature, despite the seasonal variability. Cathey *et al*. (2014) proved to be possible determine a chemical signature in early stages of *M. Mercenaria* larval shells with the spatiotemporal stability of trace and minor elements. In some studies such as Sorte *et al*. (2013) revealed three elements which are common between seven species Mg, Mn and Sr. In Becker *et al*., (2005) the elements Mn, Pb and Ba allowed to distinguish among mussels collected between two major bays and open coast sites. Trace elements signature improvement in several fingerprint studies is supported by the frequent presence of elements such as Sr, Zn and Mn according to with the local characteristics (Sorte *et al*., 2013; Norrie *et al*., 2016).

The main limitation of geochemical methods for seafood traceability is the hard mineralised structures, their absence in partitioned products, bounding the access over the food chain (Leal *et al*., 2015). The trace elemental fingerprinting proved to be an accurate, fast and reliable method to determine bivalve's geographic origin at different scales distinguishing

specimens from close populations (Carson *et al*., 2013; Sorte *et al*., 2013; Ricardo *et al*., 2015; Norrie *et al*., 2016).

b) Soft tissue success

Otherwise testing the method in soft tissue overcome this matter with high accuracy, effectiveness, low-cost reproducibility and can be closely related to consumption products. In order to assess trace elements is important to avoid parameters fluctuations choosing specimens with the same size, by preventing any bias associated to different ages and physiologic requirements (Yu Li *et al*., 2006; Costas - Rodríguez *et al*., 2010; Ricardo *et al*., 2015). Until now researches were mainly focus in soft tissue's metal bioaccumulation studies, in order to assess heavy metal pollution (Ji *et al*., 2006; Wang *et al*., 2012; Moschino *et al*., 2012), instead to identify trace elements fingerprint (H. Zhao & Zhang, 2016a). Yu Li et al. (2006) determine Zn, Mn and Cu as the most abundant trace elements in bivalve's tissues seeking to know better the chemical composition of suspended particles and clams.

1.2.3. Biochemical technique

a) ICP-MS

The ICP-MS is a technique with a high sensitivity that can measure almost any element at ppt to ppm levels in a wide variety of samples. Can measure several elements in a single analysis (multi-element analysis), distinguishing different element species and isotopes. The main requirements to ensure the best precision and accuracy of analytical analysis are increased instrument sensitivity, an proper matrix tolerance, low levels of interferences, wide linear dynamic range and isotopic information (Thomas R., 2004; Balcaen *et al.,* 2015).

b) ICP-MS compounds

The ICP- MS system begins with the sample introduction section which is responsible for several criteria due to the high performance of the instrument detection. In this method, the samples are usually in liquid form, and they are pumped with a peristaltic pump into a nebuliser where are converted into a fine aerosol by the presence of argon gas. The liquid is transformed into fine droplets through the pneumatic action of the gas flow (carrier gas). In the spray chamber the finest droplets which represent only 1-2% of the sample, are separated from largest being the only ones to emerge to the Plasma Torch Section. The Plasma torch is used to generate positively charged ions during the development of argon plasma at a high temperature. The argon gas is released in the torch and is exposed to an intense magnetic field, produced by radiofrequency (RF) passing through the copper coil (RF coil). The highly energised argon ions, which produce the plasma discharges, are used to interact with analyte

ground state atoms from the sample, removing an electron and generating positively charged ions. Once the analyte ions are produced, they will pass through an interface section maintained at a vacuum by a mechanical roughing pump. The interface section is composed by two metallic cones called Sampler cone and Skimmer cone which promotes an efficient passage of the analyte ions beamed through the ion optics section and guided into the mass separation device (MS) without losing their electrical integrity. The ion optic section which is positioned between the Skimmer cone and the quadrupole MS, electrostatically focus the ion beam producing a flat signal toward the mass separation device stopping photons, particulates and neutral species from reaching the detector section which is responsible for the background noise and the signal instability (Thomas R., 2004).

The mass separation device with quadrupole technology has four cylindrical or hyperbolic metallic rods that allow the separation of analyte ions according to their mass-to-charge ratio and filter out the main nonanalyte interferences and matrix ions. The detection of some elements using the traditional quadrupole mass analyser is compromised because the formation of polyatomic spectral interferences generated by a combination of argon solvent and sample-based ionic species, however, the collision cell reaction technology can reduce these harmful species. This scanning process promotes the correct analyte ions arrive at the detector, where are converted to an electrical signal and processed the data by handling system. The data is converted into analyte concentration using ICP-MS calibration standards and certified referenced material (Thomas R., 2004).

c) Types of Interferences

In ICP-MS analysis there are two main spectral interferences isobaric and polyatomic. The polyatomic interferences are the combination of two or more isotopes from different elements and can be caused by Argon gas from the plasma, matrix components, sample solvents or accidental entrance of oxygen/nitrogen during the analysis procedure. The combination of argon ions ($^{40}Ar^{+}$) with other species can result in the double positive charge ions with the same mass charged (m/z) as the isotopes from different elements. Examples of the most common polyatomic interferences are $^{40}Ar+ + {}^{16}O+ \rightarrow {}^{56}Fe+$ and $^{40}Ar^{+} + {}^{35}Cl+ \rightarrow {}^{75}As^{+}$, Argon gas can combine with the acid from sample matrix or diluents such as HNO_3 or HCl. Other common interferences are related with the combination of H^{+}, $^{16}O^{+}$ and $^{16}OH+$ and analyte ions which can form molecular hybrids (Thomas R., 2004; Balcaen *et al*., 2015).

Isobaric interferences are due to different elements whose isotopes have a common mass (m/z), *e.g*. Fe and Ni have isotopes at mass 58, and therefore any signal measured at m/z 58 will have contributions from both elements Fe and Ni. Analyte and interfering ions have the same nominal mass but exhibit small differences in their exact masses. Remove the effects

of interferences have been the main issue to ICP-MS analysis precision, including the development of new instruments with high-resolution ICP-MS and cell-based ICP-MS. Meanwhile, several approaches to coping with spectral overlap have been suggested such as matrix matched, blank subtraction, mathematical corrections, cold plasma conditions among others, revealing accurate results for a wide range of analyte elements and samples types (Balcaen *et al*., 2015).

d) ICP-MS/MS or ICP-QQQ equipment

ICP-MS instrument was developed to achieve a higher mass resolution with free interference measurements, so Agilent launched the 8800 ICP-MS/MS system also known as ICP-QQQ a triple quadrupole based. The instrument is equipped with a collision/reaction cell between the two quadrupoles (Q1 and Q2), the system can be used as single quad mode with Q1 as mass filter, or an ion guide, or as a bandpass and the Q2 as the second scan of the target product ions, selecting analyte with a specific m/z value. The different options allowed to search for the most accurate method, meanwhile the operation instrument in MS/MS mode clearly revealed an added value because it was possible to exclude all plasma based sample and matrix ions with a mass that differs the analyte ions from the cell (Balcaen *et al*., 2015; Bolea-Fernandez *et al*., 2017).

e) Collision Reaction Cell (ORC)

The collision/reaction cell is an octopole arrangement cell which is pressurised with a gas (reactive or non-reactive) in order to remove interferences by a selective reaction of the analyte and interfering ions. With the reactive gas, typically used H_2, O_2, NH_3 is possible to eliminate polyatomic ions and reduce isobaric overlap, otherwise, unwanted product ions can appear leading to new interferences (Tanner & Baranov, 1999). Researchers showed to be possible recreate interference-free conditions converting the interfering species into new species maintaining the natural isotopic mass from analyte ions (on-mass mode) or converting the analyte ion into a reaction product ion with a different mass-to-charge ratio from the original analyte ion (mass-shift mode) (Balcaen *et al*., 2015).

The no-reactive gas usually used is He, provides kinetic energy discrimination of the analyte ions. Inside the cell, the chemistry follows the laws of kinetics and thermodynamics, all ions collide with He molecules. The larger ions undergo more collisions than molecular ions losing more kinetic energy. Before they enter into the analyser quadrupole, an energy barrier is established bounding the passage of ions with specific kinetic energy to exit the cell. Interferences are removed based upon differences in atomic size, collision mode cannot remove isobaric or doubly charged interferences. Nevertheless, the reaction mode approach

is more suitable for applications where the matrix is relatively constant within a batch of samples (Yamada, 2015; Bolea-Fernandez *et al*., 2017). The selection of the gas mode for the octopole reaction system (ORS) is related to the analyte element's nature and behaviour (Bolea-Fernandez *et al*., 2017).

1.3 Development of elemental profile in estuarine ecosystem

Estuaries are transitional environments witch connect the seawater and freshwater by tidal influence and freshwater inflow. They are highly disturbed with several inputs, mostly aggravated by the anthropogenic loads they are due to urbanization development resulting in a high amount of discharges of domestic effluents, industry, fossil fuel burning, mining, groundwater use, surface runoff, soil erosion and mobilization of historically contaminated sediments (Serafim *et al*., 2013; Machado *et al*., 2016).

All of these disturbances can influence chemical profile achievement. The environmental approach for metals and toxicity effects to aquatic organisms in estuaries is essential to improve the knowledge of biogeochemical mechanisms processes, metal chemistry and organism physiology (Machado *et al*., 2016).

1.3.1 Trace Element Fingerprint - Seasonal variations

a) Biotic parameters

The bivalve's trace element fingerprint can be successfully accurate beyond seasonal variations (Carson *et al*., 2013; L. Zhao & Yang, 2013; H. Zhao & Zhang, 2016a) and biological defensive responses to metal exposure, reflecting biochemical, metabolic and behavioural alterations (*e.g*. synthesis of antioxidant enzymes and metallothionein protein - MT) (Chaharlang *et al*., 2012; Wang *et al*., 2012; Oaten *et al*., 2016; Santana *et al*., 2017).

Benthic marine species have a natural bias to absorb metals; the bivalves absorb it at much higher concentration than the concentrations found in water and sediments. During the feeding they filter a large number of suspended particles aggregated with a high concentration of metals, leading to a toxic state representing a higher risk to consumers (Figueira *et al*., 2012; Moschino *et al*., 2012; Figueira & Freitas, 2013). The tolerance and resistance mechanisms induced by exposure promotes the accumulation of certain metals in tissues and avoid the entry of others in order to survive and cope with stressors from the surrounding environment (Santana *et al*., 2017; Piló *et al*., 2017).

Metal bioaccumulation, in aquatic specimens tissues depends on several factors such as magnitude, duration and frequency of exposure, metal type, and vary between species (Abdullah *et al*., 2007; Chaharlang *et al*., 2012; Carson *et al*., 2013; Oaten et al., 2016; Santana

et al., 2017). The bivalve *Ruditapes philippinarum* mainly absorbs metals from the overlying water, as dissolved and particulate forms, which appears to be a passive and common process (Marques *et al*., 2017).

Current knowledge confirmed a positive correlation between residence time, specimen's age and metal allocation and mobilisation (Yu Li *et al*., 2006; Duarte *et al*., 2014). The regulatory mechanisms trigger several biological processes characterising the organisms and population fitness. The enhancement of enzyme antioxidant synthesis (Wang *et al*., 2012), lipid peroxidation levels (Figueira *et al*, 2012) promotes a high loss of energy and a reduction of other metabolic processes such as sexual maturity and growth (Santana *et al*., 2017), revealing a negative influence to several reproduction stages (Yu Li *et al*., 2006; Oaten *et al*., 2016; Santana *et al.*, 2017). The gonad development at reproduction period also affect the individual weight and influence the concentration of metals in soft tissues (Wang *et al.,* 2010). All of these responses allows maintaining the organism's homeostasis when exposed to contaminants (Marques *et al*., 2017).

Metal bioaccumulation differences can be relevant in specimen's different sizes, sex and ages (Ji *et al*., 2006). Amisah *et al*. (2010) with Volta clam *G. Paradoxa* (Born, 1778) research and Chaharlang *et al*. (2012) with oyster revealed that some metals as Cu and Zn are essential elements with an important role in growth and cell metabolism, despite being one of the most abundant at aquatic systems.

Bivalves are often chosen for biomonitoring studies, because they are sedentary organisms, with a long live cycle, easily identified and sampled, reasonably abundant, tolerant of natural environmental fluctuations and pollution, being considered suitable bioindicators of trace metal contamination, although this is not consensual at researches community (Abdullah *et al*., 2007; Chaharlang *et al*., 2012; Carson *et al*., 2013).

b) Abiotic parameters

The main difficulty of metal assessment is determining the real metal concentration rate bioaccumulated in bivalve's tissues since there is a high number of biotic and abiotic factors that act simultaneously (Wang *et al*., 2012; Moschino *et al*., 2012; Piló *et al*., 2017). These interactions can reveal no significant spatial pattern if the environments are similar (Sorte *et al*., 2013; Carson *et al*., 2013; Norrie *et al*., 2016). Bivalve's trace element accumulation patterns are not solely derived from the external bioavailability, but also from the toxicokinetics which is trace element-specific, available in aquatic environments (Amisah *et al*., 2010; Serafim *et al*., 2013; Velez *et al*., 2015a; Marques *et al*., 2017). Figueira & Freitas (2013) determine in poorly contaminated areas a high concentration of metal in *Ruditapes*

philippinarum and *Ruditapes decussatus* which easily exceed the maximum known limits of bioaccumulation to several elements.

The environmental parameters such as pH, potential redox, salinity gradient influenced by tidal variations, temperature, sediment characteristics, and organic content contribute to metal bioaccumulation, metal bioavailability and toxicity rates (Amisah *et al*., 2010; Oaten *et al*., 2016; Duarte & Caçador, 2012; Piló *et al*., 2017; Norrie *et al*., 2016; Machado *et al*., 2016).

Tidal variations are related to hydrological mechanisms that influence all estuarine environment and geomorphology. The organism bioaccumulation is mainly due to metal availability which depends of environmental parameters such as sediment geochemical composition (mostly prevalence in muddy), redox potential modifications (Eh) in anoxic environments and the metal speciation (Jacqueline Eggleton & Kevin V. Thomas, 2004; Norrie *et al*., 2016, Machado *et al*, 2016). Many elements are strongly affected by the salinity and temperature levels mixed with tidal turbidity which is responsible by the metal chelation and mobilisation with organic ligands (Duarte & Caçador, 2012; Duarte *et al*., 2014).

The riverine discharges also have several consequences, with the high amounts of freshwater inputs, particulate matter and sediments into estuarine environments affecting their chemistry modifying metal concentration, and hydrology (Duarte & Caçador, 2012; Mil-Homens *et al*., 2014). Freshwater flows have a strong influence on these ecosystems, specifically in their geomorphology, salinity gradient, dissolved oxygen, turbidity and nutrient availability which when is limited it can bound the organism's distribution and abundance patterns (Chainho *et al*., 2013). The river's flood variation not only depends on seasonal events but also from their manipulation by several types of dams and reservoirs transporting high levels of inert sand material contained inorganic fraction which influence the river and estuarine communities (Duarte & Caçador, 2012).

Furthermore to TEF determination, the interaction of the several parameters may affect the identification of a geochemical signature. Is imperative to select the less affected elements for geographic origin achievement in order to provide a good indicator of elemental signature (Dunphy *et al*., 2011; Carson *et a*l., 2013; H. Zhao & Zhang, 2016b) since the spatial variability approach, can predict the population connectivity over a small within and between estuary scales (Carson *et al*., 2013; Norrie *et al*., 2016).

1.3.2 Trace Element Fingerprint - Spatial variations

a) Spatial scale approach and accuracy

Population connectivity researches provide a better understanding of population dynamics and their ecology be relevant to local fishery and provide better management activities for these natural resources with a high economic impact.

Ricardo *et al*. (2015) and Ricardo *et al*. (2017) in collected sites with a less than 1 km apart, achieved distinct elemental signatures with 99% success. That reveal TEF is a reliable, accurate method, distinguishing specimens from geographically close populations beyond time and seasonal variations. Norrie *et al*. (2016), Carson *et al*. (2013) and H. Zhao & Zang (2016a), differentiated chemical signatures from individuals collected at numerous locations within a single estuary as well as between closely located estuaries. The geographical accuracy range can be over 20 Km, *e. g*. Southern Californian mussels in Becker *et al*. (2005) and at Gulf of Maine in Sorte *et al*. (2013) research. The several signatures can be explained by the high environmental variability observed in estuaries, lagoons and open-coast sites (Carson *et al*., 2013; Ricardo *et al*., 2015; H. Zhao & Zang, 2016a). To the wide-scale approach, over 100 km, the methods commonly used are molecular, in order to assess the genetic population differentiation and structuration (Norrie *et al*., 2016).

b) Sediment as geochemical reference

Sediment assessment is an important reference of benthic organism habitat. As it is known bivalves have a filter feeding mechanism which uptake suspended sediment particles from the water column, especially from fine-grained bottom sediments accumulating several metals according to their sorptive nature (Yu Li *et al*., 2006; Abdullah *et al*., 2007; Wang *et al*., 2012). Metals easily chelate with suspended solid and bottom sediments, settling as metal-charged particles which are deposited and stabilise for an extended period. Any sediment disturbance event can have a significant effect on metal bioavailability, promoting desorption and transformation of contaminants into more toxic chemical forms (Eggleton & Tomas, 2004).

In H. Zhao & Zhang (2016b) research, the sediments were considered a match factor to define a geographical origin with trace elements. Some trace elements have specific patterns geologically related to sediments (Amisah *et al*., 2010; H. Zhao & Zhang, 2016b). The metal contaminated water bodies may quickly lead to bioaccumulation in the estuarine food web (Figueira *et al*., 2011). In contrast, previous studies as Moschino *et al*. (2012), Velez *et al.* (2015a) and Chiesa *et al*. (2018) obtained an absence of a direct correlation of elemental concentrations between clams and sediments.

The chemical concentrations knowledge requires a deeper understanding of metals paths transferred from sediments and water into bivalve's tissues (H. Zhao & Zhang, 2016b).

1.3.3 Metal pathways in estuarine environments

The bioaccumulation in organism's tissues, metal bioavailability and toxicity are highly related to the environmental parameters. The dynamics are due to hydrologic mechanisms such as tidal flows, river discharges, flocculation phenomena and residence time.

a) Metal bioavailability and toxicity

Metal bioavailability and toxicity in estuaries are highly related to multiple chemical and environmental factors such as pH, redox potential, salinity, temperature, organic matter contents. The geochemical proprieties of the fine-grained bottom sediments are highly influenced by the contaminant's affinity (sorption processes) and competitive metal interactions that promote the speciation process (Eggleton & Thomas, 2004; Allen & Janssen, 2006).

Along estuary physicochemical gradients, metals can assume several state forms, such as the particulate form (insoluble fraction) or dissolved form (soluble fraction) depending on hydrodynamics, water chemistry and mechanisms of toxicity (Duarte *et al*. 2014; Machado *et al*., 2016). Metal toxicity is evaluated by the proportionality of the total biotic ligand sites fraction occupied by the toxic metal (Allen & Janssen, 2006). The flocculation process is typical in these environments, tidal and river drainage influence can change metal behaviour by settling metal-charged particles (sedimentation), or the inverse processes of riverbed sediments re-suspension by sorption processes of the fine particulate matter (Duarte & Caçador, 2012; Norrie *et al*., 2016; Machado *et al*., 2016). Other critical processes in the water column are related with metal spatial distributions and biogeochemical cycles such as solubilisation, speciation, precipitation, diffusion and advection (Duarte *et al*., 2014; Machado *et al*., 2016).

During the organic matter, decomposition occurs redox potential and pH modification that promotes the enhancement of several processes such as desorption, partitioning, bacterial degradation and organic contaminants oxidation (Eggleton & Thomas, 2004). The decomposition process metals can be fractioned in several phases, exchangeable, bounded to carbonates, bounded to Fe/Mn oxide/hydroxides at a different degree of crystallisation, bounded to organic matter and sulfides and residual (Breda *et al*., 2018).

Elements such as Ca, Mn, Ni and Fe are released upon CO_2 accumulation. In contrast, the Fe/Mn oxide reduction complex which is triggered in the presence of sulfide (ions S^{2-}) developing flocs with clay particles that are deposited. The reduced solubility of sulfide bound the metal bioavailability and stabilise most of the elements in sediments, reducing their potential toxicity to biota (Vinagre *et al.,* 2008; Machado *et al*., 2016; Norrie *et al*., 2016; Chiesa *et al*., 2018). The metal toxicity can decrease in the presence of high concentrations of Ca and Mg, this organic ligands can reduce the metal bioavailability if the affinity of the toxic metal to the ligand is high enough to promote an aggregation complex, *e.g.* the Cu acute toxicity mitigation with the presence of Ca^{2+}, Mg^{2+}, Na^{2+} and K^{2+} ions (Allen & Janssen, 2006). Whereas the fast deposition of the most metals is confined to particles during the sinking process, some dissolved metals establish affinity bonds with ligands over the water column depending on estuarine residence time (Duarte *et al*., 2014).

b) Physical parameters influencing metal speciation

Metals usually have non-conservative behaviour in the presence of multiple abiotic and biotic factors such as salinity, pH, redox potential, temperature, bacterial degradation and oxidation of organic contaminants. Salinity is responsible for several modifications in water chemistry and the ionic strength between metals and the particulate matter. Salinity gradient promotes the water pH buffering effect by the increasing presence of carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-) ions. This process triggers the metal desorption from sediment and suspended matter, promoting the metal complexation with chloride and sulphate ions forming soluble inorganic complexes. In tidal flats, metal is continuously remobilised by the interaction with organic matter, water level, redox conditions and water chemistry modifications (Vinagre *et al*., 2008; Machado *et al*., 2016). The pH influence on metal behaviour is more evidenced at lower salinities. Riverine discharges result in a high concentration of organic matter available which increase the metal mobilisation with the accumulation of particulate metal (Machado *et al*., 2016; Norrie *et al*., 2016).

Turbidity zones along the estuary are often, and they are related to the highest concentration of suspended particles promoted by tidal and rivers flows mixture responsible by the physical dynamic of sediment and estuary stratification. Usually is found a high concentration of aggregated particles and colloids from river source. High turbidity spots are biased towards microbial metabolic processes and several biogeochemical impacts on organic matter and metal no-conservative behaviour (Duarte *et al*., 2014; Machado *et al*., 2016).

Disturbance events can also have a significant effect on metal bioavailability. Several human activities such as dredging, dredge disposal and fishing, promotes the sediment resuspension and contaminants transfer from sediment to biota. The shellfish dredges have a high ecological impact, they disrupt the benthic substrate, alter their composition, enhance turbidity and the resuspension of contaminated sediment particles (*e.g*. metals, tributyltins, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and pesticides) becoming available to the trophic web (Eggleton & Thomas 2004).

c) Biotic factors influencing metal behaviour

The most important biotic factors that can change metal behaviour in the water column is phytoplankton which mostly uptake metals at dissolved fractions. The deposition of dead phytoplankton and the presence of organic matter contribute to particulate metal input since dead cells accumulate more metals than living ones (Machado *et al*., 2016). Phytoplankton and other aquatic plants are known to modify their surrounding environment, contributing in mineralisation processes, enhancing sedimentation and metal allocation by speciation mechanisms (Caçador *et al*., 1996). Estuarine environments led to the organism's adaptation

through the exposing of different salinity gradients, *e.g.* halophytes (Caçador *et al*., 1996; Vinagre *et al*., 2008). Salt marshes areas have proved to be more efficient in metal mobilisation than no-vegetated areas due to roots presence and activity. The existence of complex organic compounds in sediment and the development of oxic microenvironments surrounding the roots are the main contributors for metal speciation and mobilisation (Caçador *et al*., 1996).

The temporal and spatial scale approaches become crucial to metal mobilisation, transport and toxicity processes. Most variables and processes vary across orders of magnitude depending on estuary dimensions, riverine discharges, coastal hydrodynamics, and the organism size and physiology (Machado *et al*., 2016; Norrie *et al*., 2016).

1.3.4. Metal toxicity effect in aquatic organisms

The metal availability and toxicity in sediments and living organism are the main issues to assess the environmental effect in aquatic systems. Metals are the most dangerous group of pollutants (*e.g*. Hg, Pb and Cd and metalloid As) because their high toxicity and accumulation are related to a low metabolization process (Figueira & Freitas, 2013; Velez *et al*., 2015a). The element Cd is one of the most abundant in aquatic environments and one of the most toxic to bivalves and other benthic life forms. To macro-benthic organisms, the tolerance of Cd only is possible with the presence of halophytes which can mobilise the metal in their roots system. Otherwise, the metal presence in sediment represents a lack of macro-benthic life (Vinagre *et al.,* 2008). Yu Li *et al*. (2006) study observed that there is no regulation of Cd concentration to a constant level in *R. philippinarum* like it was observed in other species. The Cd accumulation can lead to DNA and lipid damages, enzyme inhibition, among others (Figueira *et al*., 2012). Figueira & Freitas (2013) and Marques *et al*. (2017) researches also confirm the absence or limited capacity of *R. philippinarum* to regulate the concentration of Pb.

The most common elements up taken are stored in different cellular organelles. Element's soluble fraction is mainly found in the cytosol with proteins for detoxification (metallothionein-like proteins). Elements in the insoluble fraction are associated with metal-rich granules, organelles and cellular debris, their absorption dependent on the consumer digestive capacity. The bioaccumulation and subcellular partitioning are different between species that is why the metal uptake and the species vulnerability to contamination are different (Wallace *et al.*, 2003, Velez *et al*., 2015a).

The metal toxicity assessment in estuarine organisms is complex because the organism's physiology, metal bioavailability and potential for remobilisation alter with salinity gradient and temperature variation (Eggleton & Thomas, 2004; Machado *et al*., 2016; Chiesa *et al*. 2018).

1.4 Tracing the origin of *R. philippinarum*

1.4.1 Societal problem

The introduction of Manila clam in Tagus estuary increased fishery interest leading to an intensive exploration of this resource mainly due to the economic success combined with their wide distribution. The lack of specific regulation in Tagus estuary led to the enhancement of illegal harvestings (17.000 - 47.000 Kg/day) (Ramajal, 2016). In order to commercialise the clams, they are labelled with false origin passing by from clam collected at Sado estuary, where there's no regulation. The primary goal of the current regulation in Tagus estuary is to avoid the risk of human consumption of clams collected in polluted areas with microbiological or chemical agents which promotes the commercialisation restrictions appliance (Dispatch nº 4022/2015).

The intensive fishery and commercial exploitation of Manila clam led to a daily limitation establishment of 80 kg per catcher or fishers (ordinance 1228/2010; concierge Nº. 85/2011). For recreational fishing, the ordinance regulation is nº14 / 2014, article 12 and authorises the daily capture of 5 kg per trainer.

1.4.2 Societal challenge

The societal challenge issue is determining the clam's origin in the market chain in order to increase food security reducing the consumption risk and catcher's physical integrity risk promoting the intermediaries selling price control enhancing the catcher selling opportunities.

1.4.3 Scientific question

The societal challenge supported the development of the followed scientific question:

What are the differences of *R. philippinarum* chemical profile between and within Tagus and Sado estuaries?

1.5 Finality and main objectives

This study development is framed in NipoGes project in order to distinguish the *R.philippinarum* populations from Tagus and Sado estuaries with chemical fingerprint determination. To achieve this goal were established the following objectives:

- ✓ Apply ICP-MS technique as an important analytical tool to determine elemental concentrations and identify the chemical profile of *R. philippinarum* in Tagus and Sado estuaries

- ✓ Identify and quantify the clam's trace elements in Tagus and Sado estuaries and relate with physic-chemical parameters.
- ✓ Identify spatial distribution pattern of clam's chemical profiles along the Tagus and Sado estuaries.
- ✓ Identify significant differences of chemical profiles between sites within and between estuaries.
- ✓ Relate the sediment chemical profile of the sampled sites with the chemical profile of the sampled clams in Tagus and Sado estuaries.
- ✓ Discuss the relationship between clam's chemical profile and chemical elements of lithological influence and anthropogenic activities influence.

1.5.1 Work hypothesis

To determine distribution patterns in clam's chemical profiles will be tested the following working hypotheses:

- ✓ There are distinct patterns in clam's chemical profile between Tagus and Sado estuaries.
- ✓ There are significant differences in clam's chemical profile between sites within each estuary.
- ✓ There is a relationship between the sediment and clams chemical profiles.

Multivariate discriminant approaches were applied in order to know whether the combination of elemental concentrations could be used to distinguish *R. philippinarum* from different locations of collection sites at various space scale and answer the scientific question developed.

Chapter II: Material and Methods

2. Material and Methods

2.1 Study areas

2.1.1 Tagus estuary

Tagus estuary is considered one of the largest in Europe, near to 320 km^2 as total area, has an extremely high socio-economic importance, supporting several industries, agriculture, a high industrialised harbour and intensive urban activities from the large metropolitan area of Lisbon for decades (Caçador *et al*., 2009). The several activities promoted high levels of contamination from chemical, petrochemical, metallurgic, shipbuilding, cement manufacture industries, and agriculture fertilisers/pesticides wastes. Through the years, several untreated effluents were released in the estuary without restrictions recently the effort to increase urban and industrial effluent treatments led to the construction of several wastewater purification stations (Duarte & Caçador, 2012). In southern margin there is an inactive chemical-industrial complex, [e.g. Margueira (Almada), Quimiparque (Barreiro) and Siderurgia Nacional (Seixal)] which during the 1960-1970s produced several fertilizers, food products, nitric, hydrochloric and sulphuric acid, ore processing for metallurgy, among others (Monteiro *et al*., 2016). Recent studies reveal a high concentration of several metals such as Zn at mudflat sediments and high levels of Pb, Cu and Cd at Rosario saltmarshes followed of a high amount of organic matter (Vinagre *et al*., 2008). The increasing presence of Cr, Ni, Co, Hg, Al and the metalloid As are mainly due to anthropogenic effects rather than the grain size effect or lithological effects (Chainho *et al*., 2008; Mil-Homens *et al*., 2014). At the Tagus estuary northern margin near to Trancão River there is a concentrated industrial area that produces metals, wood factoring and food products (Chainho *et al*., 2013).

Tagus river represents the estuary main freshwater input with water volume drained of 86,629 km^2 total area, is considered the second most important hydrographic basin of the Iberian Peninsula (Duarte & Caçador, 2012) and the second largest in Portuguese territory (Cruzeiro *et al*., 2016). The dam construction in the Tagus River increased the effluent impact by the reduction of freshwater flows bounding the hydrographic basin regeneration and the presence of several fish communities (Chainho *et al*., 2013). The fluvial drainage is influenced by seasonal variations the average monthly value varies between 120 $m^3.s^{-1}$ in summer and 653 $m^3.s^{-1}$ in winter [Published in (INAG) Water National Institute public database]. The estimated residence time varies from 6 to 65 days including river discharges in winter and summer, respectively (Duarte & Caçador, 2012).

This estuary has a semi-diurnal mesotidal system, with tidal amplitudes from 1.0 m (neap tide) to 3.5 m (spring tide) at the estuary mouth (Cruzeiro *et al*., 2016; Brito *et al*., 2018). Tagus estuary has asymmetric tidal currents, where floods are longer than ebbs (Fortunato *et*

al., 1999), leading to higher velocities during ebbs enhancing fine-grained sediments exportation. The southern margin has a low altitude variation (up to 60 m) followed by an irregular shape which promoted the development of extensive outcrops of sand, gravel, sandstones, conglomerates, silt, and clay leading to water and sediment retention sites (Brito *et al*., 2018). The occurrence of several morphological alterations of margins easing their erosion. The destruction of several riparian habitats with the construction of infrastructures, and the constant dredging operations in order to help the intense port activity, change the hydromorphologic characteristics without knowing the specific impacts through biological communities (Duarte & Caçador, 2012; Chainho *et al*., 2013).

2.1.2 Sado estuary

Sado estuary is the second largest estuary in Portugal, with an area near to 240 km^2 located at 40 km south of Lisbon (Lillebø *et al*., 2011, Caeiro *et al*., 2017). The Sado estuary is included in NATURA 2000 (PTCON0011) therefore protected under EU legislation (Directives 2009/147/EC and 92/43/EEC, respectively the Birds and Habitats Directives) (Caeiro *et al*., 2017). The intertidal area has approximately 78 km^2 (Lillebø *et al*., 2011), in which 30% are salt marshes and intertidal flats. The estuary has a wide bay partially separated by intertidal sandbanks where is Troia beach and is linked to the ocean by a channel with 50 m deep (Gonçalves *et al*., 2015). Sado estuary hydrodynamics is influenced by semidiurnal tides which vary between 1.6 m as spring tides and 0.6 m as neap tides. Sado river drainage represents an annual mean flow of 40 $m^3.s^{-1}$ with a high seasonal influence, at summer time there are low freshwater discharges, and the estuary hydrology is overcome by tidal flows. This aquatic system is also referred to as a lagoon-type estuary with a total water volume of 500.106 m^3 and a mean water residence time of 21 days (Gonçalves *et al.*, 2015).

Sado estuary represents a distinct model of conflict and competition between the nature conservation and human activity. This estuary belongs to Arrábida Natural Park which is characterised by the wide biogeographical diversity, with a high ecological and socioeconomic importance, which is influenced by a highly industrialised and urbanised area of Setúbal city (Caeiro *et al*., 2003). The north of estuary represents a shallow specific area, mostly occupied by extensive intertidal flats and salt marshes and is near to the harbour area of Setúbal city where are generally threatened by several sources of anthropogenic influence. The neighbouring area is composed by heavy industries such as the large paper mill, shipyards, pesticide and fertiliser factory, copper mining activities, thermoelectric plant, aquaculture among others (Caeiro *et al*., 2003; Carreira *et al*., 2013). In the south margin of Sado estuary near to Sado river mouth and their small tributaries are influenced by an intensive agricultural exploration, mainly rice fields, draining high amounts of organic matter, fertilisers and aggregate insecticides (Caeiro *et al*., 2009). At Carrasqueira village the fishermen traditionally

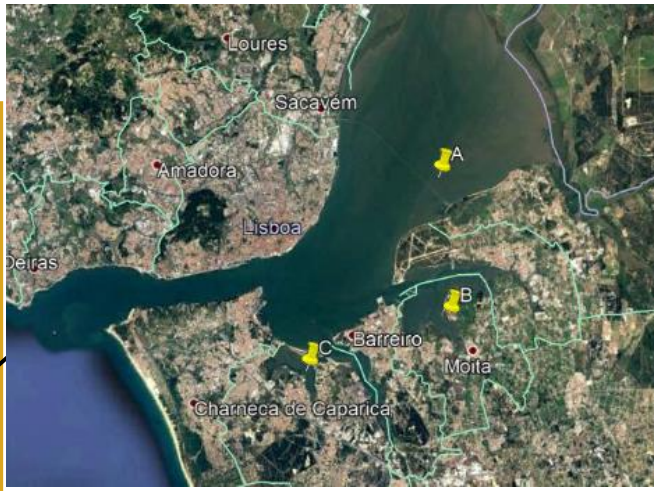
use trawl nets in the area to capture estuarine species that inhabit the sedimentary environment representing an important natural resource to preserve (Caeiro *et al*., 2017). The increasing levels of many contaminants, both organic and inorganic, are revealing adverse toxicological consequences to biota that have been reported in recent studies (Caeiro *et al*., 2009; Costa *et al*., 2012; Carreira *et al*., 2013). Several inputs are related to heavy metal contents by the historic existence of pyrite mines in river Sado, especially copper discard mining waste directly into the river without treatment following the high levels of Cd, Cu, Zn, Hg and Pb in specific areas (Lillebø *et al*., 2011). The north area is highly industrialised and responsible for the highest level of Pb, Cu, Cr and Ni (Caeiro *et al.*, 2005; Serafim *et al*., 2013).

The enhanced presence of trace elements in coastal waters of the southern region of Iberian Peninsula are highly related with the lithological influence of the river basins by weathering of rocks and soils that cross the Peninsula Belt and bring out the abundance of Cu, Cr, Ni and Co (Mil-Homens et al., 2014).

2.2 Sampling strategy

The sampling survey was performed in flat intertidal areas of the Tagus and Sado estuaries. At Tagus estuary were selected three sites, Alcochete (A), Montijo bay (B) and Seixal bay (C) (Figure 1). Alcochete is located at northern part of the estuary near to Tagus Natural Reserve which is composed by intertidal mudflats and salt marshes and is influenced by Tagus River discharges. Montijo bay and Seixal bay are considered high polluted areas with a high amount of metals accumulated in sediments previewing a historic and intensive anthropogenic pressure followed by a low tidal influence and increasing time residence.

In Sado estuary it was selected three sites, at north margin the sampling site Gâmbia - Pontal dos Musgos (D) near to an oyster nursery, at south margin the sampling site Herdade do Pinheiro (E) near to Alcácer channel, and the sampling site Cabeço do Ratão (F) near to Carrasqueira rice field and near to the Sado river mouth (Figure 1).



a) Sampling sites of *Ruditapes philippinarum* in Tagus estuary: Alcochete (A), Montijo bay (B) and Seixal bay (C).

b) Sampling sites of *R. philippinarum* in Sado estuary: Gâmbia (D: 38°27'39.06"N 8°43'19.57"W); H. Pinheiro (E: 38°27'39.06"N 8°43'19.578"W) and C. Ratão (F: 38°26'18.5532"N 8°45'26.013"W).

Figure 1: Sampling sites of *Ruditapes philippinarum* in a) Tagus estuary and b) Sado estuary. Samples location and maps from Google Earth.

The selection procedure of the sampling area in each estuary was performed with the following criteria: the occurrence of *Ruditapes philippinarum*; different hydrological (sub-basins influences) and geomorphological characteristics (type of sediment). The clams were collected in May 2018, which matched with the fertile period of manila clams. To guarantee a representative sample approach they were randomly collected in the sampling sites at low tide conditions. In each sampling site it was manually collected thirty specimens adults with a similar size (ranged between 3.5 cm and 4.5 cm) (n= 3x30 = 90 total specimens/estuary) and sediment were collected (n=3 recipients /estuary) to determine the contents of 16 trace elements Sc, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Sr, Cd, Cs, Ce, Eu and Th with inductively coupled plasma mass spectrometer (ICP-MS) analysis.

After sampling, the specimens and sediment recipients were transported on ice (0 °C) to the laboratory and stored at −20 °C for later processing.

2.3 Laboratory analysis

2.3.1 Metals quantification in clams

a) Clam sample pre-treatment

In ICP-MS analyses were used 10 specimens from each sampling site which were replicated. Clams soft tissue was removed from the shell with a plastic knife, rinsed between 2 - 3 times with H_2O Milli-Q (18.2 MΩ.) and stored overnight at -20 °C. Then all samples were freeze-dried over -80 °C during 72h in a vacuum-freeze dryer and grinded with a ceramic mortar and pestle.

b) Hotplate acid digestion

The analytical analysis used to quantify trace elements in clam soft tissues by ICP-MS required previous sample digestion. The hotplate digestion procedure was performed based Ashoka *et al*. (2009), which is a combination of nitric acid (HNO_3) with hydrogen peroxide (H_2O_2) on a hotplate, in order to digest the clam organic compounds over a cyclic thermic reaction.

Was weighed 0.1 g of dry tissue directly into PFA containers then it was added 3 mL of HNO_3 (65% v/v) (Suprapur, Merck®), the caps were tightened and stood overnight at room temperature as a pre-digestion procedure. Afterwards, they remained in a hotplate at 125°C for 3 hours. At this step, the acid solution starts to develop an intense orange colour atmosphere due to the organic matter digestion supported by the acid cycling resuspension. The caps were opened and remained at 150°C until incipient dryness. It was added 3 mL of HNO_3 (65% v/v) (Suprapur, Merck®) and it was maintained overnight at 125°C. After cooling,

the caps were opened and in each container it was added 2 mL of H_2O_2 (30% v/v) (Suprapur, Merck®) for further oxidation, remaining in the hotplate at 60 °C until completed reaction and it was followed of the evaporation process until incipient dryness at 150°C. All samples were homogenised with 3 mL of H_2O Milli-Q (18.2 MΩ.) and 1.6 mL of HNO_3 (65% v/v) (Suprapur, Merck®) remaining overnight at 125 °C. After cooling, they were transferred to 50 mL volumetric flask and made up with H_2O Mili-Q (18.2 MΩ.) to a final concentration of HNO_3 (2% v/v) (Suprapur, Merck®) and stored in Falcon tubes at - 20°C until ICP-MS analyses.

2.3.2 Metals quantification in sediments

a) Sediment sample pre-treatment

After unfreezing process, sediments were placed on the oven a constant temperature of 50 °C until completely dryness, it was removed all shells remnant using two sieves with different mesh sizes (500µm and 1mm) and a plastic tweezer. Small portions of each sample (~ 30 – 40 g) were grinded to a fine powder using the Planetary Ball Mill PM 100 (Retsch®).

b) Hotplate acid digestion

Sediment digestion method it was based on Ottley *et al*., (2003) procedure which apply a combination of ($1HNO_3$ + 4HF) to achieve a total digestion of silicates, then use aqua regia solution ($1HNO_3$ + 3HCl), followed by HNO_3 65% (v/v) (Suprapur, Merck®) and H_2O_2 (30% v/v) (Suprapur, Merck®) to complete the digestion eliminating the organic contents.

For digestion procedure, was weighted 0.1 g of sediment dried powder directly in the digestion containers, then it was added 0.5 mL HNO_3 (65% v/v) (Suprapur, Merck®) and 2 mL HF (50% v/v) (OPTIMA Grade®) remaining on a hotplate at 125 °C for 42h. The PFA containers were opened to evaporate near dryness avoiding the stabilisation of insoluble fluorides. After the initial dry procedure, samples are taken up with 2 mL of aqua regia solution (HNO_3 (65% v/v) (Suprapur, Merck®) + 3HCl (37% v/v) (Suprapur, Merck®)) remaining overnight at 150 °C. After cooling, the PFA containers were opened to evaporate near dryness on the hotplate and to end digestion process it was repeated the same overnight at 125°C with 2 mL HNO_3 (65% v/v) (Suprapur, Merck®). After cooling, the PFA containers were opened, and it was added 1 mL of H_2O_2 (30% v/v) (Suprapur, Merck®) for further oxidation, remaining in the hotplate at 125 °C until solution transparency and evaporation process to incipient dryness. All samples were homogenised with 3 mL of H_2O Milli-Q (18.2 MΩ.) and 1.6 mL of HNO_3 (65% v/v) (Suprapur, Merck®) remaining overnight on a hotplate at 125 °C. Following cooling and near dryness processes, samples were made up with H_2O Milli-Q (18.2 MΩ.) to a final concentration of HNO_3 (2% v/v). They were stored in 50 mL Falcon tubes at - 20°C until ICP-MS analyses.

Digestion of certified reference materials and blanks was applied using the same analytical procedure and reagents to ensure the assays quality control.

2.3.2 ICP-MS Analysis

a) Operating conditions

After digestion procedure, the determination of all elements concentrations was obtained with ICP-MS equipment from Agilent Technologies, 8800 ICP-MS triple quad.

To ensure the best equipment performance it was used ^{101}Ru, ^{103}Rh and ^{193}Ir (400 ppb) as internal standards in order to control and compensate the possible instrument drift due to matrix suppression and other spectral overlaps such as double charged ions, polyatomic and isobaric interferences.

In the octopole reaction system (ORS) the gas was selected according to the natural behaviour of isotopes from each analyte ions. It was used no-reaction gas He, reaction gas O_2 and NH_3 and without gas (Table 1).

The ICP-MS analysis precision is determined with relative standard deviation (RSD) calculations, which is related to the repeatability of the assays. The equipment sensibility and quality control were evaluated with the limit of detection (LOD) and QL quantification limit (QL) calculations.

The LOD is the sample lowest quantity detected from the instrument which can allow distinguishing one isotope to another. For the LOD calculations, it was required the standard deviation of 11 blank measurements (cps) and QL is 10 * LOD for each element at the beginning of the ICP-MS analysis (see LOD and QL values Table 2 and Table 3).

Determination of element concentrations was based on a calibration graph obtained from several dilutions (0, 1, 5, 10, 20, 60, 100, 200, 600, 1000 and 2000 ppb's) of 100 ppm of stock multi-element standard solution from High Purity Standards® ICP-MS-68B solution A. All standards and samples were measured in triplicate, the coefficient of determination ($R^2 \geq 0,999$) guaranteeing the highest prediction values.

The RSD is defined by the ratio of standard values and means it was considered values <10% to ensure a good precision.

Table 2: Instrument conditions

ICP-MS 8800 QQQ Agilent Technologies		
Scan type	MS/MS	
Plasma	RF Power	1550 W
	RF Matching	1.70 V
	Carrier Gas	1.20 L/min
	Neublizer Pump	0.10 rps
	Gas Switch	Makeup Gas
	He Use gas	He flow 4.0 mL/min
	O_2 Use gas	4th Gas Flow 50 %
	NH_3 Use Gas:	He flow 1.0-4.0 mL/min 3rd gas Flow 15%
OctP Bias	-8.0 V	
Acq Parameters	Acq mode	Spectrum
Spectrum Mode Option	Q2 Peak pattern	1 point
	Replicates	3
	Sweeps/replicate	10
Isotope / ORS	^{45}Sc [NH3];^{52}Cr [He]; ^{55}Mn [He]; ^{56}Fe [NH3]; ^{57}Fe [NH3]; ^{59}Co [He]; ^{60}Ni [He]; ^{63}Cu [He]; ^{66}Zn [He]; $^{75\ ->\ 91}$As [O2]; $^{78\ ->\ 94}$Se [O2]; ^{88}Sr [He];^{111}Cd [No Gas]; ^{133}Cs [No Gas]; ^{140}Ce [No Gas]; ^{153}Eu [No Gas]; ^{232}Th [No Gas]	
Internal standards	^{103}Rh; ^{101}Ru; ^{193}Ir	
Calibration standard	ICP-MS-68B (100 mg/L, HNO_3 4% (v/v))High Purity Standards®	
DL Solution	400 ppb solution A	

b) ICP sample preparation

Before ICP-MS analysis, the digested samples remained at room temperature for 30 minutes in order to reduce viscosity and ensure the homogeneity of ICP-MS reading solutions. Some samples were diluted 100 fold with HNO_3 2% (v/v) (Suprapur, Merck) in order to determine the concentration of major elements. The final results for each element were expressed as mg. Kg^{-1} or ppm of dry weight.

The multi-element analysis accuracy it was evaluated with standard reference material for clam, mussel tissue SRM - 2976 certified by NIST (Supplementary information, Table 10 and 11) and standard reference material for estuarine sediment BCR - 667 from Institute for Reference Materials and Measurements (IRMM) (supplementary information Table 12 and Table 13). For sediment analysis, it was added standard referenced material with elements referenced for ceramics compounds, for quality control (supplementary information Table 14 and Table 15).

The measured values of analyte elements for CRM sample and referenced values certified by NIST were expressed as ppb, and they were used to calculate the % recovery values. The analyte concentrations obtained from the experiment reveal a good approach with certified values with acceptable recoveries range from 82% to 120% (Table 2 and table 3).

Table 5: Evaluation of ICP-MS analytic parameters. Reference material mussel tissue SRM – 2976 with certified values (ppb), recoveries (%) values, LOD (ppb) and QL (ppb) determinations, for clam samples.

Elements	ORS mode	[CRM]measured (ppb)	[CRM] Ref (ppb)	Recovery (%)	LOD (ppb)	QL (ppb)
Sc	NH_3	0,04	0,03	147	0,004	0,04
Cr	He	82,8	1,0	83	0,04	0,4
Mn	He	65,7	66,0	100	0,08	0,8
Fe	NH_3	328,6	342,0	96	0,06	0,6
Co	He	1,18	1,2	96,6	0,007	0,07
Ni	He	1,6	1,9	88,6	0,004	0,04
Cu	He	7,9	8,0	98,0	0,04	0,4
Zn	He	280,5	274,0	102	0,6	5,8
As	O_2	28,5	26,6	107	0,03	0,3
Se	O_2	3,9	3,6	109	0,05	0,5
Sr	He	144,4	186,0	78	0,05	0,5
Cd	No Gas	1,7	1,6	102	0,004	0,04
Cs	No Gas	0,3	0,05	548	0,002	0,02
Ce	No Gas	0,2	0,2	91	0,001	0,01
Eu	No Gas	0,005	0,005	101	0,001	0,01
Th	No Gas	0,1	0,02	560	0,01	0,1

The elements Sc, Cs and Th were out of detection limit range calculated during the ICP analysis and Sr measured had a low recovery value. These elements were not considered for further data treatment.

Table 8: Evaluation of ICP-MS analytic parameters. Reference material BCR - 667 with certified values (ppb), recoveries (%) values, LOD (ppb) and QL (ppb) determinations, for sediment samples.

Elements	ORS mode	[CRM]measured (ppb)	[CRM] Ref (ppb)	Recovery (%)	LOD (ppb)	QL (ppb)
Sc	NH_3	25	27,4	92	0,005	0,05
Cr	He	342	356	96	0,07	0,7
Mn	He	1697	1840	92	0,04	0,4
Fe	NH_3	84732	89600	95	0,1	1,4
Co	He	43	46	94	0,009	0,09
Ni	He	241	256	94	0,04	0,4
Cu	He	118	120	98	0,05	0,5
Zn	He	309	0,35	88274	0,3	3
As	O_2	34	28,6	121	0,01	0,1
Se	O_2	1	3,18	32	0,05	0,5
Sr	He	438	412	106	0,07	0,7
Cd	No Gas	1	1,34	86	0,002	0,02
Cs	No Gas	16	15,6	100	0,001	0,01
Ce	No Gas	107	113,4	95	0,002	0,02
Eu	No Gas	1,98	2	98,8	0,0004	0,004
Th	No Gas	18	20	92	0,002	0,02

The sediment samples were diluted 100 fold to measure Fe which is considered as a major element in estuarine environments. The measured Zn and Se it was out of the detection limit range calculated during ICP analysis. These elements were not considered for further data treatment.

2.4 Statistical Analyses

The elemental data of clam samples were used as chemical descriptors, applying statistical methods using the PRIMER v6 software, to evaluate spatial patterns and their significant differences along and between estuaries.

The descriptive statistical analysis it was applied to characterise the element´s spatial disposal. Then was applied the PCA analysis (principal component analysis) in order to reduce the dimensionality of original variables maintaining the highest variance as possible and determine the primary evolution between class similarities. PCA can determine which variables contribute most to this difference building a discriminatory pattern for clams with different geographical origins.

PCA ordination was performed for Cr, Mn, Fe, Co, Cu, Zn, As, Se, Cd and Ce, to examine patterns in multidimensional data by reducing the number of dimensions, with minimal loss of information. The PCA ordination was based on the average of the elements concentrations (mg/kg) measured in clams from different ‘sites’ and ‘estuaries’. Previously, the calculation of the elements concentrations resemblance matrix based on Euclidean distances, and if necessary were log (X+1) transformed before analysis, then normalised by subtracting the mean and dividing by the standard deviation for each variable.

Significant differences of chemical patterns in clams and sediment within and between estuaries were assessed testing the hypothesis with PERMANOVA analysis (permutational multivariate analysis of variance), using the following models, two-factor Permanova test with “Estuary” (2 levels fixed), “Sites” (6 level fixed and nested in Estuary) for all variables analysed at $p < 0.05$ level. Monte Carlo test was performed as the complement of permutation level.

Chapter III: Results

3. Results

3.1. Trace elements quantification in *Ruditapes philippinarum*

The mean values and standard error of elemental concentrations (mg/kg, DW "*Dry Weight*") in *R. philippinarum* soft tissues from Tagus and Sado estuaries are summarised in Table 4 and Figure 2 and 3.

The concentrations of Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Cd, Ce and Eu of the clams collected in three sampling sites of Tagus estuary: Alcochete, Montijo bay and Seixal bay and also in three sampling sites of Sado estuary: Gâmbia, Herdade do Pinheiro and Cabeço do Ratão were analysed to determine the spatial patterns of the clam's chemical profile among sites from the same estuary and between estuaries.

Table 11: Elements content in the clams of the Tagus and Sado estuaries.

Estuary	Tagus			Sado		
Sites / Elements	Alcochete	Montijo bay	Seixal bay	Gâmbia	H. Pinheiro	Cabeço Ratão
Cr	2,673 ± 0,12	2,84 ± 0,41	2,72 ± 0,46	2,45 ± 0,03	3,69 ± 0,19	3,45
Mn	58,25 ± 0,32	33,52 ± 4,94	22,34 ± 4,4	27,96 ± 0,96	31,81 ± 1,71	29,59
Fe	1648,92 ± 63,07	2168,99 ± 383,32	2080,89 ± 427,71	1567,8 ± 85,97	2831,72 ± 4,82	2173,29
Co	5,75 ± 0,23	4,24 ± 0,21	4,55 ± 0,23	2,13 ± 0,1	4,18 ± 0,19	3,64
Ni	18,72 ± 1,35	12,61 ± 0,03	12,94 ± 0,85	6,69 ± 0,18	6,47 ± 0,17	9,28
Cu	13,42 ± 0,45	10,68 ± 0,16	14,24 ± 1,58	14,24 ± 0,63	13,37 ± 1,22	22,54
Zn	199,13 ± 8,02	225,31 ± 2,33	223,21 ± 12,17	146,5 ± 2,6	91,36 ± 2,22	143,53
As	22,97 ± 0,54	40,81 ± 5,21	38,68 ± 2,51	21,05 ± 0,5	61,81 ± 3,78	33,54
Se	13,76 ± 0,3	8,1 ± 0,62	8,05 ± 0,31	4,9 ± 0,08	7,85 ± 0,17	5,55
Cd	0,5 ± 0,051	0,85 ± 0,02	0,74 ± 0,009	0,16 ± 0,0008	0,83 ± 0,16	0,64
Ce	4,11 ± 0,21	4,93 ± 0,34	4,23 ± 0,74	2,2 ± 0,006	4,03 ± 0,046	3,02
Eu	0,1 ± 0,005	0,1 ± 0,009	0,1 ± 0,01	0,06 ± 0,0003	0,11 ± 0,0001	0,08

Mean ± SE, n=2 of elemental concentrations (mg/kg, DW) in clams collected in Tagus and Sado estuaries. Collection sites in Tagus estuary: Alcochete, Montijo bay and Seixal bay and Sado estuary: Gâmbia, Herdade do Pinheiro, and Cabeço do Ratão, n=1.

At both estuaries studied the *R. philippinarum* samples registered the highest concentration values for the elements Fe, Zn, As and Mn. At Tagus estuary, the elemental concentration in the clam's soft tissues decreased in the following order Fe > Zn > As > Mn and at Sado estuary the order was Fe >Zn > Mn > As (Table 4). The lowest concentration were registered at Tagus estuary for the elements Ni, Cu, Se, Co, Ce, Cr, Cd and Eu, ranked in decreasing order Ni > Cu > Se > Co > Ce > Cr > Cd > Eu and at Sado estuary were Cu > Ni > Se > Cr > Ce > Co > Cd > Eu. The highest and the lowest trace elements concentration were similar at both estuaries, although ranked in different order.

At Tagus estuary the sampling site Alcochete, registered the highest values of the elements Mn (58,25 ± 0,32 mg/kg), Ni (18,72 ± 1,35 mg/kg) , Se (13,76 ± 0,3 mg/kg) and Co (5,75 ± 0,23 mg/kg). While, the lowest values were observed for the elements Fe (1648,92 ± 63,07 mg/kg, Zn (199,13 ± 8,02 mg/kg), As (22,97 ± 0,54 mg/kg), Ce (4,11 ± 0,21 mg/kg), Cr (2,67 ± 0,12 mg/kg) and Cd (0,5 ± 0,051 mg/kg).

At sampling site Montijo bay, the highest values were Fe (2168,99 ± 383,32 mg/kg), Zn (225,31 ± 2,33 mg/kg), As (40,81 ± 5,21 mg/kg), Ce (4,93 ± 0,34 mg/kg), Cr (2,84 ± 0,41 mg/kg) and Cd (0,85 ± 0,02 mg/kg). The lowest values observed were Ni (12,61 ± 0,03 mg/kg, Co (4,24 ± 0,21 mg/kg) and Cu (10,68 ± 0,16 mg/kg).

At sampling site Seixal bay, the highest value observed was for Cu (14,24 ± 1,58 mg/kg), the elements Mn (22,72 ± 0,46 mg/kg) and Se (8,05 ± 0,31 mg/kg) registered the lowest values.

The clams sampling at Alcochete site registered the highest values of Mn, Ni, Se and Co, on contrary at Montijo bay were obtained the lowest values. The elemental Cu content at Seixal bay is presenting the highest value.

At Sado estuary, the sampling site Gâmbia, registered the highest values of the elements Zn (146,5 ± 2,6 mg/kg) and Ni (6,69 ± 0,18 mg/kg). Whilst, the lowest values were obtained for the elements Fe (1567,8 ± 85,97 mg/kg), Mn (27,96 ± 0,96 mg/kg), As (21,05 ± 0,5 mg/kg), Se (4,9 ± 0,08 mg/kg), Cr (2,45 ± 0,03 mg/kg), Ce (2,2 ± 0,006 mg/kg), Co (2,13 ± 0,1 mg/kg) and Eu (0,06 ± 0,0003 mg/kg).

At sampling site Herdade do Pinheiro, the highest values were Fe (2831,72 ± 4,82 mg/kg), As (61,81 ± 3,78 mg/kg), Mn (31,81 ± 1,71 mg/kg), Se (7,85 ± 0,17 mg/kg), Co (4,18 ± 0,19 mg/kg), Ce (4,03 ± 0,046 mg/kg), Cd (0,83 ± 0,16 mg/kg) and Eu (0,11 ± 0,0001 mg/kg). Whilst, the lowest values were observed for the elements Zn (91.36 ± 2,22 mg/kg), Cu (13,37 ± 1,22 mg/kg) and Ni (6,47 ± 0,17 mg/kg).

At sampling site Cabeço do Ratão Cu registered the highest value (22,54 mg/kg).

The clams sampling at Gâmbia site registered the highest values of Zn and Ni. On contrary at Herdade do Pinheiro were obtained the lowest values.

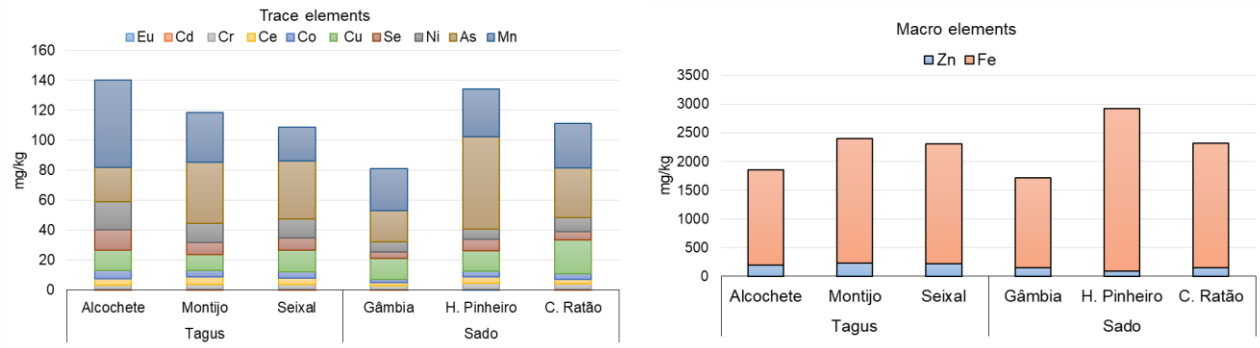
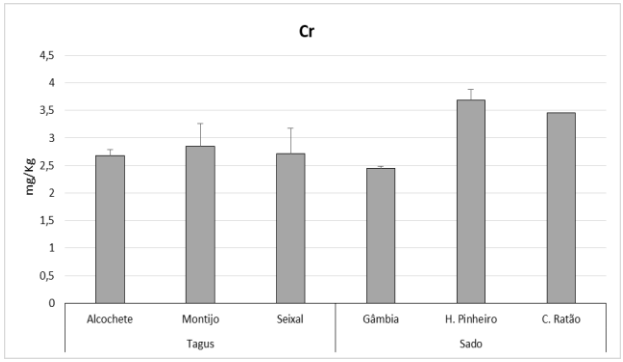
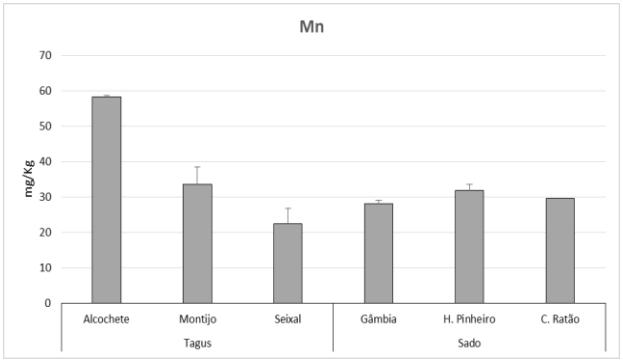
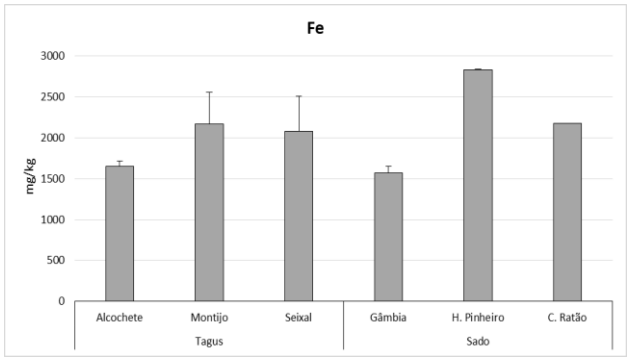
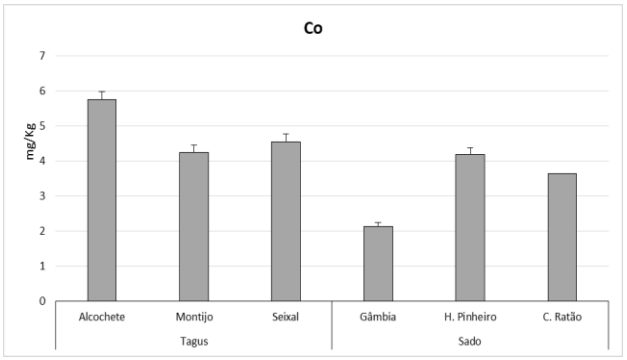


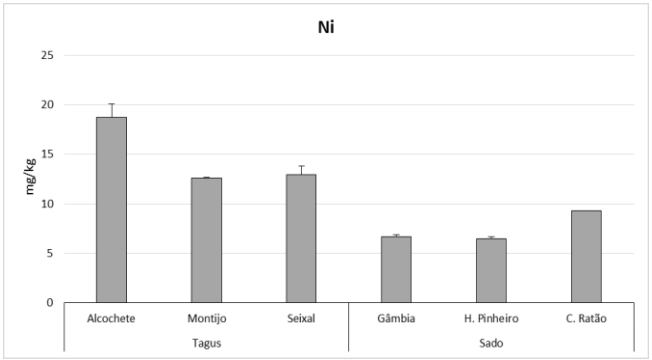
Figure 4: Elemental concentration values mean of the trace and major elements (mg/kg) determined for clam samples between sites in Tagus and Sado estuaries.

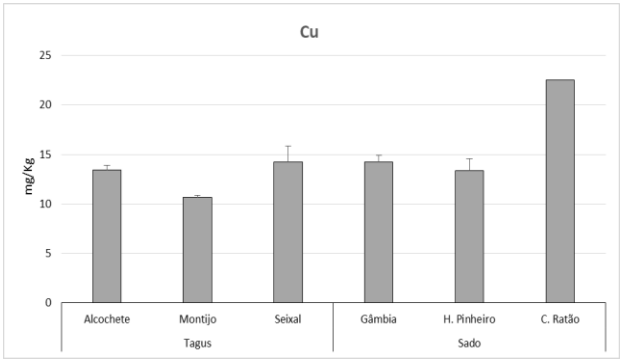












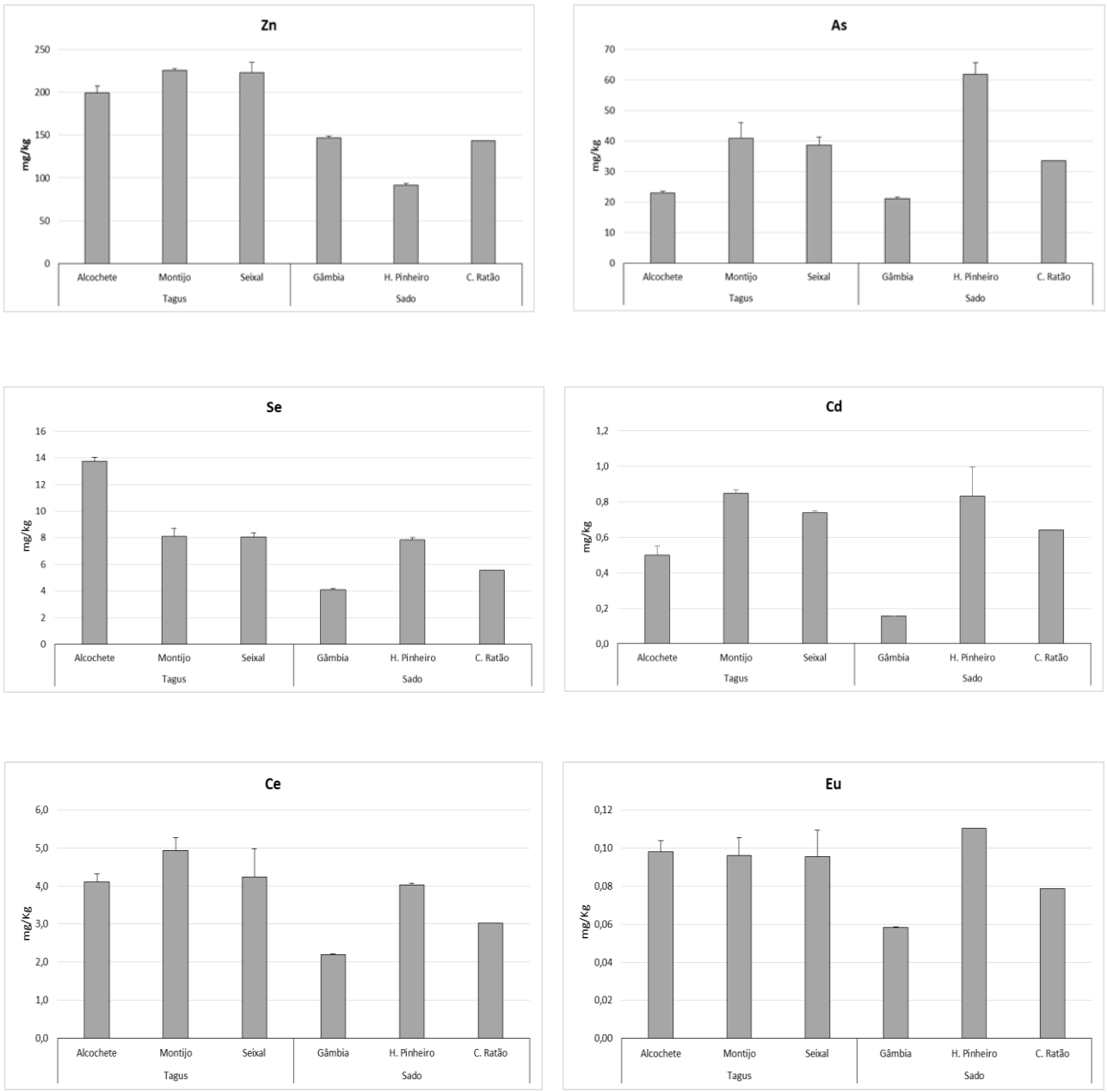


Figure 5: Mean ± SE, (n=2) of elemental concentrations (mg/kg, DW) from Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Cd, Ce and Eu in clam samples collected in Tagus estuary at the sites Alcochete, Montijo bay and Seixal bay and Sado estuary at sites Gâmbia, H. do Pinheiro and Cabeço do Ratão, n=1.

The elements Ni and Co; Fe and Zn; Mn and Se; Cd and Ce; Cr and Eu describes an identical pattern in all clams collected in Tagus estuary. The element Fe, As, Co, Ce, Cr, Cd, Eu and Se describes an identical pattern in clams collected in Sado estuary (Figure 3).

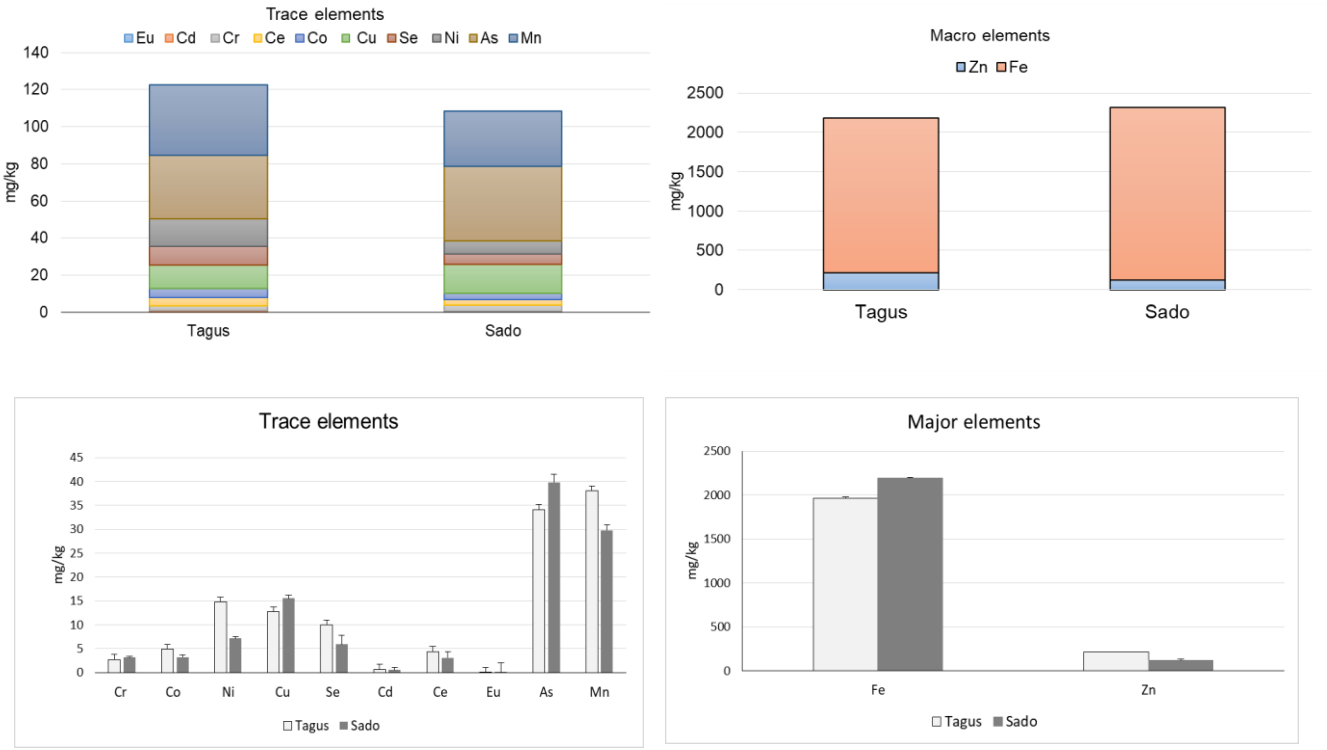


Figure 6: Elemental concentration values mean ± SE, (n=2), of the trace and major elements (mg/kg, DW) determined for clam samples between estuaries.

Comparing the elemental composition of clams collected in both estuaries, the clams collected in Tagus estuary showed the highest concentrations for the elements Zn, Mn, Ni, Se, Co, Ce, Cd and Eu, while the clams sampling in Sado estuary showed the highest concentration for the elements Fe, As, Cu and Cr (Figure 4). The elements Zn, Mn, Ni, Co, Cu, Se and Ce, were considered the elements with the highest variability between estuaries.

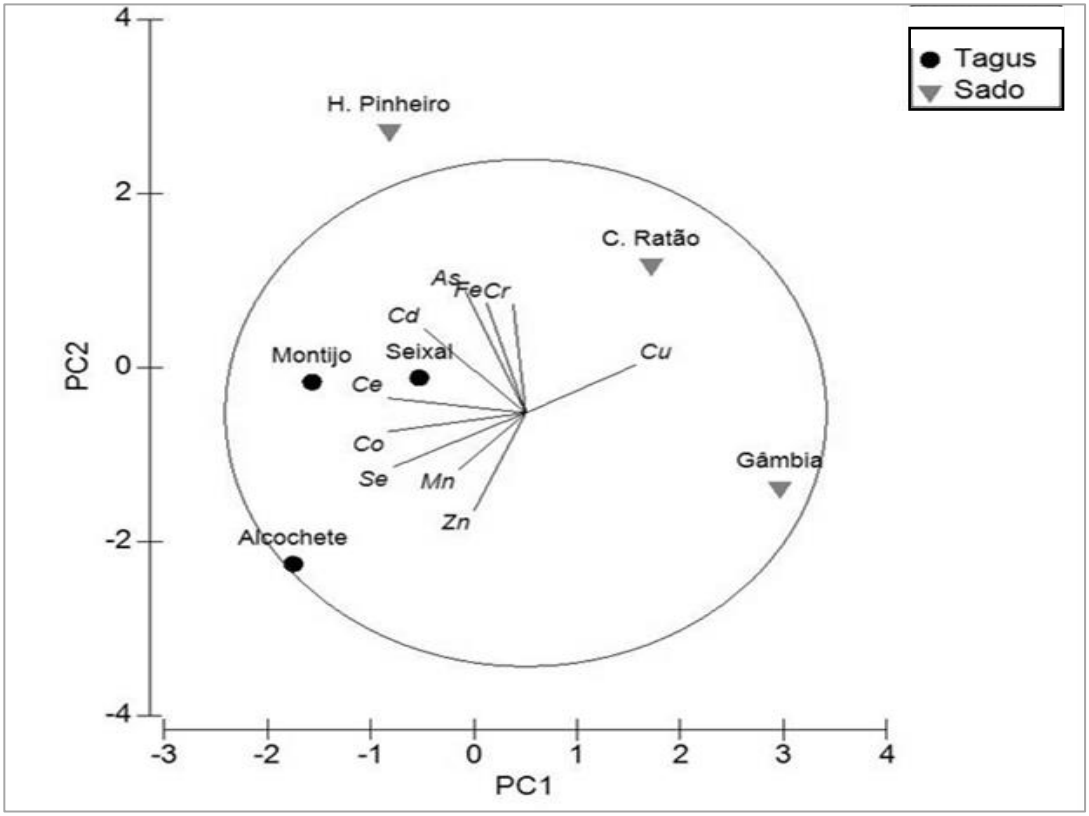


Figure 7: Principal Component Analysis (PCA) plot based on the elemental concentrations measured in clams collected at different sampling sites. The dots represent sampling sites in Tagus estuary (Alcochete, Montijo bay, and Seixal bay) and the triangles represent sampling sites in Sado estuary (Gâmbia, Herdade do Pinheiro, and Cabeço do Ratão), (PC1 38,8 %, PC2 33,8%).

PCA was based on results of the concentration from 10 elements Cr, Mn, Fe, Co, Cu, Zn, As, Se, Cd and Ce, measured in Manila clam soft tissue. The ordination of the sampling sites shows a clear separation of the clams collected in both, Tagus and Sado estuaries. The PCA ordination of the elemental concentration showed that the first two components (PC1, 38,8 %, and PC2, 33,8 %) accounted for about 72,6 % of the variability in the Figure 5. The collected clams in Tagus estuary were separated from those of Sado estuary, mainly by the predominance of the elements Zn, Se, Co, Ce, Mn and Cd in Tagus estuary and the elements As, Fe, Cr and Cu in Sado estuary.

At Tagus estuary, the elements Zn, Se, Co and Mn can describe the clams collected in Alcochete, and the elements Ce and Cd can describe the clams collected in Montijo and Seixal which revealed high similarity for these elements. Although the elemental concentration of Cu and Cr revealed to be important to the clams collected in H. Pinheiro and Gâmbia (Sado estuary).

The significant statistical differences on the clam´s chemical profile from different sampling sites were assessed by the hypothesis testing using PERMANOVA in order to

determine if there are distinct patterns between clams from different origins, Tagus and Sado estuaries, are summarised in Table 5.

Table 5: Details of two-factor PERMANOVA test with Estuary (Es) (2 levels fixed), Sites (Si) (6 level fixed and nested in Estuary) for all variables analysed. Bold values highlight significant effects (p<0.05). MC is Monte Carlo test. (p<0.05). MC is Monte Carlo test.

Source variation	df	SS	MS	Pseudo-F	P(perms)	perms	P(MC)
Es	1	33,446	33,446	9,7216	**0,0001**	9512	**0,0005**
Sites(Es)	4	67,844	16,961	4,9299	**0,0015**	9460	**0,0011**
Res	5	17,202	3,4404				
Total	10	120					

The PERMANOVA showed significant differences for clam's chemical profile between sites of the Tagus and Sado Estuaries (p< 0.05).

Pairwise test of PERMANOVA summarised in Table 6, revealed significant differences only between Gâmbia and Herdade do Pinheiro (p< 0.05), which are consistent with ordination results.

Table 6: Details of pairwise test of PERMANOVA Sites nested in Estuary "Si(Es)" within level 'Tagus' of factor 'Estuary' and within level 'Sado' of factor 'Estuary' for all variables analysed. Bold values highlight significant effects (p <0.05).

Level Tagus	t	P(perm)	perms	P(MC)
Alcochete x Montijo	2,1684	0,33	3	0,0885
Alcochete x Seixal	1,8003	0,3278	3	0,148
Montijo x Seixal	0,69244	0,6645	3	0,6479
Level Sado	t	P(perm)	perms	P(MC)
Gâmbia x H. Pinheiro	5,6261	0,3321	3	**0,0122**
Gâmbia x C. Ratão	6,1648	0,3278	3	0,0697
H. Pinheiro x C. Ratão	2,4375	0,3296	3	0,1823

3.1. Trace elements quantification in estuarine sediment

The mean values and standard error of elemental concentrations (mg/kg, DW) in sediment from Tagus and Sado estuaries are summarised in Table 7 and Figure 6.

The concentrations of Sc, Cr, Mn, Fe, Co, Ni, Cu, As, Sr, Cd, Cs, Ce, Eu and Th of the sediment collected in the same clam's sampling sites in Tagus estuary: Alcochete, Montijo bay and Seixal bay and also in the same clam's sampling sites in Sado estuary: Gâmbia, Herdade do Pinheiro, and Cabeço do Ratão were analysed in order to establish a relationship between the sediment and clams chemical profiles.

Table 7: Elements content in the sediment of the Tagus and Sado estuaries.

Estuary	Tagus			Sado		
Sites / Elements	Alcochete	Montijo	Seixal	Gâmbia	Herdade Pinheiro	Cabeço Ratão
Sc	11,16 ± 0,39	5,76 ± 0,09	0,21 ± 0,01	8,34 ± 0,40	2,38 ± 0,11	0,76 ± 0,03
Cr	60,33 ± 0,54	30,49 ± 0,96	2,12 ± 0,68	45,89 ± 2,69	12,28 ± 0,49	3,91 ± 0,16
Mn	559,37 ± 8	213,85 ± 2,81	14,19 ± 0,31	252,6 ± 12,75	92,85 ± 5,79	56,35 ± 6,06
Fe	32922,24 ± 371,28	17852,41 ± 451,79	1075,76 ± 93,23	23470,16 ± 842,62	6851,05 ± 299,65	1880,56 ± 134,82
Co	11,94 ± 0,18	6 ± 0,17	0,28 ± 0,01	8,15 ± 0,35	2,55 ± 0,06	1,17 ± 0,15
Ni	25,86 ± 0,45	12,33 ± 0,35	0,56 ± 0,02	17,83 ± 0,75	5,2 ± 0,13	1,65 ± 0,05
Cu	24,16 ± 0,29	16,19 ± 0,41	0,64 ± 0,02	33,1 ± 1,16	9,87 ± 0,37	3,16 ± 0,18
As	17,01 ± 0,23	13,65 ± 0,27	1,11 ± 0,08	12,18 ± 0,47	4,23 ± 0,10	2,07 ± 1,44
Sr	104,59 ± 5,99	93,38 ± 1,86	33,96 ± 1,25	77,51 ± 3,19	51,81 ± 2,13	48,5 ± 0,0001
Cd	0,36 ± 0,005	0,28 ± 0,01	0,01 ± 0,002	0,14 ± 0,005	0,05 ± 0,001	0,02 ± 0,07
Cs	10,6 ± 0,36	6,81 ± 0,15	1,26 ± 0,09	4,82 ± 0,29	3 ± 0,12	1,87 ± 1,31
Ce	58,61 ± 1,67	33,36 ± 2,05	5,79 ± 0,45	42,29 ± 2,88	14,38 ± 1,04	8,39 ± 1,31
Eu	0,98 ± 0,03	0,65 ± 0,01	0,14 ± 0,01	0,72 ± 0,04	0,36 ± 0,02	0,22 ± 0,01
Th	11,11 ± 0,38	5,76 ± 0,45	1,1 ± 0,09	7,12 ± 0,69	2,31 ± 0,22	1,41 ± 0,23

Mean ± SE, n=3 of elemental concentrations (mg/kg) in sediment collected in Tagus and Sado estuaries. Collection sites in Tagus estuary: Alcochete, Montijo bay and Seixal bay and Sado estuary: Gâmbia, Herdade do Pinheiro, and Cabeço do Ratão, n=2.

The sediments collected in Tagus estuary, registered the highest values in the following decreased order Fe > Mn > Sr > Ce > Cr, while the sediments collected in Sado estuary, registered the highest values in the following decreased order Fe > Mn > Sr > Cr > Ce > Cu. The lowest values in the sediment collected in Tagus estuary were ranked in decreasing order Ni > Cu > As > Co > Sc > Th > Cs > Eu > Cd and the elements Ni > As > Sc > Co >Th > Cs >Eu > Cd in the sediment collected in Sado estuary (Table 7, Figure 6 and 7).

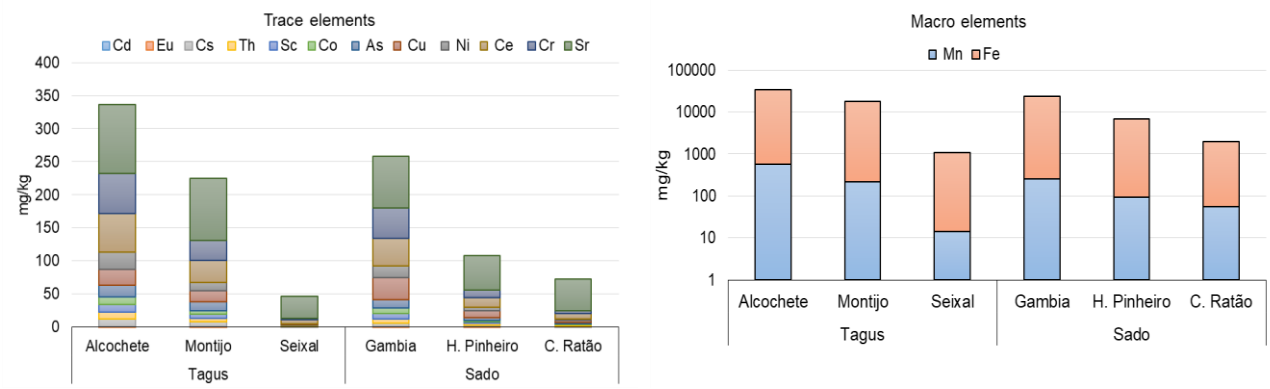
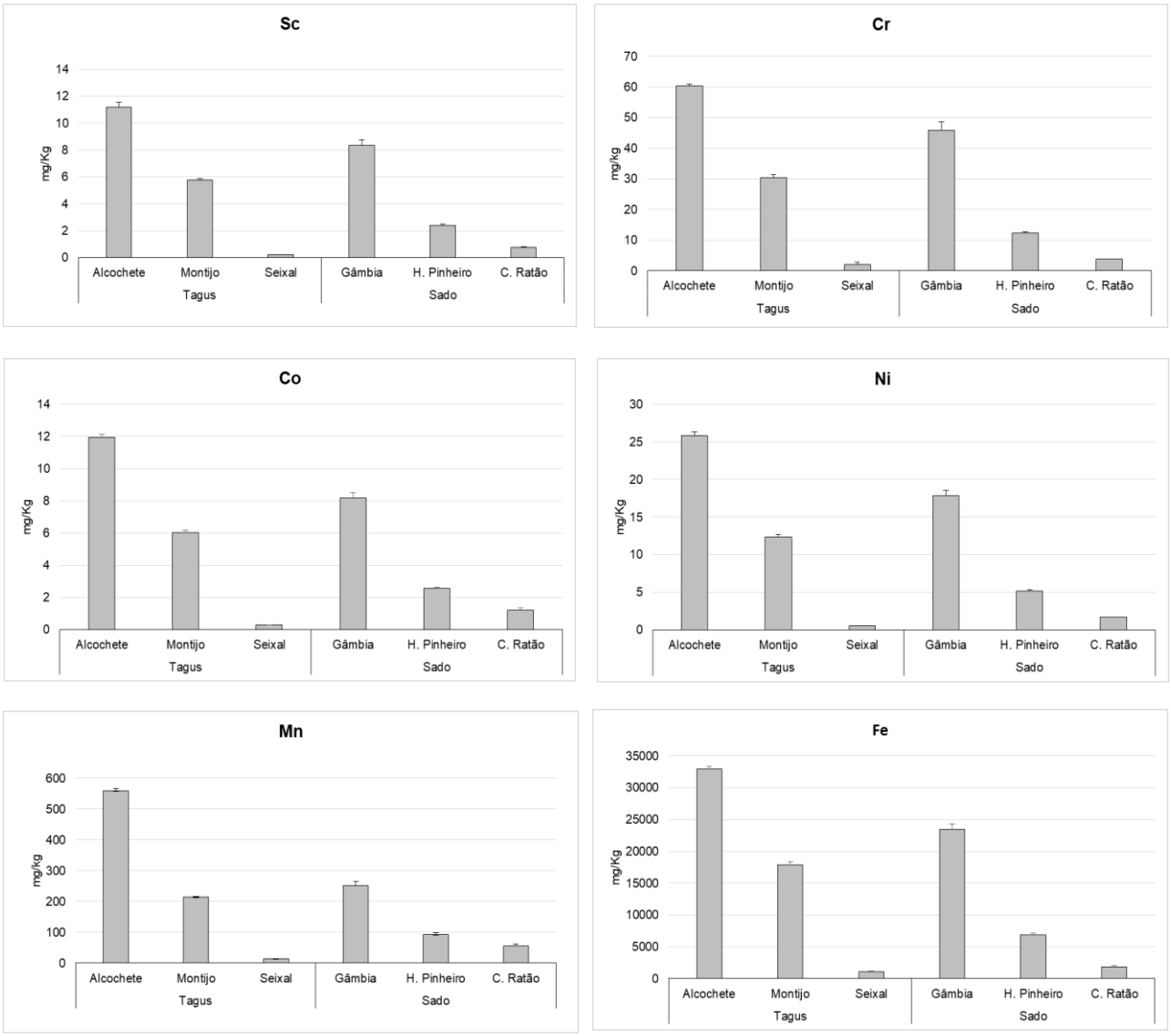


Figure 9: Elemental concentration values mean of the trace and major elements (mg/kg) determined for clam samples between sites in Tagus and Sado estuaries. The major elements graphic the y-axis are log-transformed values.



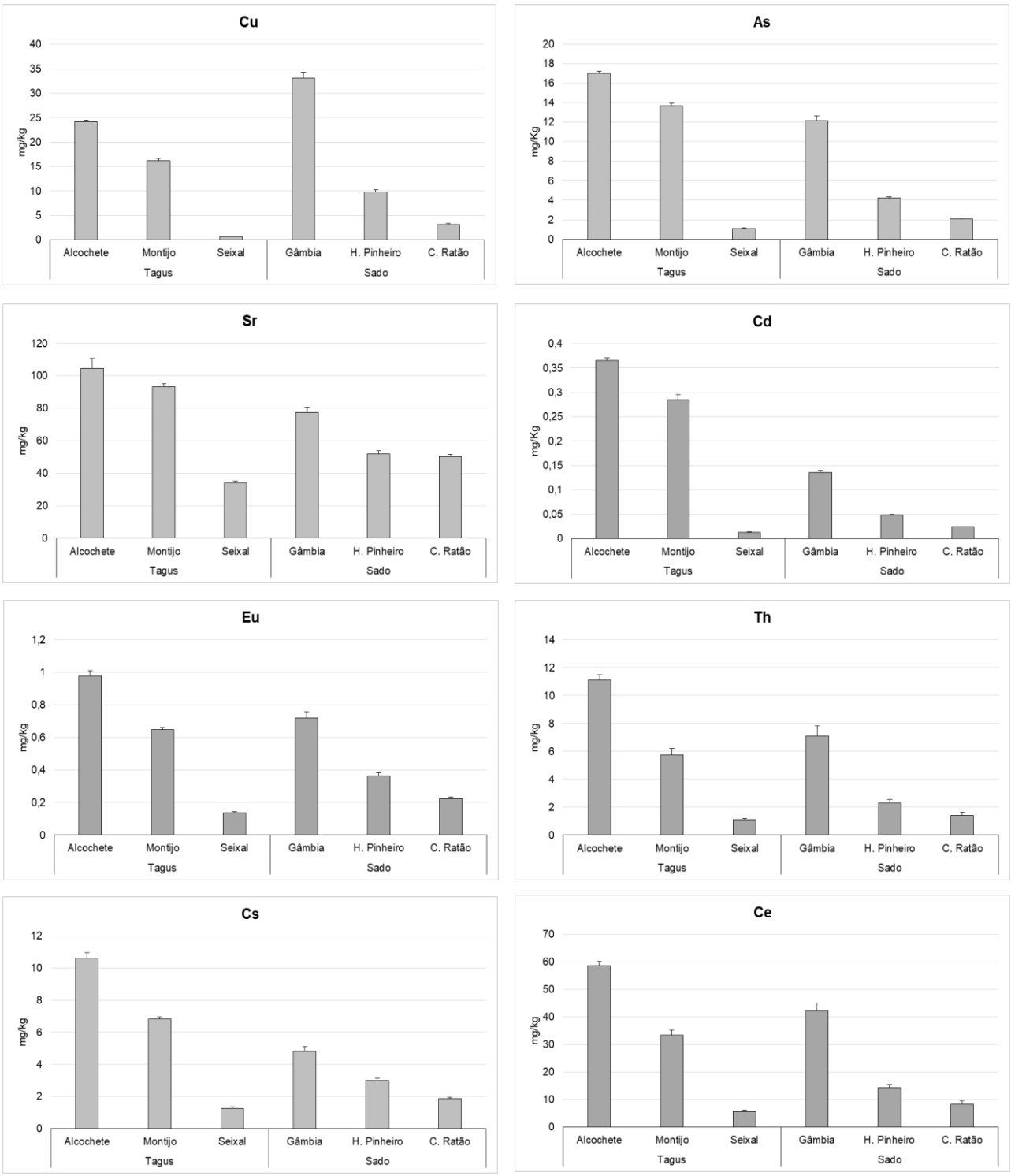


Figure 10: Mean ± SE, (n=3) of elemental concentrations (mg/kg DW) from Sc, Cr, Mn, Fe, Co, Ni, Cu, As, Sr, Cd, Cs, Ce, Eu and Th in sediment samples collected in Tagus estuary at sites Alcochete, Montijo bay and Seixal bay and Sado estuary at sites Gâmbia, Herdade do Pinheiro, and Cabeço do Ratão.

The sediment collected in Alcochete site (Tagus estuary) and Gâmbia (Sado estuary) registered the highest concentrations for all elements analysed, while the lowest values were observed in sediments of Seixal bay (Tagus estuary) and Cabeço do Ratão (Sado estuary). It is evident a similar spatial distribution pattern of the elements within sediments of the sampling sites at both estuaries (Figure 6 and Figure 7).

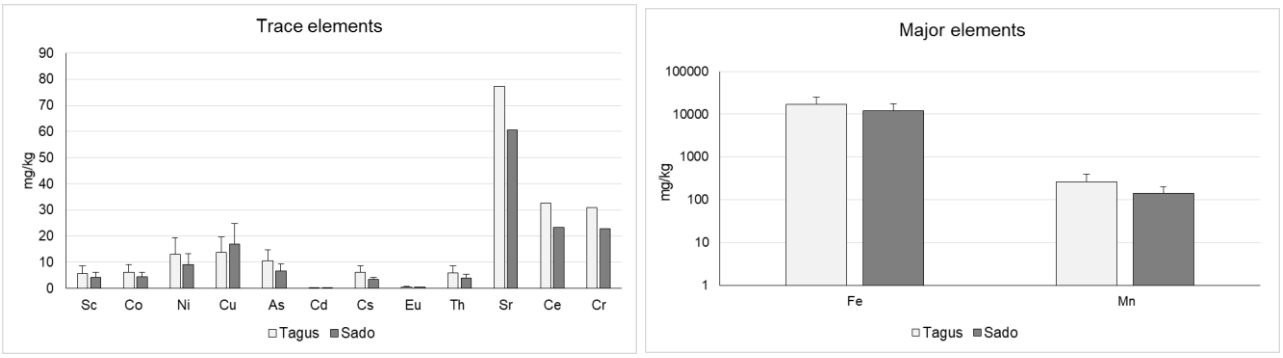


Figure 11: Elemental concentration values mean ± SE, (n=3), of the trace and major elements (mg/kg) determined for sediment samples within and between estuaries. The major elements graphic the y-axis are log-transformed values.

Several elements in Tagus estuary registered the highest concentration values except for Cu (33,1±1,16 mg/kg), which the highest value was observed in Sado estuary sampling site Gâmbia (Figure 7 and 8).

Significant statistical differences on the sediment chemical profile from different sites were assessed using PERMANOVA are summarised in Table 8.

Table 8: Details of two-factor PERMANOVA test with Estuary (Es) (2 levels fixed), Sites (Si) (6 level fixed and nested in Estuary) for all variables analysed. Bold values highlight significant effects (p<0.05). MC is Monte Carlo test.

Source variation	df	SS	MS	Pseudo-F	P(perms)	perms	P(MC)
Es	1	19,234	19,234	102,24	**0,001**	998	**0,001**
Si(Es)	4	205,94	51,484	273,69	**0,001**	998	**0,001**
Res	11	2,0693	0,18811				
Total	16	224					

PERMANOVA showed significant differences for sediment chemical profile between estuaries (Es) and among sites nested in Estuary Si(Es) (p< 0.05).

Pairwise test of PERMANOVA are summarised in Table 9 and clearly showed significant differences between sampling sites in both estuaries, on the contrary of the clam chemical profile.

Table 9: Details of pairwise test of PERMANOVA sites fixed and nested in Estuary "Si(Es)" within level 'Tagus' of factor 'Estuary' and within level 'Sado' of factor 'Estuary' for all variables analysed. Bold values highlight significant effects (p<0.05).

Level Tagus	t	P(perm)	perms	P(MC)
Alcochete x Montijo	10,072	0,114	10	**0,001**
Alcochete x Seixal	28,687	0,096	10	**0,001**
Montijo x Seixal	26,543	0,097	10	**0,001**
Level Sado	t	P(perm)	perms	P(MC)
Gâmbia x H. Pinheiro	11,398	0,096	10	**0,001**
Gâmbia x C. Ratão	11,932	0,104	10	**0,002**
H. Pinheiro x C. Ratão	5,7355	0,089	10	**0,004**

Chapter IV: Discussion and Conclusion

4. Discussion and conclusion

A methodological approach to investigate the geographical origin of the non-indigenous bivalve species *R. philippinarum* is an urgent need due to the human health risk enhancement involved with the increasing harvest in Tagus estuary, and the false labelling of Sado estuary as commercial benefits. Differences in clam's chemical profile to determine a chemical *fingerprint* between populations could be a practical tool to identify the geographical origin of the specimens. The ICP-MS technique is an analytical tool which determines the bulk elemental concentrations in a single analysis, distinguishing different isotopes with high accuracy and precision. Applying this tool was possible to identify and quantify the clam's trace elements contents and characterise the chemical *fingerprint* within and between Tagus and Sado estuaries.

Estuaries are transitional microenvironments which provide many environmental conditions bringing out different metal availability and different clam's bioaccumulation behaviour. All these disturbances can influence elemental traceability achievement as certain elements Zn, Fe, and Mn are highly related with local characteristics and are easily assimilated by the specimens. (Sorte *et al*., 2013, Cathey *et al*., 2014; Ricardo *et al*., 2015; Norrie *et al*., 2016). The lithological influence of the Peninsula Belt explained the significant availability of Cu, Cr, Ni and Co in estuaries located in the south of Portugal (Mil-Homens *et al*., 2014).

The trace elemental contents of the clams collected in Tagus estuary showed significant chemical differences of the fingerprint between the sampling sites selected: At Alcochete and Montijo bay an opposite chemical profile was obtained and at Seixal bay was characterised by the high value of Cu.

The Alcochete area is characterised by a high abundance of Ni and Co, this is a typical pattern in Southern estuaries. Other elements as Mn and Se are strongly influenced by the fine-grained fraction of organic contents from the freshwater inputs, nutrients runoff of Tagus River and the saltmarshes of Tagus Estuary Natural Reserve, which plays an essential role in metal affinity and allocation (Vinagre *et al*., 2008; Duarte & Caçador, 2012; Mil-Homens *et al*., 2014). The highest concentrations of Mn, Ni, Co, and Se registered in clams collected in Alcochete sampling site seems to be related to local influences determining a distinct element distribution pattern.

Manganese is commonly assimilated by bivalves which explained the high concentration of Mn in the clams collected in Alcochete. Moreover, the high concentration of Mn in clams could result from the ingestion of Mn-rich particles due to diatom blooms (Ricardo *et al.*, 2015). This oligo-element has enzymatic structural functions, being an essential element to the organism's metabolic functions.

The presence of pesticides strongly impacts Tagus estuary, surpass the levels limited by EU Directive for transitional surface waters (2013/39/EU) (Cruzeiro *et al*., 2016). Arsenic and Se are considered chemical constituents of pesticides, herbicides, and insecticides. The clams collected in Montijo and Seixal bays registered high concentrations of these elements, which could be explained by their easy mobilisation and the increased presence of pesticides and herbicides in Tagus estuary.

The clams sampled in Montijo bay registered high concentration of several elements as Fe, Zn, As, Ce, Cr, and Cd. This area is high polluted presenting muddy and silk sediments with high organic contents and low hydrodynamic, providing a high resident time condition and the metal speciation. These characteristics support the bias of Montijo clams to assimilate high amounts of different elements (Machado *et al*., 2016; Chiesa *et al*., 2018). Besides, the sampling sites Montijo and Seixal bays are strongly influenced by tidal flows and less by the river discharges which contribute with a periodic metal dilution (Duarte & Caçador, 2012).

Seixal bay is characterised by intertidal areas including an important salt marshes (Corroios) and an intensive urban occupation, small factories (fertilisers and pesticides), industrial fish processing and naval construction (Caçador *et al*., 2009). The clams sampled in Seixal bay registered highest concentrations of Cu, probably due to Cu speciation influenced by the tidal flows, Cu natural affinity to organic compounds and the local high retaining capacity.

Arsenic, Cu, and Cd registered higher concentration in clams than in sediments, and seem to be related with the enhanced prone to be bioaccumulated by bivalves, mainly by Manila clams (Velez *et al*., 2015a; Marques *et al*., 2017; and Chiesa *et al*., 2018). Arsenic and Cd are considered the most toxic for several aquatic organisms, without a known biological role, and high mobility represents an increased risk to the organism's health. The absence or limited capacity to regulate these elements concentration in their tissues is the main difficulty to maintain the homeostasis (Figueira & Freitas, 2013; Velez *et al*., 2015a).

The obtained results in clams collected in Sado estuary also showed that is possible to differentiate chemical distribution pattern between the sampling sites. The Gâmbia and Herdade do Pinheiro clams registered an opposite chemical profile, and the high content of Cu characterised the Cabeço do Ratão clams.

Gâmbia and Herdade do Pinheiro areas are characterised by shallow hydrodynamics and limited depth, with high organic loads due to non-point pollution, runoff from aquacultures (oysters nurseries) and rice fields located upstream of the Águas de Moura channel and Alcaçer channel (Caeiro *et al*., 2005 and Caeiro *et al*., 2009).

Clams collected in Gâmbia site registered the highest concentrations for Zn and Ni. Zinc, Ni, Cu, and Co, are considered essential elements in the bivalve’s metabolic activities, being permanently assimilated. The abundance of these elements in the surrounding

environment is associated with the lithologic influence typical at southern estuaries and the occurrence of heavy minerals, past mining activities and shipyard station Lisnave (Caeiro *et al*., 2005; Caeiro *et al*., 2009; Mil-Homens *et al*., 2014).

The low hydrodynamics conditions, fertilisers, pesticides and nutrient runoff of the intensive rice field's activities, characterises the sampling site of Herdade do Pinheiro, which explain the high concentrations of Fe, As, Mn, Se, Co, Cd, and Eu in clam's tissues (Caeiro *et al*., 2005). These clams registered the highest concentrations of As and Se which are considered as chemical constituents of pesticides due to the intensive agriculture activities.

Gâmbia and Herdade do Pinheiro sites are characterised by the increased organic contents from the inputs fluvial channels, being less influenced by the tidal flows than Cabeço do Ratão. The lowest values of Cu registered in clam's tissues are due to the Cu high organic affinity promoting the metal allocation and the reduced availability.

As previously observed in the Tagus estuary, at Sado estuary As, Cu, and Cd registered higher concentrations in clams than sediments, highlighting the prone of bioaccumulation of these elements by Manila clams (Velez *et al*., 2015a; Marques *et al*., 2017; Chiesa *et al*., 2018).

All the elemental influences in the environment contributed to defining clam's chemical profile within and between estuaries. The ordination demonstrated that collected clams in Tagus estuary were separated from those of Sado estuary by the elements Zn, Mn, Ni, Co, Cu, Se and Ce. Although the significant differences were only obtained between Gâmbia and Herdade do Pinheiro.

Sediment has been considered as a match factor to define clam's geographical origin. Clams are benthic organisms that filter the sediment particles. Therefore, the determination of sediment chemical profile could be useful to relate to clam's specific chemical patterns (Amisah *et al*., 2010; H. Zhao & Zhang, 2016b).

The opposite, was obtained in this study, although of being an important reference to benthic organism habitat the absence of a clear and proportional relationship between the elemental concentrations in sediments and clams tissues was already observed (Moschino *et al*., 2012; Velez et al., 2015a; Chiesa *et al*., 2018).

The elemental contents of sediment collected in Tagus estuary are generally higher when compared with other Portuguese estuarine systems (Chiesa *et al*., 2018). In the current study, it was observed higher elemental contents in sediments collected in Tagus estuary comparing with those obtained in Sado estuary.

Both estuaries presented similar spatial distribution patterns, the upstream sites presented high element concentrations compared to downstream sites. The significant differences between sampling sites are explained by the lithological and hydrodynamic

characteristics from each site influencing environmental conditions and the elements speciation.

The present study shows that is possible to obtain a chemical profile of the geographical origin “fingerprint” between Tagus and Sado estuaries. However, no significant differences were detected between all sites in each estuary. The clam´s geographic origin in Tagus estuary is characterised by the dominance of the elements Zn, Co, Se and Ce and clam´s geographical origin in Sado estuary is characterised by the dominance of Cu and Cr.

Identifying the most differentiating elements between sites and estuaries, was possible to define trace elements that describe clam´s geographical origin. To develop the precision, accuracy and sensibility of the traceability tool is essential to assess the elemental stability through time, beyond intrinsic and extrinsic factors that influence the presence of the elements in clams.

Chapter V: References

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Chapter VI: Supplementary information

6. Supplementary information

Table 16: Standard reference material SRM - 2976 Mussel tissue from NIST, certified elements values and standard deviation values (mg.Kg^{-1}).

SRM 2076 Mussel tissue								
Certified elements	As	Cd	Cu	Fe	Pb	Se	Zn	Hg
Values (mg/kg)	13,3	0,82	4,02	171	1,19	1,8	137	0,06
Stdev	1,8	0,16	0,33	4,9	0,18	0,15	13	0

Table 11: Standard reference material SRM - 2976 Mussel tissue from NIST, referenced elements values and standard deviation values (mg.Kg^{-1}).

SRM 2076 Mussel Tissue							
Ref. elements	Al	Cr	Ni	Ag	Sn	Ca	Cl
Values (mg/kg)	134	0,5	0,93	0,011	0,096	7600	57000
Stdev	34	0,16	0,12	0,005	0,039	30	500
Ref. elements	Mg	K	Na	Br	Ce	Cs	Co
Values (mg/kg)	5300	9700	35000	329	0,109	0,027	0,61
Stdev	50	50	100	15	0,008	0,001	0,02
Ref. elements	Eu	Mn	Rb	Sc	Sr	Th	
Values (mg/kg)	0	33	4,14	0,0146	93	0,011	
Stdev	0	2	0,09	0,0003	2	0,002	

Table 12: Standard reference material BCR-667 Estuary sediment from IRMM, certified elements values and standard deviation values (mg.Kg^{-1}).

BCR-667 Estuary sediment									
Certified elements	Ce	Dy	Er	Eu	Gd	Ho	La	Lu	Nd
Values mg/kg	56,7	4,01	2,35	1	4,41	0,8	27,8	0,325	25
Stdev	2,5	0,14	0,15	0,05	0,12	0,06	1	0,02	1,4
Certified elements	Pr	Sc	Sm	Tb	Tm	Yb	Th	U	
Values mg/kg	6,1	13,7	4,66	0,682	0,326	2,2	10	2,26	
Stdev	0,5	0,7	0,2	0,017	0,025	0,09	0,5	0,15	

Table 13: Standard reference material, BCR-667 Estuary sediment from IRMM, referenced elements values and standard deviation values (mg.Kg^{-1}).

BCR-667 Estuary sediment									
Ref. elements	Br	Cd	Co	Cr	Cs	Cu	Fe	Info. values	As
Values mg/kg	99,7	0,67	23	178	7,8	60	44800	Values mg/kg	14,3
Stdev	2,5	0,11	1,3	16	0,7	9	1000	Stdev	19,9
Ref. elements	Mn	Ni	Pb	Sb	Se	Ta	Zn		
Values mg/kg	920	128	31,9	0,96	1,59	0,876	0,175		
Stdev	40	9	1,1	0,05	0,08	0,017	13		

Table 14: Standard reference material, Andesite, AGV-2 from U.S. Geological Survey Certificate of Analysis, referenced values and standard deviation values (µg.g^{-1}).

AGV-2 Cerâmica									
Certified elements	Al	Ca	Fe	K	Mg	Na	P	Si	Ti
Values µg/g	89500	37200	46800	23900	10800	31100	2100	277000	6300
Stdev	110	90	90	90	20	90	10	350	130

Table 15: Standard reference material, Andesite, AGV-2 from U.S. Geological Survey Certificate of Analysis, certified values and standard deviation values (µg.g^{-1}).

AGV-2 Cerâmica													
Certified elements	Ba	Be	Ce	Co	Cr	Cu	Dy	Ga	La	Mn	Nb	Nd	Ni
Values µg/g	1140	2,3	68	16	17	53	3,6	20	38	770	15	30	19
Stdev	32	0,4	3	1	2	4	0,2	1	1	20	1	2	3
Certified elements	Pb	Pr	Rb	Sc	Sr	Th	U	V	Y	Yb	Zn	Zr	
Values µg/g	13	8,3	68,6	13	658	6,1	1,88	120	20	1,6	86	230	
Stdev	1	0,6	2,3	1	17	0,6	0,16	5	1	0,2	8	4	