Biological Trace Element Research (2022) 200:385–401
https://doi.org/10.1007/s12011-021-02629-x

Spatial and Temporal Distribution of the Multi-element Signatures of the Estuarine Non-indigenous Bivalve *Ruditapes philippinarum*

Soraia Vieira[1] · Pedro Barrulas[2] · Paula Chainho[3,4,5] · Cristina Barrocas Dias[2,6] · Katarzyna Sroczyńska[1] · Helena Adão[1]

Received: 10 December 2020 / Accepted: 7 February 2021 / Published online: 15 February 2021

Abstract

Filter-feeder bivalves such as non-indigenous *Ruditapes philippinarum* absorb and accumulate metals, resulting in multi-element profiles. The goal of this study was to analyse spatial and temporal distributions of the multi-element signatures in *R. philippinarum* populations of the Tagus and Sado estuaries (SW coast, Portugal). The clam and sediment samples were collected at three sampling sites in each estuary, on three sampling occasions, and the analysis were done by ICP-MS (inductively coupled plasma mass spectrometry). The chemical elements were categorized according to estuarine geomorphology sources (Se, Co, Ni and Cu), elements with function in metabolic processes of the clams (Mn, Fe, Zn and Cr) and elements derived from the anthropogenic inputs (As, Pb and Cd). Zinc, Co, Ni and Pb were the main contributors for the chemical signatures of Tagus estuary populations, whilst for the Sado estuary populations were Cu, Fe, Cr, As and Cd. They were representative of all elemental categories and proved to be spatial and temporal habitat discriminators of bivalves' estuarine populations. The multi-element signatures of *R. philippinarum* as a natural tag derived from the physical and chemical conditions of its habitat is a potential rapid tool to use in ecological monitoring and habitat assessment.

Keywords Multi-element signatures · *R. philippinarum* · Spatial and temporal distributions · Traceability · Elemental discriminators

Introduction

The non-indigenous benthic bivalve *Ruditapes philippinarum* (Adams & Reeve, 1850), commonly named as Manila clam, is a well-succeeded invasive species in worldwide estuaries and coastal systems. It represents one of the most commonly consumed bivalve species extensively cultivated all over the world [1]. Originally native to the Indo-Pacific region, it was introduced into the west coast of North America, the East Atlantic (Portugal, France, Spain, Ireland, England) and Mediterranean European coasts (France, Italy) [2]. In Portugal, it was introduced in the 1980 in Ria Formosa (South of Portugal), rapidly invading estuaries and coastal systems over all of the country, including the Sado estuary [3] and the Tagus estuary [2, 3]. With a large geographical spatial distribution and increasing abundance in recent years, *R. philippinarum* gained a significant economic relevance for fisheries and aquaculture, being intensively harvested in Europe [4–7]. In Portugal, it is one of the most important commercial bivalves [1], with an intensive exploitation, particularly in the Tagus estuary, though it is an illegal harvesting, involving a serious risk for human consumption due to microbiological and metal contamination.

The Manila clam has also been recognized as an appropriate bioindicator and/or sentinel species of environmental quality in aquatic systems [8, 9]. Filter-feeding species have a natural bias to absorb and accumulate metals, resulting on

✉ Helena Adão
hadao@uevora.pt

[1] MARE, Marine and Environmental Sciences Centre, University of Évora, School of Sciences and Technology, Apartado 94, 7002-554 Évora, Portugal

[2] Laboratório HERCULES, Universidade de Évora, Palácio do Vimioso, Largo Marquês de Marialva 8, 7000-09 Évora, Portugal

[3] MARE, Marine and Environmental Sciences Centre, Lisbon University, Faculty of Sciences, Campo Grande, 1749-016 Lisbon, Portugal

[4] Department of Animal Biology, Faculty of Sciences, University of Lisbon, Lisbon, Portugal

[5] Polytechnic Institute of Setúbal, Setúbal, Portugal

[6] Chemistry Department, School of Science and Technology, Évora University, Rua Romão Ramalho, 59, 7000-671 Évora, Portugal

multi-element signature profiles, which can be used as a traceability tool to identify the habitat origin and to assess the ecological status in coastal and estuarine systems [10–12]. High ability of Manila clam to accumulate elements in its soft tissues and shells has supported its use as a bioindicator of pollution for specific elements in estuarine environments, e.g. Pb, As, Hg and Cd [13–15]. High filtration rates and the large feeding spectrum of this bivalve [16] allow the accumulation of large number of metals aggregated in a suspended particles, revealing a higher predisposition to uptake metals and metalloids than other bivalve species [4, 8]. Nevertheless, the bioaccumulation behaviour is influenced by the bivalve' intrinsic species features, such as sex, age and physiological conditions (e.g. the reproduction physiology), and by the interactions with the abiotic conditions, particularly within the period of chemical exposure [8, 17–19]. The bivalve multi-element signature profiles are deeply dependent on the estuarine environmental conditions, namely, the salinity gradient, pH, temperature and tidal variations and turbidity [20–24].

The chemical profile that successfully discriminate specimens from different sampling sites is based on the link between species and their environmental conditions: trace elements such as Fe, Cu, Zn, Mn, Mg and Cr are related with bivalves' growing and cell metabolism [18, 25, 26]; the presence of xenobiotic elements as Hg, Pb, Cd and As are related with the toxicity levels and the tolerance in the specimens that are influenced by anthropogenic disturbances [7, 27, 28]; Ni, Cu and Co are mainly related to the estuary lithological nature, becoming important to determine the organisms' geographic origin [29]; and the trace elements such as Sr, Sc, Se, Cs, Ce, Eu and Th are geologically related to sediment morphogenesis [29, 30]. Bivalve chemical profiles are also related to their life cycle, reflecting different clams' physiological conditions for bioaccumulation [31]. In the Tagus estuary, the reproductive cycle showed a synchronous gender gonadal development occurred in April and May, followed by an extensive spawning until December [32].

The ICP-MS (inductively coupled plasma mass spectrometry) is a highly sensitive analytical technique, and its high accuracy, precision and sensitivity allows the multi-elemental measurement at trace levels (ranging from part per trillion (ppt) to part per million (ppm)) as a multi-element screening [33, 34]. This analytical tool allows to distinguish different element species and isotopes in a single analysis, being effective to determine the specimens' multi-element composition profiles with a high cost-effectiveness [25, 35, 36]. This technique performed in bivalve soft tissues revealed to be useful to identify the bioaccumulation throughout the multi-element composition of populations under metal pollution disturbances [7, 37], generally reflecting local environmental conditions [26]. The applicability of this technique in soft tissues could be explored to determine the bivalves' origin by using trace elements as site-specific descriptors. The multi-element tag to discriminate the spatial distribution patterns of the non-indigenous *R. philippinarum* populations could be detected using the ICP-MS technique, a high sample throughput tool for geographic origin determination [38].

The main aim of this study was to analyse the spatial and temporal distribution of the multi-element signatures of the *Ruditapes philippinarum* populations collected in the Tagus and Sado estuaries, located in the SW coast of Portugal, to assess the trace elements as a traceability tool to support management decisions of the human activities in coastal ecosystems. The following research question was addressed: Are there significant differences on the *R. philippinarum* multi-element signatures between populations of the Tagus and Sado estuaries, within sampling sites of each estuary and between sampling occasions? Based on expected differences of the environmental conditions in both estuaries and the differences in bivalves' life cycle, we hypothesized to find significant differences in clam's chemical profile between both estuaries, between sampling sites within each estuary and between sampling occasions. We hypothesize that differences between estuaries and sampling sites should mainly be explained by chemical elements related with geomorphological and anthropogenic inputs, while the temporal differences should arise from the biogenic processes of the bivalves.

Material and Methods

Study Areas

Sampling sites were located in intertidal mudflats of the Tagus and Sado estuaries (southwest coast of Portugal). Both estuaries are geologically related to the Iberian Pyrite Belt and earlier mining activities, which explain the high concentration of the elements Fe, Cu, Ni, Co and rare earth elements (REE). Despite the proximity of the estuaries and the common hydrologic basins, there are differences in the distribution patterns of those elements in sediments among estuaries [29, 39, 40].

The Tagus estuary (38° 44′ N; 09° 08′ W) is one of the largest estuarine systems in Europe, with approximately 320 km^2 and is located in the most populated area of Portugal. Nearly 40% of this area consists of intertidal flats and saltmarshes, which are partly included in the protected area designated as "Tagus Estuary Natural Reserve" [41]. The Tagus estuary is a semi-diurnal mesotidal system, with a tidal amplitude varying between 4 and 1 m during spring and neap tides, respectively [42]. The salinity gradient is strongly influenced by the Tagus river flows (mean value of 400 $m^3\ s^{-1}$) changing with seasonal and inter-annual conditions [43, 44]. Water temperature ranges from 8 to 26°C [41]. The lower Tagus estuary southern branch has small enclosed bays (e.g. Montijo, Barreiro and Seixal bays) with wide intertidal areas, including important saltmarsh ecosystems, characterized by a

high water residence time (ranging from 6 to 65 days) but strongly influenced by tides. This large estuary has a high socio-economic importance, supporting one the major Portuguese international harbours and several chemical, petrochemical, metallurgic, shipbuilding and cement manufacture industries [9]. Industrial and urban pollution sources have been mitigated through the construction of sewage treatment plants [22]. Despite intense anthropogenic disturbances observed in this estuarine ecosystem, it provides suitable environmental conditions to support a high abundance of *R. philippinarum*, which is currently a dominant bivalve species in the Tagus estuary [45]. The economic importance of the Manila clam harvesting at the Tagus estuary combined with high microbiological contamination levels triggered public health hazards related to the bivalve consumption [5].

The Sado estuary (38° 31′ 14″ N, 8° 53′ 32″ W) is the second largest estuarine system in Portugal, with an area of approximately 240 km^2 and, similar to the Tagus estuary, with a high socio-economic importance, supporting important industrial and harbour-associated activities [46], with copper mines; heavy industries such as a paper mill, shipyards, pesticide and fertilizer factories; thermoelectric plant and intense aquaculture production [46, 47]. The upstream areas of the Sado estuary are intensively explored with rice fields, which are responsible for high inputs of fertilizers and pesticides discharged in the river [48]. Most of the estuarine area is classified as a protected area, designated as "National Reserve of the Sado estuary". The Sado estuary has a semi-diurnal mesotidal system with tidal amplitude varying between 1.6 and 0.6 m during spring and neap tides, respectively. Salinity is influenced by the Sado river flow (annual mean of $40m^3.s^{-1}$) changing with seasonal and inter-annual conditions [49] and with temperature ranging from 10 to 26°C. This system is partially separated by intertidal sandbanks (*Troia beach*) and linked to the ocean by a 50-m deep channel [49]. The intertidal area has approximately 78 km^2 [50], and 30% of this area consists of salt marshes and intertidal flats and the water residence time of the estuary being approximately 21 days. Bivalves' harvesting activity is residual when compared to the Tagus estuary. *R. philippinarum* abundance is much lower, most likely because the Sado estuary might be in an early expansion progress for this invasive species [45].

Sampling Design

The selection of the sampling sites to compare spatial and temporal distribution patterns of clam chemical profile signatures was based on the following combined criteria: (*i*) the occurrence of *R. philippinarum* populations in the Tagus and Sado estuaries [2, 5, 51] and (*ii*) the gradient of hydrological and geomorphological characteristics of both estuaries. Accordingly, three sampling sites were selected in the Tagus estuary: *Alcochete (AL)* located at the estuary uppermost section, next to the "Tagus Estuary Natural Reserve", and strongly influenced by the Tagus river flow; the *Montijo bay (MT)*, located in the surroundings of heavy industrialized areas; and the *Seixal bay (SE)*, characterized by the intertidal important salt marshes [52] (Fig. 1a). Three sampling sites were selected in the Sado Estuary: *Gâmbia (GA)*, located at the northern branch of the estuary, with an important oyster nursery production and a large paper mill and shipyards in its vicinity; *Herdade do Pinheiro (HP)*, located in the northern branch of the estuary within the "Sado Estuary Natural Reserve", is highly influenced by the Sado river flow and classified as a Special Protection Area for the conservation of wild birds; and *Cabeço do Ratão (CR)*, located in a small sand bank at the southern branch of the estuary, next to Tróia peninsula (Fig. 1b). The sampling design and GPS locations are summarized in Table 1.

Environmental Data

Temperature (°C) and salinity of the overlying water above the sediment were measured in situ, at each sampling site and sampling occasion to assess the environmental conditions of the collection sites (Supplementary material - Table S1). Data of the sediment grain size and organic matter content (OM) in the sediments is also included in the Supplementary information (Table S2).

Biological Data

R. philippinarum specimens were randomly collected at each sampling site during spring low tide at three sampling occasions: (1) *May 2018* and *May 2019*, corresponding to the reproduction period for this species, and (2) *January 2019*, which is representative of the resting phase for this species in Portuguese estuaries [32]. Thirty adult specimens were collected at each sampling site and sampling occasion. Collected specimens length varied between 3.5 and 4.5 cm, representing adults with sexual maturity [32]. Sediments associated with the clam specimens were sampled (approx. 5 cm deep, which represents the bivalves living area) at each site and sampling occasion, to relate the multi-element signature of the clams' soft tissue with that of the sediments. The samples were transported on ice to the laboratory and stored at −20°C.

Sample Preparation for ICP-MS Analysis

Due to the different matrix and complexity of the studied samples, two independent procedures for sample preparation and digestion were established in this work, in order to determine the concentration of 18 trace and major elements (Sc, Sr, Se, Cs, Ce, Ni, Sr, Co, Cu, Cr, Mn, Fe, Zn, As, Cd, Pb, Eu and Th).

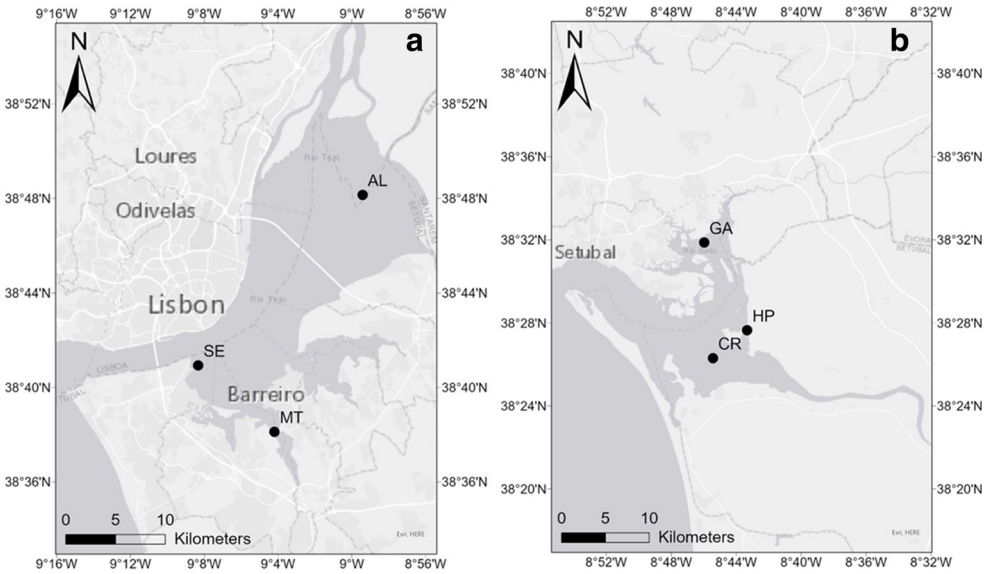


Fig. 1 Location of the sampling sites of *R. philippinarum* and associated sediments. **a** Tagus estuary: Alcochete (AL), Montijo (MT) and Seixal (SE). **b** Sado estuary: Gâmbia (GA); Herdade do Pinheiro (HP) and Cabeço do Ratão (CR)

Bivalves Sample Preparation and Digestion

A set of 10–15 clams were used to minimize the chemical variability of the clams and to ensure the reproducibility of the data of each clams site sampling. Specimens were rinsed with Milli-Q water (18.2 MΩ.cm) in order to avoid any contamination due the presence of other species like algae or other epibionts and sediments. After the pre-treatment, the bivalves' soft tissues were removed from the shell with a plastic knife, rinsed between 2 and 3 times with Milli-Q water (18.2 MΩ.cm) and stored overnight at −20°C. All samples were freeze-dried at −80°C for approximately 72h, after which they were milled to homogenize the sample and to enhance the digestion performance.

The acidic digestion procedure of the clams was based on the method previously described in the literature [53], with some modifications. Briefly, approximately 100 mg of powdered clams, previously freeze-dried, were weighted in PFA

Table 1 Sampling design: estuaries, sites and sampling occasions. GPS locations and sample code are also indicated

Estuary	Sampling Site	GPS Location	Sampling occasions	Sample code	
				Bivalves	Sediment
Tagus	Alcochete (AL)	38° 48′ 9.000″ N	May 2018	TM18AL	TM18ALS
		8° 59′ 25.200″ W	January 2019	TJ19AL	TJ19ALS
			May 2019	TM19AL	TM19ALS
	Montijo (MT)	38° 38′ 7.080″ N	May 2018	TM18MT	TM18MTS
		9° 4′ 11.340″W	January 2019	TJ19MT	TJ19MTS
			May 2019	TM19MT	TM19MTS
	Seixal (SE)	38° 40′ 56.100″ N	May 2018	TM18SE	TM18SES
		9° 8′ 19.740″ W	January 2019	TJ19SE	TJ19SES
			May 2019	TM19SE	TM19SES
Sado	Gâmbia (GA)	38° 31′ 52.080″ N	May 2018	SM18GA	SM18GAS
		8° 45′ 58.500″ W	January 2019	SJ19GA	SJ19GAS
			May 2019	SM19GA	SM19GAS
	Herdade do Pinheiro (HP)	38° 27′ 39.060″ N	May 2018	SM18HP	SM18HPS
		8° 43′ 19.578″ W	January 2019	SJ19HP	SJ19HPS
			May 2019	SM19CR	SM19CRS
	Cabeço do Ratão (CR)	38° 26′ 18.553″ N	May 2018	SM18CR	SM18CRS
		8° 45′ 26.014″ W	January 2019	SJ19CR	SJ19CRS
			May 2019	SM19HP	SM19HPS

Savillex® beakers and exposed to a pre-digestion step with 3 mL of HNO_3 (65%, Suprapur, Merck®) at room temperature for 24h (note: this first step proved to be essential to allow for a full and safe digestion of the organic material). After the pre-digestion period, samples were digested in closed beakers on a hotplate at 125 °C for 3 h (during this time the digestion could be monitored through the formation of an orange atmosphere). Subsequently, all samples evaporated until dryness over a hotplate at 150°C, followed by addition of 2 mL of concentrated HNO_3. The second digestion step was then started by heating up the samples at 125°C over 24h. The second digestion step was completed through addition, at room temperature, of 2 mL of H_2O_2 (30 %, OPTIMA grade, Fisher Chemicals). When the digestion of the organic matter was complete, the samples were allowed to dry on a hotplate and were re-suspended in 3 mL of Milli-Q H_2O and 1.6 mL of concentrated HNO_3, followed by dilution in PFA volumetric flasks with Milli-Q H_2O up to 50 mL, yielding a final matrix of 2% HNO_3 for analysis. Data reproducibility was ensured by means of digestion triplicates for all samples. A Certified Reference Material (CRM) of mussel tissue (SRM – 2976) was used and fully digested according to the described method, together with a blank control, for validation of the method.

Sediment Sample Preparation and Digestion

Prior to any sample treatment, sediments were placed in an oven and dried at 50°C, over 24h. Sediments were then sieved using two different mesh sizes (500μm and 1mm) in order to remove possible solid contaminants like shells and other associated debris. Finally, the sediments were milled using the Planetary Ball Mill PM 100 (RETSCH®) to yield a fine and homogeneous powder.

Sediments were digested under acidic hotplate attack, following the methods previously described in the literature (Makombe et al. 2017), with some adaptations. In PFA Savillex® beakers, sediments samples were weighted (≅ 100 mg), and a mixture of 0.5 mL HNO_3 (65% v/v) (Suprapur, Merck®) and 2 mL of HF (50% v/v) (OPTIMA Grade®) was added. Afterwards, the closed beakers were placed over a hotplate at 125°C for 48h for a first cycle of digestion, after which the caps were removed and the solutions were allowed to concentrate almost until complete dryness (note: complete dryness will provoke the stabilization and precipitation on insoluble fluorides). A second acid attack was performed by adding 2 mL of aqua regia solution (freshly prepared) and left overnight at 150°C with closed vessels. After a second drying cycle was performed and 2 mL of concentrated nitric acid was added, the mixture was allowed to digest at 130°C, over more than 24h. The last digestion step was finished by adding, at room temperature, 1 mL of H_2O_2 (OPTIMA grade®), followed by a last drying cycle. The residue was then re-suspended in 3 mL of Milli-Q H_2O and 1.6 mL of concentrated HNO_3, followed by dilution in PFA volumetric flasks with Milli-Q H_2O up to 50 mL, yielding a final matrix of 2% HNO_3 ready for analysis. As mentioned before for the clam samples, data reproducibility was ensured by means of digestion triplicates for all the samples. A CRM of estuarine sediment BCR-667 was used and fully digested according to the described method, together with a blank control, for validation of the method. According to the concentration range of the analytes, the samples were diluted 10 or 100 fold when necessary.

ICP-MS Analysis: Operating Conditions and Analytical Parameters

Multi-elemental quantification was performed on an Agilent 8800 ICP Triple Quad (ICP-QQQ). Prior to the analysis, the equipment was calibrated with a tune solution from Agilent Technologies, and the sensitivity and resolution were optimized as well as the doubly charged ions (< 2.07%) and oxides species (< 1.04%) were minimized.

The external calibration method was used for the quantification, and a calibration curve with 8 levels (0, 50, 100, 200, 400, 800, 100 and 3000 ppb) was prepared using a multi-elemental solution from High Purity Standards (solution A, ICP-MS-68B). Along ICP-MS analysis, a solution containing three internal standards (^{101}Ru, ^{103}Rh, ^{193}Ir) was added online to correct the data for possible drifts and matrix effects. Method precision was evaluated running each solution in triplicate reporting for each sample the relative standard deviation (RSD). The method was validated through the accuracy determination by means of analysis for the chosen CRM, every 10 samples measurements, which is presented and summarized in Table S3 and S4.

Experimental detection limits (LOD) were performed by measuring 11 replicates of a blank solution and 11 replicates of 400 ppb standard solution. Quantification limits (LOQ) were calculated from 10 times the detection limit obtained before (Table S3 and S4). The operational conditions are described in Table 2.

Data Analyses

Multivariate ordination analyses were applied to identify the temporal and spatial changes of trace-elements concentrations (mg kg^{-1}) in clams soft tissues and sediment samples, between "estuaries" and between "sites" under different physiological conditions, reproduction and resting stage, represented by "sampling occasions". A principal component analysis (PCA) was performed using all multi-elements variables data. The elements were "grouped" based on (i) geomorphologic characteristics of each estuary (Se, Co, Ni and Cu), (ii) importance of clams metabolic processes (Mn, Fe, Zn and Cr) and (iii) elements derived from the anthropogenic inputs (As, Pb, and Cd). All elements (Sc, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se,

Table 2 ICP-MS operating conditions and instrument parameters

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^{-1}
	Nebulizer pump	0.10 rps
Collision reaction cell	He	4.0 mL min^{-1}
(CRC) gases	O_2	0.50 mL min^{-1}
	NH_3	2.5 mL min^{-1}
Acq parameters		Acq mode spectrum
Spectrum mode option	Q2 peak pattern	1 point
	Replicates	3
	Sweeps/replicate	10
Isotope /CRC gas mode	NH_3	^{45}Sc; ^{56}Fe
	He	^{52}Cr; ^{55}Mn; ^{59}Co; ^{60}Ni; ^{63}Cu; ^{66}Zn; ^{88}Sr
	O_2	^{75}As; ^{78}Se
	No gas	^{111}Cd; ^{133}Cs; ^{140}Ce; ^{153}Eu; ^{208}Pb; ^{232}Th

Sr, Cd, Cs, Ce, Pb, Eu and Th) obtained from clams and sediment samples at different "estuaries", "sites" and "sampling occasions" were expressed as mg kg^{-1}. The PCA was applied separately for clam and sediment scaled datasets using the function "fviz pca_biplot" in the library "FactoMineR" using "R package" [54, 55]. To determine the contribution of each variable the square cosine (COS^2) was used. The 3-factor permutational analysis of variance (PERMANOVA) add-on package [56] was applied to determine the significant differences between estuaries, sites and sampling occasions, using PRIMER v6 software package [57]. The design test included the following factors: "estuary" (2 levels, fixed), "sites" (6 level random and nested in estuary) and "sampling occasion", (3 levels, fixed) for all variables analysed at $p < 0.05$ level. The analysis was performed for the 3 groups representing geomorphologic, metamorphic and anthropogenic elements concentrations resemblance matrix calculated based on Euclidean distances. Data was checked for a normal distribution and, when necessary, was log (X+1) transformed before analysis then normalized by subtracting the mean and dividing by the standard deviation for each variable. Redundancy analysis (RDA) was performed to answer how the geomorphological, metabolic and anthropogenic clam's profile can be explained by the concomitant geomorphological, metabolic and anthropogenic sediment characteristics. Response data consisted of three (geomorphological, metabolic and anthropogenic) normalized clam's element matrices, and explanatory matrix consisted of the same set of geomorphological, metabolic and anthropogenic sediment's element profiles. Variables of the explanatory matrix were log10 transformed. A forward selection procedure, using function ordiR2step() was used to select only significant variables ($p<0.05$). Variation inflation factors (VIF) was calculated to check for linear dependencies and to ensure that only variables with small VIFs (<10) were included. RDA analysis was performed in R [55] using "vegan" and "BiodiversityR" packages [58, 59].

Results

Spatial and Temporal Variability of Multi-elements in *R. philippinarum*

The mean ± SE of multi-element concentrations measured in *R. philippinarum* soft tissues are expressed in mg kg^{-1}, DW ("dry weight") and are summarized in Fig. 2 and Table S5 and S6. The concentrations of multi-elements analysed in clam's soft tissues (Se, Co, Ni, Cu; Mn, Fe, Zn, Cr; As, Pb and Cd) were present in the clam's soft tissues above the analytical method LOQ, allowing for the determination of the chemical signature distribution patterns in the Tagus and Sado estuaries.

The elements, aggregated by groups Cu, Co and Ni (geomorphologic); Mn (metabolic); and As and Pb (anthropogenic), registered high variability between estuaries and sampling occasions (Fig. 2). In Tagus estuary, the highest concentration were observed in *Alcochete* and *Montijo* (*TM18AL* and *TM18MT*) in *May 2018*, whereas the lowest concentration were also registered *in May 2019* in *Seixal* (*TM19SE*) and in *January* 2019 at *Alcochete* (*TJ19AL*) (see Table S5 and S6). In Sado estuary, the highest concentrations were observed in *January 2019* and *May 2019* at the *H. do Pinheiro* sampling site (*SJ19HP* and *SM19HP*), whilst the lowest concentrations were registered in *Gâmbia* (*SJ19GA*) in *January* 2019; the

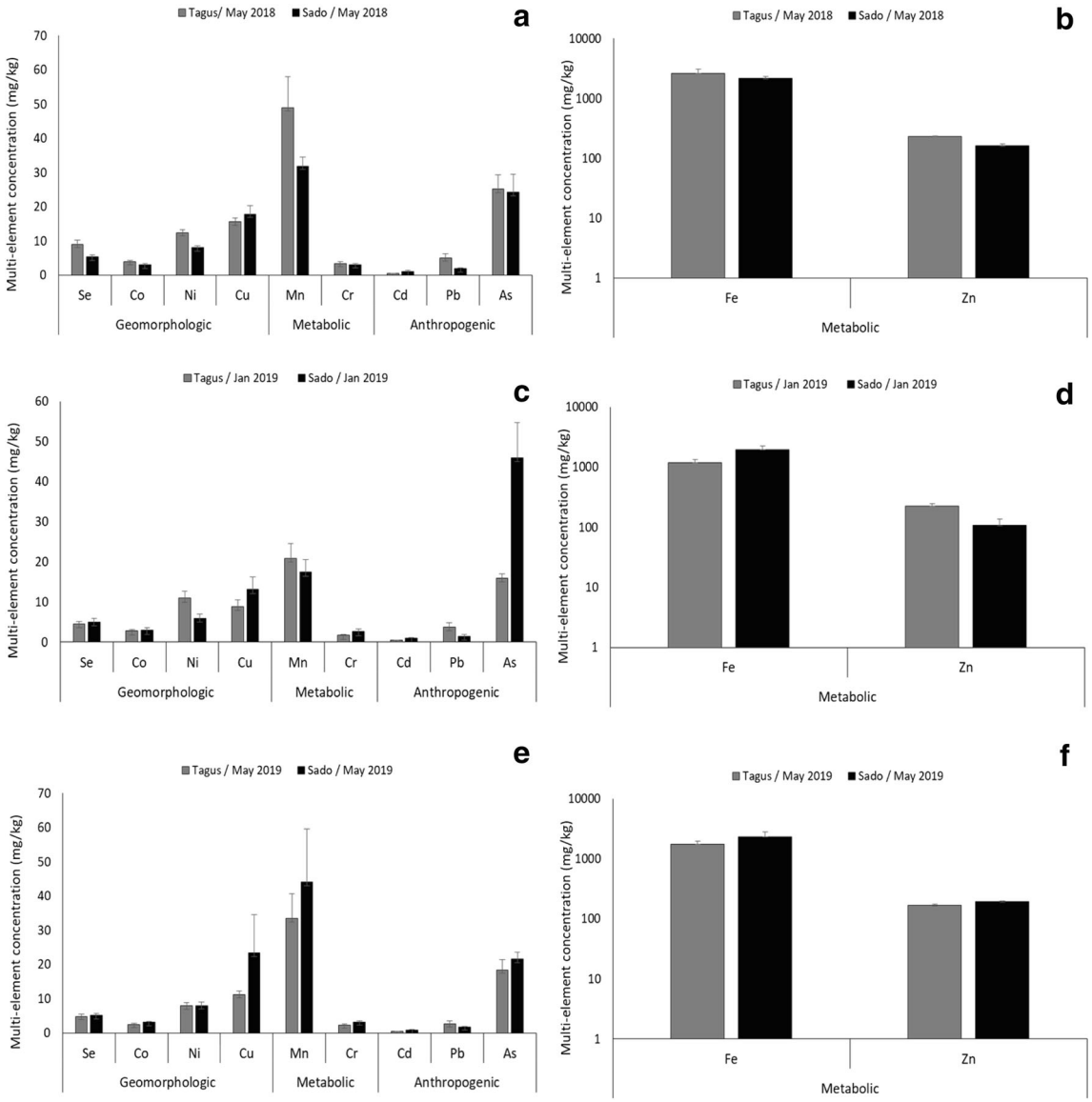
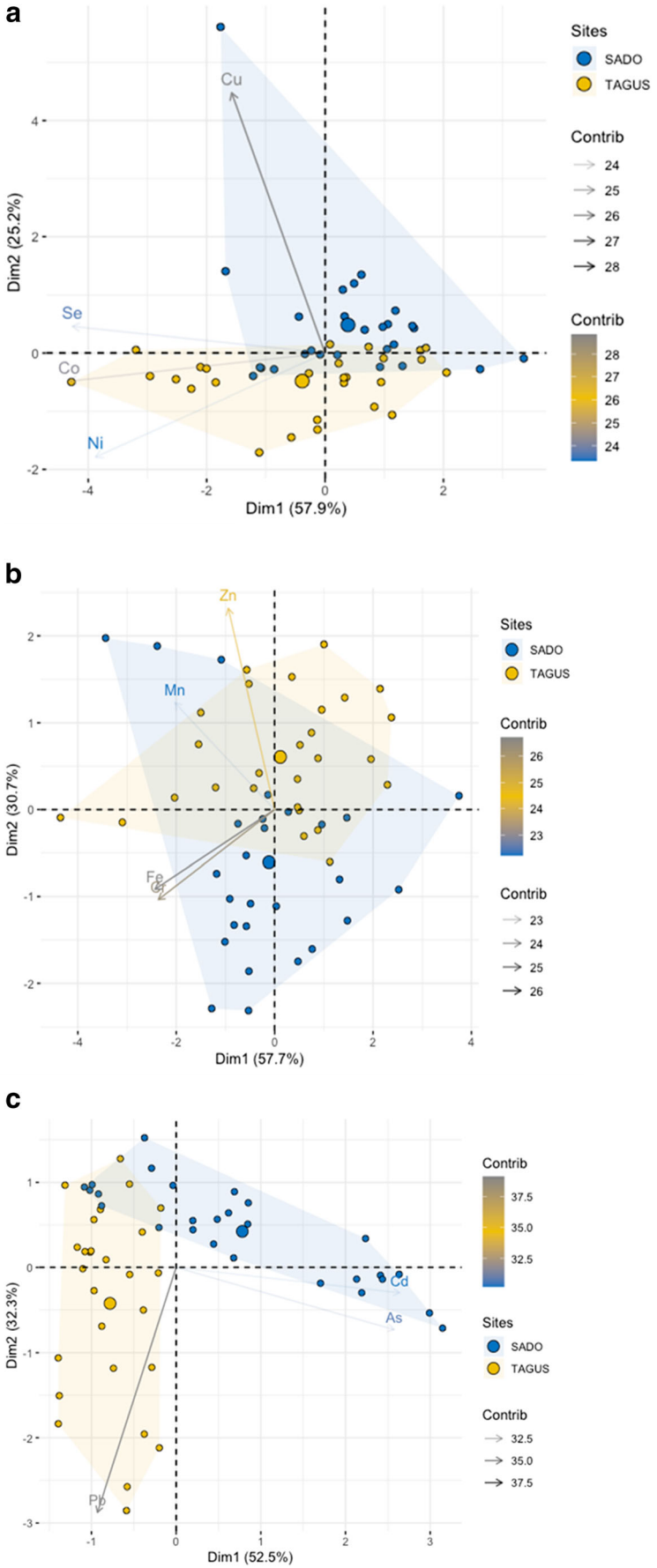


Fig. 2 Mean values ± SE, *n*=3 of trace and major elements (mg kg^{-1}) registered in clam samples of the Tagus and Sado estuaries, at each sampling occasion. (**a**)Trace elements: geomorphologic, metabolic and anthropogenic tracers and (**b**) major elements: metabolic tracers in the Tagus and Sado estuaries in May 2018; **c** Trace elements and (**d**) major elements in Tagus and Sado estuaries in January 2019; (**e**) Trace elements and (**f**) Major elements in the Tagus and Sado estuaries in May 2019. All concentration values of major elements were log-transformed.

exception was observed for the element As, which showed the lowest values in *May 2018* and *January 2019* at *C. do Ratão* (*SM18CR*) (*SJ19CR*) (see Table S5 and S6). Throughout the study period, the Tagus estuary registered the highest concentrations of the elements and the highest variability among sites. However, it was in the Sado estuary that the highest concentrations of As and Cu were detected, respectively, in *January 2019* and *May 2018* (Fig. 2).

The PCA ordination analysis based on geomorphologic and anthropogenic elements explained 83.1% and 88.4% of the variability in the first two principal coordinates, respectively, clearly discriminating estuary origin of the clams' population (Fig. 3 a, b, c). On the contrary the origin discrimination based on metabolic elements was less evident (Fig. 3b). The elements Fe, Co, Cr and Pb contribute to explain the variability in Tagus estuary, and the Cu, Cd, Fe and As presented the highest contribution in Sado estuary.

The PCA ordination applied to metabolic and geomorphologic elements clearly discriminate the clams between sampling occasions (*May 2018*, *January 2019* and *May 2019*), according to the life cycle :"May" is the reproductive period and "January" is the rested period. The elements Fe, Cr, Cu and Co present the highest contribution to explain the temporal variability (Fig. 4a, 4b, 4c).


a
Cu
Se
Co
Ni
Dim2 (25.2%)
Dim1 (57.9%)
Sites
SADO
TAGUS
Contrib
24
25
26
27
28
b
Zn
Mn
Fe
Cr
Dim2 (30.7%)
Dim1 (57.7%)
Sites
SADO
TAGUS
Contrib
23
24
25
26
c
Cd
As
Pb
Dim2 (32.3%)
Dim1 (52.5%)
Sites
SADO
TAGUS
Contrib
32.5
35.0
37.5

◀ **Fig. 3** **a** Principal component analysis (PCA) biplot based on scaled geomorphologic element concentrations measured in clams, coloured by estuary "confidence" convex type. Variable's vectors are presented based on their contributions to the principal components (gradient colours and transparency of vectors) with grey representing high contributions, yellow intermediate and blue representing very low contributions. The dots represent the cluster centroids for group variables. **b** Principal component analysis (PCA) biplot based on scaled metabolic element concentrations measured in clams, coloured by estuary "confidence" convex type. Variable's vectors are presented based on their contributions to the principal components (gradient colours and transparency of vectors) with grey representing high contributions, yellow intermediate and blue representing very low contributions. The dots represent the cluster centroids for group variables. **c** Principal component analysis (PCA) biplot based on scaled anthropogenic element concentrations measured in clams, coloured by estuary "confidence" convex type. Variable's vectors are presented based on their contributions to the principal components (gradient colours and transparency of vectors) with grey representing high contributions, yellow intermediate and blue representing very low contributions. The dots represent the cluster centroids for group variables

The PERMANOVA analysis, based on metabolic elements, showed significant differences between "estuaries", "sites" and "sampling occasions" ($p \leq 0.05$), and significant interaction ($p \leq 0.05$) was detected between the factors "sites" × "sampling occasions". Based on geomorphologic elements, significant differences were obtained between "sites" and "sampling occasions" ($p \leq 0.05$), and the anthropogenic elements showed significant differences only between "sites" and significant interaction between the 3 factors "estuary" × "sites" × "sampling occasions" ($p \leq 0.05$) (Table 3). Individual pairwise comparisons on the interaction factor "sites (estuary)" showed higher variability of the geomorphological and metabolic elements between sampling sites along the sampling occasions in Tagus estuary ($p<0.001$). The significant differences between sampling occasions were obtained between clams collected in January 2019 from those collected at May 2018 and 2019, ($p<0.001$). Individual pairwise comparisons on the interaction factor "sites (estuary)" based on anthropogenic elements showed significant differences between clams from all sites in each estuary ($p< 0.05$).

Spatial and Temporal Variability of Multi-elements in Estuarine Sediments

The mean ± SE of multi-element concentrations measured in the estuarine sediments collected simultaneously with clams, at the sampling sites and at different sampling occasions, are expressed in mg kg^{-1}, DW "dry weight" and are summarized in Fig. 5 and Tables S7 and S6.

The concentrations of 14 elements analysed in the sediment sampled (Cr, Co, Ni, Cu, Cd, Cs, Ce, Pb, Eu, Th, Sc, Fe, Mn and Zn) were above the analytical method LOQ, allowing the determination of the chemical signature distribution patterns in the Tagus and Sado estuaries. The elements were grouped according to the geomorphologic origin (e.g. Sc, Sr, Se, Cs, Ce, Ni, Sr, Co, Cu, Eu and Th) and metabolic function in bivalves' life cycle (e.g. Zn, Mn and Fe). In both estuaries, sediment multi-element concentrations registered low variability between sampling occasions. Several geomorphologic tracers such as Se, Cu and Ni revealed high variability between estuaries and sampling occasions (Fig. 5). Nevertheless, higher element concentrations in the sediments were detected in Tagus estuary than in Sado estuary.

The first two axes of the PCA ordinations accounted for 90.7 % of data variability, highlighting the discrimination between the sediment element concentrations of the upstream and downstream sampling sites (Fig. 6 a and b).

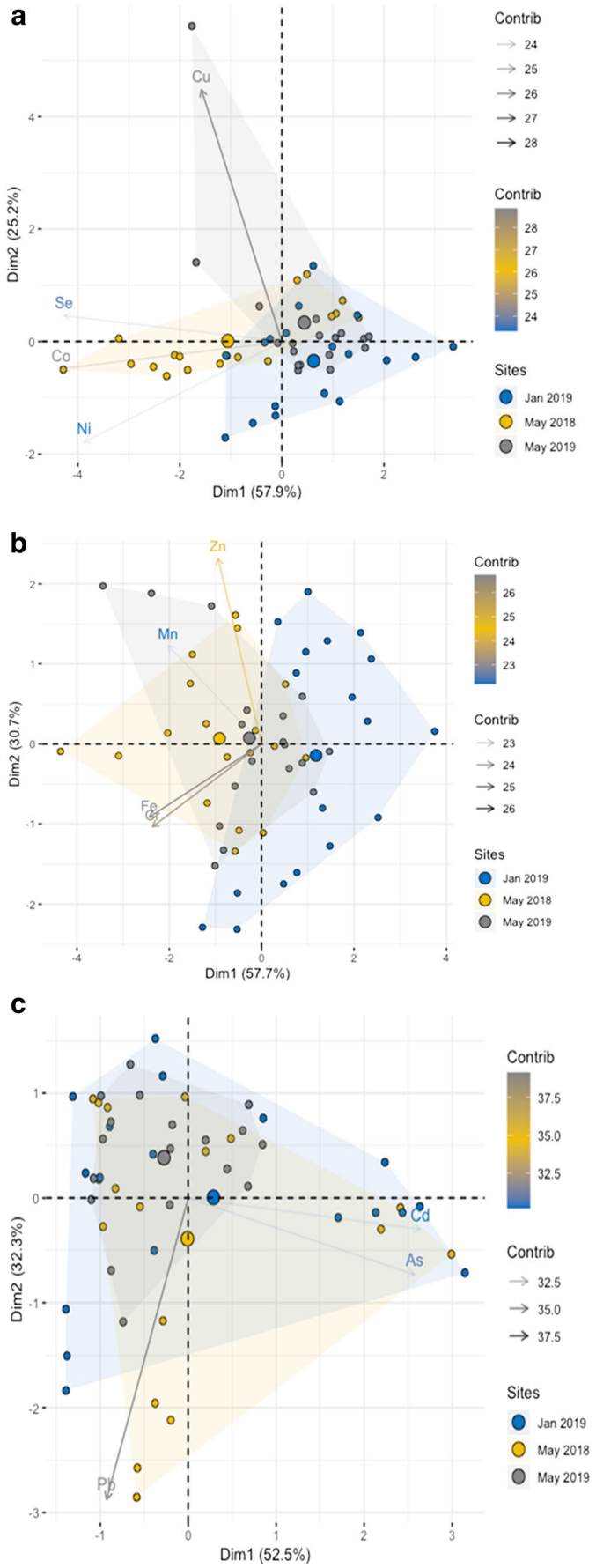
The PERMANOVA analysis detected significant differences of the geomorphologic elements between "sites" ($p \leq 0.05$) and significant interaction ($p \leq 0.05$) between the factors "sites" × "sampling occasions". In accordance with PCA results (Fig. 6a, b), individual pairwise comparisons on the interaction factor "sites (estuary)" × "sampling occasion" showed significant differences in geomorphologic elements, between sampling sites and throughout sampling occasions ($p<0.001$). No significant differences were observed across sampling occasions at each site (*May 2018, January 2019 and May 2019*).

RDA Analysis

RDA analysis was significant only for geomorphological-based dataset and indicated that the spatial distribution of bivalve's chemical signature was mainly driven by the concentration of Ni in Tagus estuary and Cu in Sado estuary. Sediment's elements, Cs, Sc and Ce, explained a significant variation in clam's chemical profile (p=0.001; Adj R^2 = 0.38 and F= 12.01) responsible for the spatial differentiation between Tagus and Sado estuaries (Fig. 7).

Discussion

Most estuaries worldwide are anthropogenically disturbed as they are considered filters of neighbouring contaminants before entering the ocean. Most of these contaminants are toxic, persistent and bio accumulative [60]. The bivalves have a natural tendency to accumulate these chemical elements, creating multi-element signatures, which reflect their habitat conditions [61]. The main aim of this study was to compare elemental distribution patterns of clams' populations within and among estuaries and between sampling occasions and to identify the most important chemical elements that


a
Cu
Se
Co
Ni
Dim2 (25.2%)
Dim1 (57.9%)
Contrib
24
25
26
27
28
Contrib
28
27
26
25
24
Sites
Jan 2019
May 2018
May 2019
b
Zn
Mn
Fe
Cr
Dim2 (30.7%)
Dim1 (57.7%)
Contrib
26
25
24
23
Contrib
23
24
25
26
Sites
Jan 2019
May 2018
May 2019
c
Cd
As
Pb
Dim2 (32.3%)
Dim1 (52.5%)
Contrib
37.5
35.0
32.5
Contrib
32.5
35.0
37.5
Sites
Jan 2019
May 2018
May 2019

◀ **Fig. 4** **a** Principal component analysis (PCA) biplot based on scaled geomorphologic element concentrations measured in clams, coloured by sampling occasions "confidence" convex type. Variable's vectors are presented based on their contributions to the principal components (gradient colours and transparency of vectors) with grey representing high contributions, yellow intermediate and blue representing very low contributions. The dots represent the cluster centroids for group variables. **b** Principal component analysis (PCA) biplot based on scaled metabolism element concentrations measured in clams, coloured by sampling occasions "confidence" convex type. Variable's vectors are presented based on their contributions to the principal components (gradient colours and transparency of vectors) with grey representing high contributions, yellow intermediate and blue representing very low contributions. The dots represent the cluster centroids for group variables. **c** Principal component analysis (PCA) biplot based on scaled anthropogenic element concentrations measured in clams, coloured by sampling occasions "confidence" convex type. Variable's vectors are presented based on their contributions to the principal components (gradient colours and transparency of vectors) with grey representing high contributions, yellow intermediate and blue representing very low contributions. The dots represent the cluster centroids for group variables.

contribute to the observed differences. According to previous studies, we expected to observe differences in clams' chemical profiles even in spatially close populations [25, 36].

This study was done to investigate if the multi-element analysis in clam soft tissues can be used in the future as a tool to distinguish clams from two neighbouring estuaries, sampling sites and from different reproductive stages. Since the elements assimilation in clam soft tissues is strongly influenced by several physical-chemical factors [11, 13], the studied elements were grouped by their main functional attributes, revealing distinct perspectives of the geomorphologic, metabolic and anthropogenic influences in the biological systems.

Spatial Elemental Distribution Between Estuaries and Sites

Metabolic element concentrations in clams' soft tissues showed significant differences between estuaries, sites and sampling occasions, and these differences were likely explained by the dynamics of the life cycle related metabolic processes. The clams' assimilation mechanism determines the fate and the potential effects of elements in their tissues; e.g. Mn, Fe, Cu and Zn are essential in the bivalves metabolic functions and energy management [18, 25, 26].

The concentration of anthropogenic elements in clams' chemical signatures did not display significant differences between estuaries but demonstrated significant differences between sites, which could be related to the influence of local unspecific pollution sources in each estuary. The presence of xenobiotic elements (e.g. Pb, Cd and As) in clam's soft tissue are the result of the efficiency of the tolerance mechanisms [18, 19, 62].

Geomorphologic elements did not display significant differences neither between estuaries nor between sites, likely due to similar lithologic origin of both estuaries [29, 50] that represent the same hydrological basin named "Tagus-Sado basin" (SNIRH) [63].

In both estuaries, the highest elemental concentration values were obtained in the clams collected in salt marshes and intertidal flats areas (Tagus estuary: *Alcochete (AL)*, *Montijo bay (MT)* and Sado estuary: *H. do Pinheiro (HP)*), characterized by the high water residence time, organic matter content and prevalence of fine sediment, that all together favour the sulphide minerals aggregation to sediment suspended particles [64].

The elements As, Cd, Cu, Cr and Fe explained between sites spatial variability of the clam multi-element profiles in Sado estuary where the highest concentration of As and Cd was registered in the clams collected at sites in the proximity of pollution sources associated to the neighbouring activities, e.g. pesticides from intensive agriculture in rice fields, shipping and aquaculture activities [45, 48, 65]. Sado estuary is also influenced by the Iberian Pyrite Belt, and earlier mining activities can account for the high abundance of Cu, Cr and Fe [29, 39, 40].

The concentrations of Co, Zn, Ni and Pb in clams' soft tissues explained the sites spatial variability of Tagus Estuary. Zinc is the most abundant element, and it was previously observed in high concentrations of salt marshes in the Tagus estuary [66], most likely due to intensification of agriculture and industrial activities [22].

Temporal Elemental Distribution

Multi-element composition of the clam soft tissues sampled at different sampling sites in both estuaries in *May 2018, May 2019* and *January 2019* showed an evident temporal pattern. The highest concentrations of elements related with metabolic processes (Mn, Zn, Fe and Cr) and geomorphologic characteristics (Cu, Se, Co) were measured during the reproduction periods (*May 2018* and *May 2019),* which coincide with higher clam's assimilation efficiency and physiologic requirements. The temperature is the main factor for gametogenesis maturity [32], which could explain differences in chemical signatures between sampling occasions (Table S1), reflecting different clams' physiological conditions for bioaccumulation. For instance, Fattorini et al. (2008) revealed the importance of inter-annual shifts in the gametogenesis as a response of trace metal variations in mussels from Adriatic Sea. These inter-annual variations are due to changes in water temperature, salinity and precipitation conditions that highly interact with organisms physiological conditions [13, 62,

Table 3 Details of three factor PERMANOVA test with "estuary" ("Tagus" and "Sado" two level, fixed), "site" ("Alcochete", "Montijo", "Seixal", "Gâmbia", "H. Pinheiro" and "C. Ratão" six level, random) and "sampling occasion" ("May 2018", "January 2019" and "May 2019" three level, fixed)

PERMANOVA	Source of variation	Degrees of freedom	Sum of squares	Pseudo-F	Perms	P(perm)	P(MC)
Clams multi-element concentrations (mg kg^{-1}) Geomorphologic influence	Estuary	1	26.021	2.2713	10	0.199	0.1484
	Sampling occasion	2	41.316	5.599	9947	0.0036	*0.0038*
	Sites (estuary)	4	45.316	11.252	9938	0.0001	*0.0001*
	Estuary × sampling occasions	2	32.666	4.4268	9951	0.0044	*0.0062*
	Sites (estuary) × sampling occasion	8	29.517	3.6237	9918	0.0001	*0.0002*
	Residual	36	36.655				
	Total	53	212				
Clams multi-element concentrations (mg kg^{-1}) Metabolic influence	Estuary	1	22.723	4.2201	10	0.1009	*0.0389*
	Sampling occasion	2	54.459	5.5303	9958	0.0105	*0.0117*
	Sites (estuary)	4	21.538	4.2462	9951	0.0003	*0.0007*
	Estuary × sampling occasions	2	28.239	2.8676	9948	0.0368	0.0534
	Sites (estuary) × sampling occasion	8	39.39	3.8828	9923	0.0001	*0.0003*
	Residual	36	45.651				
	Total	53	212				
Clams multi-element concentrations (mg kg^{-1}) Anthropogenic influence	Estuary	1	43.678	3.893	10	0.0957	0.061
	Sampling occasion	2	9.3491	1.4963	9955	0.2248	0.2268
	Sites (estuary)	4	44.879	23.3	9954	0.0001	*0.0001*
	Estuary × sampling occasions	2	18.765	3.0033	9950	0.0221	*0.0279*
	Sites (estuary) × sampling occasion	8	24.993	6.4878	9920	0.0001	*0.0001*
	Residual	36	17.335				
	Total	53	159				
Sediment multi-element concentrations (mg kg^{-1}) Geomorphologic influence	Estuary	1	59.924	1.7085	10	0.4017	0.2403
	Sampling occasion	2	85.948	2.6088	9963	0.091	0.0936
	Sites (estuary)	4	140.29	102.73	9950	0.0001	*0.0001*
	Estuary × sampling occasions	2	46.76	1.4193	9955	0.2655	0.2664
	Sites (estuary) × sampling occasion	8	131.78	48.249	9922	0.0001	*0.0001*
	Residual	36	12.291				
	Total	53	477				

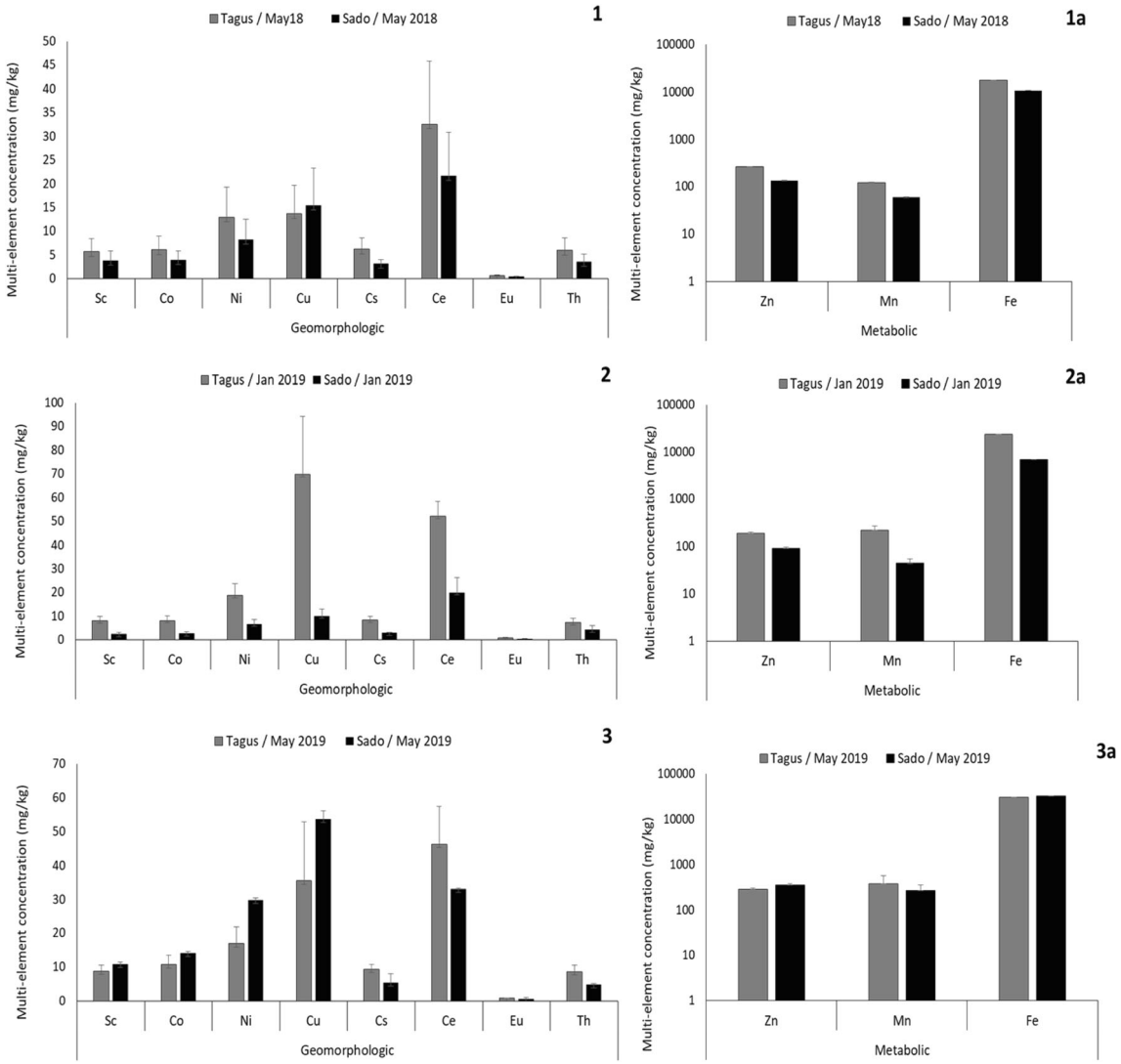


Fig. 5 Mean ± SE, n=3 of trace and major elements concentration values (mg kg^{-1}) in sediments collected at sampling sites in the Tagus and Sado estuaries, at each sampling occasion. (1) Trace elements geomorphologic tracers and (1b) major elements metabolic tracers in the Tagus and Sado estuaries in May 2018; (2) trace elements geomorphologic (2b) major elements metabolic tracers in the Tagus and Sado estuaries in January 2019; and (3) trace elements geomorphologic and (3b) major elements metabolic tracers in the Tagus and Sado estuaries in May 2019. All concentration values of major elements were log-transformed

67]. A similar temporal pattern of the clam chemical signatures was observed by Zhao et al. (2013), which was described as a response to shifting environmental conditions and clams' different element assimilation.

The high variability in clams' chemical signatures between sampling occasions *May 2018* and *January 2019* was mainly associated to Se, Co, Ni and Mn concentrations. These elements are known to be highly influenced by the seasonal and inter-annual variations promoted by the freshwater inputs, precipitation and anthropogenic sources [22, 65], whilst there were significant differences in clams' elemental composition patterns between sampling occasions. Spatial patterns were more evident and they are likely associated with different environmental conditions in each site.

Multi-element Profiles—Sediment vs Clams

Sediment properties such as sediment grain size, organic matter and chemical contents indirectly determine the clam chemical signatures [6, 13, 21, 68]. With the results obtained in clams and sediment chemical profiles, it was possible to establish a similar distribution pattern only between sites within estuaries and across sampling occasions. However, with these results, we would expect that the sediment's chemical profile would be

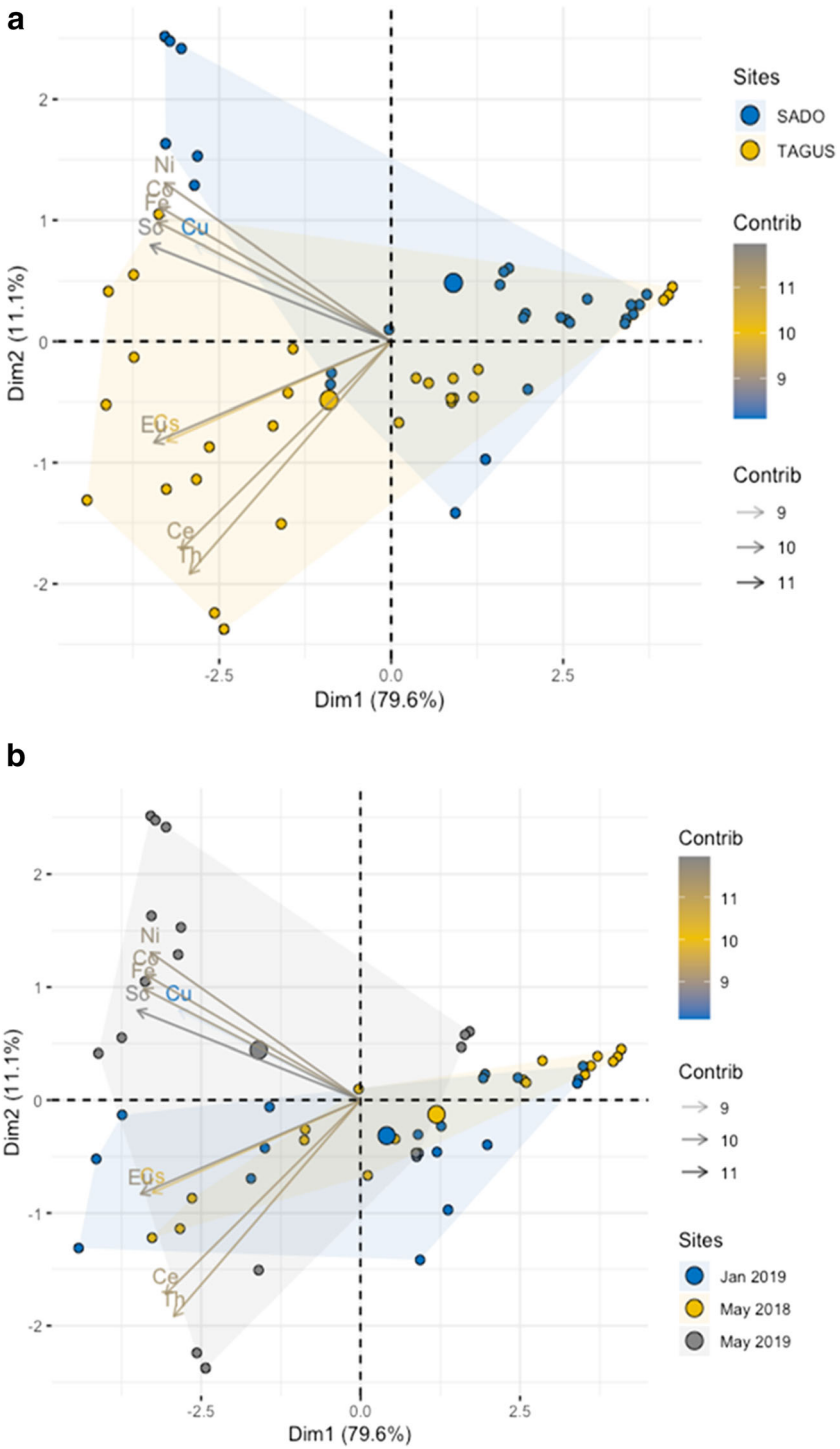


Fig. 6 **a** Principal component analysis (PCA) biplot based on scaled geomorphologic elements concentrations measured in sediments, coloured by estuaries with "confidence" ellipse type. Variable's vectors are presented by their contributions to the principal components (gradient colours and transparency of vectors) with grey representing high contributions, yellow intermediate and blue representing very low contributions. The dots represent the cluster centroids for group variables. **b** Principal component analysis (PCA) biplot based on scaled geomorphologic elements concentrations measured in sediments, coloured by sampling occasions with "confidence" ellipse type. Variable's vectors are presented by their contributions to the principal components (gradient colours and transparency of vectors) with grey representing high contributions, yellow intermediate and blue representing very low contributions. The dots represent the cluster centroids for group variables

highly related with the clams' signature profile, allowing the identification of the populations' origin. Meanwhile, it was solely possible to establish a direct link between some specific geomorphological elements of sediment and clams in both estuaries, being Cu and Ni the potential drivers of the origin of clams' populations and Cs, Sc and Ce being responsible for distinct sediment geomorphologic profiles between estuaries. As most of all rare earth element (REE) [69], these elements showed low temporal and spatial variability among sites revealing to be a good geographical tracer to differentiate estuaries, although these elements are difficult to detect in a measurable level in clam soft tissues, being considered non-essential elements for the physiologic requirements of the specimens.

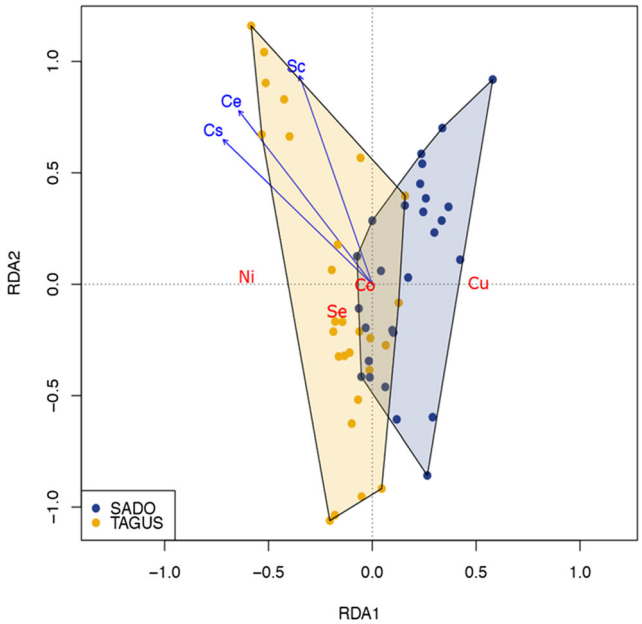


Fig. 7 Redundancy analysis (RDA) biplot based on scaled geomorphologic elements concentrations measured in sediments and clams, coloured by estuaries with "confidence" convex hull type. Variable's vectors are presented by their contributions. Only significant vectors are displayed. On red there are displayed clam's elemental scores.

Conclusions

The spatial and temporal distribution of the multi-element signatures of the non-indigenous bivalve *Ruditapes philippinarum* of the Sado and Tagus estuaries were analysed considering different categories of the chemical elements: estuarine geomorphology sources (Se, Co, Ni and Cu), elements with function in metabolic processes of the clams (Mn, Fe, Zn and Cr) and elements derived from the anthropogenic inputs (As, Pb and Cd). This approach allowed to explore the contribution of each elemental category as a tool to identify the habitat conditions and the geographical origin of the clams. The elements that most contributed for the spatial and temporal distribution of chemical signatures of the Tagus estuary clam populations were Zn, Co, Ni and Pb, whilst for Sado estuary were Cu, Fe, Cr, As and Cd. These elements, representative of all elemental categories (geomorphologic, metabolic and anthropogenic), showed a high spatial variability and a low temporal variability with some elements directly linked to the local anthropogenic pressures which can be useful to obtain local tracers.

The multi-element signatures of *R. philippinarum* as a natural tag derived from the physical and chemical conditions of its habitat. The ICP-MS technique applied to an ecological perspective, it is an important approach to develop a potential rapid tool to use as ecological monitoring and habitat assessment, including for spatial and temporal habitat discrimination of estuarine populations.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12011-021-02629-x.

Acknowledgements The authors also thank João Ramajal, Sara Cabral and Frederico Carvalho (MARE, Faculty of Sciences, Lisbon University) for their help with sample collection.

Funding This study had the funding support from the following projects: D4Ss (ALT20-03-0145-FEDER-029400); UIDB/04292/2020; NIPOGES (MAR-01.03.02-FEAMP-0013) and COCKLES (EAPA_458/2016), Coastnet (ALT20-03-0145-FEDER-022128).

References

1. FAO (2017) FAO Fisheries & Aquaculture - Cultured Aquatic Species Information Programme - Ruditapes philippinarum (Adams & Reeve, 1850). http://www.fao.org/fishery/culturedspecies/Ruditapes_philippinarum/en
2. Chainho P, Fernandes A, Amorim A, Ávila SP, Canning-Clode J, Castro JJ, Costa AC, Costa JL, Cruz T, Gollasch S, Grazziotin-Soares C, Melo R, Micael J, Parente MI, Semedo J, Silva T, Sobral D, Sousa M, Torres P, Veloso V, Costa MJ (2015) Non-indigenous species in Portuguese coastal areas, coastal lagoons, estuaries and islands. Estuar Coast Shelf Sci 167:199–211. https://doi.org/10.1016/j.ecss.2015.06.019
3. Gaspar BM. CP. CJL (2010) Distribuição, abundância e estrutura demográfica da amêijoa Japonesa (Ruditapes philippinarum) no Rio Tejo
4. Moschino V, Delaney E, Da Ros L (2012) Assessing the significance of Ruditapes philippinarum as a sentinel for sediment pollution: bioaccumulation and biomarker responses. Environ Pollut 171:52–60. https://doi.org/10.1016/j.envpol.2012.07.024
5. Ramajal J, Picard D, Costa JL, et al (2016) Amêijoa-japonesa, uma nova realidade no estuário do Rio Tejo: Pesca e pressão social e impacto socio-económico. In: Entre Rios e Mares: um Património de Ambientes, Histórias e Saberes. Tomo V da Rede BrasPor. pp 17–30
6. Zhao H, Zhang S (2016) Effects of sediment, seawater, and season on multi-element fingerprints of Manila clam (Ruditapes philippinarum) for authenticity identification. Food Control 66:62–68. https://doi.org/10.1016/j.foodcont.2016.01.045
7. Chiesa S, Chainho P, Almeida Â, Figueira E, Soares AMVM, Freitas R (2018) Metals and As content in sediments and Manila clam Ruditapes philippinarum in the Tagus estuary (Portugal): impacts and risk for human consumption. Mar Pollut Bull 126:281–292. https://doi.org/10.1016/j.marpolbul.2017.10.088
8. Ji J, Choi HJ, Ahn I-Y (2006) Evaluation of Manila clam Ruditapes philippinarum as a sentinel species for metal pollution monitoring in estuarine tidal flats of Korea: effects of size, sex, and spawning on baseline accumulation. Mar Pollut Bull 52:447–453. https://doi.org/10.1016/j.marpolbul.2005.12.012
9. Chainho P (2013) El Tajo. História de un río ignorado., Colección. Fundación Nueva Cultura del Agua, Zaragoza, 2013
10. Eggleton J, Thomas KV (2004) A review of factors affecting the release and bioavailability of contaminants during sediment

disturbance events. Environ Int 30:973–980. https://doi.org/10.1016/j.envint.2004.03.001
11. Li Y, Yu Z, Song X, Mu Q (2006) Trace metal concentrations in suspended particles, sediments and clams (Ruditapes philippinarum) from Jiaozhou Bay of China. Environ Monit Assess 121:491–501. https://doi.org/10.1007/s10661-005-9149-6
12. Wang Z, Yan C, Vulpe CD, Yan Y, Chi Q (2012) Incorporation of in situ exposure and biomarkers response in clams Ruditapes philippinarum for assessment of metal pollution in coastal areas from the Maluan Bay of China. Mar Pollut Bull 64:90–98. https://doi.org/10.1016/j.marpolbul.2011.10.017
13. Zhao L, Yang F, Wang Y, Huo Z, Yan X (2013) Seasonal variation of metals in seawater, sediment, and Manila clam Ruditapes philippinarum from China. Biol Trace Elem Res 152:358–366. https://doi.org/10.1007/s12011-013-9628-5
14. Ricardo F, Génio L, Costa Leal M, Albuquerque R, Queiroga H, Rosa R, Calado R (2015) Trace element fingerprinting of cockle (Cerastoderma edule) shells can reveal harvesting location in adjacent areas. Sci Rep 5:11932
15. Velez C, Leandro S, Figueira E, Soares AMVM, Freitas R (2015) Biochemical performance of native and introduced clam species living in sympatry: the role of elements accumulation and partitioning. Mar Environ Res 109:81–94. https://doi.org/10.1016/j.marenvres.2015.06.005
16. Nizzoli D, Welsh DT, Viaroli P (2011) Seasonal nitrogen and phosphorus dynamics during benthic clam and suspended mussel cultivation. Mar Pollut Bull 62:1276–1287. https://doi.org/10.1016/j.marpolbul.2011.03.009
17. Abdullah MH, Sidi J, Aris AZ (2007) Heavy metals (Cd, Cu, Cr, Pb and Zn) in Meretrix meretrix roding, water and sediments from estuaries in Sabah, North Borneo. International Journal of Environmental and Science Education
18. Chaharlang BH, Bakhtiari AR, Yavari V (2012) Assessment of cadmium, copper, lead and zinc contamination using oysters (Saccostrea cucullata) as biomonitors on the coast of the Persian Gulf, Iran. Bull Environ Contam Toxicol 88:956–961. https://doi.org/10.1007/s00128-012-0591-1
19. Santana LMBM, Blasco J, Abessa DMS, Campana O (2017) Bioaccumulation kinetics of copper in Ruditapes philippinarum exposed to increasing, continuous and pulsed exposure: Implications for growth. Sci Total Environ 595:920–927. https://doi.org/10.1016/j.scitotenv.2017.03.020
20. Delgado M, Pérez-Camacho A (2007, Sci Mar) Comparative study of gonadal development of Ruditapes philippinarum (Adams and Reeve) and Ruditapes decussatus (L.) (Mollusca: Bivalvia): Influence of temperature. 71(3). https://doi.org/10.3989/scimar200771n3471
21. Amisah S, Obirikorang KA, Adjei Boateng D (2011) Bioaccumulation of heavy metals in the Volta clam, Galatea paradoxa (Born, 1778) in relation to their geoaccumulation in benthic sediments of the Volta estuary, Ghana. Water Qual Expo Health 2:147–156. https://doi.org/10.1007/s12403-010-0032-5
22. Duarte B, Caçador I (2012) Particulate metal distribution in Tagus estuary (Portugal) during a flood episode. Mar Pollut Bull 64:2109–2116. https://doi.org/10.1016/j.marpolbul.2012.07.016
23. Serafim A, Company R, Lopes B et al (2013) Evaluation of sediment toxicity in different Portuguese estuaries: ecological impact of metals and polycyclic aromatic hydrocarbons. Estuar Coast Shelf Sci 130:30–41. https://doi.org/10.1016/j.ecss.2013.04.018
24. Duarte B, Silva G, Costa JL, Medeiros JP, Azeda C, Sá E, Metelo I, Costa MJ, Caçador I (2014) Heavy metal distribution and partitioning in the vicinity of the discharge areas of Lisbon drainage basins (Tagus Estuary, Portugal). J Sea Res 93:101–111. https://doi.org/10.1016/j.seares.2014.01.003
25. Becker BJ, Fodrie FJ, McMillan PA, Levin LA (2005) Spatial and temporal variation in trace elemental fingerprints of mytilid mussel shells: aprecursor to invertebrate larval tracking. Limnol Oceanogr 50:48–61. https://doi.org/10.4319/lo.2005.50.1.0048
26. Carson HS, López-Duarte PC, Cook GS, Fodrie FJ, Becker BJ, DiBacco C, Levin LA (2013) Temporal, spatial, and interspecific variation in geochemical signatures within fish otoliths, bivalve larval shells, and crustacean larvae. Mar Ecol Prog Ser 473:133–148
27. Figueira E, Freitas R (2013) Consumption of Ruditapes philippinarum and Ruditapes decussatus: comparison of element accumulation and health risk. Environ Sci Pollut Res 20:5682–5691. https://doi.org/10.1007/s11356-013-1587-z
28. Marques A, Piló D, Carvalho S, Araújo O, Guilherme S, Santos MA, Vale C, Pereira F, Pacheco M, Pereira P (2017) Metal bioaccumulation and oxidative stress profiles in Ruditapes philippinarum – insights towards its suitability as bioindicator of estuarine metal contamination. Ecol Indic 95:1087–1099. https://doi.org/10.1016/j.ecolind.2017.10.072
29. Mil-Homens M, Vale C, Raimundo J, Pereira P, Brito P, Caetano M (2014) Major factors influencing the elemental composition of surface estuarine sediments: the case of 15 estuaries in Portugal. Mar Pollut Bull 84:135–146. https://doi.org/10.1016/j.marpolbul.2014.05.026
30. de Souza Machado AA, Spencer K, Kloas W, Toffolon M, Zarfl C (2016) Metal fate and effects in estuaries: a review and conceptual model for better understanding of toxicity. Sci Total Environ 541:268–281. https://doi.org/10.1016/j.scitotenv.2015.09.045
31. Fattorini D, Notti A, Di Mento R et al (2008) Seasonal, spatial and inter-annual variations of trace metals in mussels from the Adriatic sea: a regional gradient for arsenic and implications for monitoring the impact of off-shore activities. Chemosphere 72:1524–1533. https://doi.org/10.1016/J.CHEMOSPHERE.2008.04.071
32. Moura P, Vasconcelos P, Pereira F, Chainho P, Costa JL, Gaspar MB (2018) Reproductive cycle of the Manila clam (*Ruditapes philippinarum*): an intensively harvested invasive species in the Tagus Estuary (Portugal). J Mar Biol Assoc UK 98:1645–1657. https://doi.org/10.1017/S0025315417001382
33. Thomas R (2013) Practical Guide to ICP-MS. CRC Press, Boca Raton
34. Balcaen L, Bolea-Fernandez E, Resano M, Vanhaecke F (2015) Inductively coupled plasma – Tandem mass spectrometry (ICP-MS/MS): a powerful and universal tool for the interference-free determination of (ultra)trace elements – a tutorial review. Anal Chim Acta 894:7–19. https://doi.org/10.1016/J.ACA.2015.08.053
35. Cathey AM, Miller NR, Kimmel DG (2014) Spatiotemporal stability of trace and minor elemental signatures in early larval shell of the northern quahog (hard clam) Mercenaria mercenaria. J Shellfish Res 33:247–255. https://doi.org/10.2983/035.033.0124
36. Norrie CR, Dunphy BJ, Baker JA, Lundquist CJ (2016) Local-scale variation in trace elemental fingerprints of the estuarine bivalve Austrovenus stutchburyi within and between estuaries. Mar Ecol Prog Ser 559:89–102
37. Breda S, Chiesa S, Freitas R, Figueira E, Becherini F, Gobbo L, Soares AMVM, Argese E (2018) Biogeochemical dynamics and bioaccumulation processes in Manila clam: implications for biodiversity and ecosystem services in the Ria de Aveiro Lagoon. Estuar Coast Shelf Sci 209:136–148. https://doi.org/10.1016/j.ecss.2018.04.029
38. Zhao H, Zhang S (2016) Identification of Jiaozhou bay clams (Ruditapes philippinarum) by multi-element fingerprinting technique. Food Anal Methods 9:2691–2699. https://doi.org/10.1007/s12161-016-0461-2
39. Brito P, Prego R, Mil-Homens M, Caçador I, Caetano M (2018) Sources and distribution of yttrium and rare earth elements in surface sediments from Tagus estuary, Portugal. Sci Total Environ 621:317–325. https://doi.org/10.1016/j.scitotenv.2017.11.245

40. Freitas MC, Andrade C, Cruces A et al (2008) Anthropogenic influence in the Sado estuary (Portugal): a geochemical approach Influencia antrópica en el estuario de Sado (Portugal): una aproximación geoquímica. J Iber Geol 34:271–286
41. Cabral H, Costa M, Salgado J (2001) Does the Tagus estuary fish community reflect environmental changes? Clim Res 18:119–126. https://doi.org/10.3354/cr018119
42. Cruzeiro C, Rocha E, Pardal MÂ, Rocha MJ (2016) Seasonal-spatial survey of pesticides in the most significant estuary of the Iberian Peninsula – the Tagus River estuary. J Clean Prod 126:419–427. https://doi.org/10.1016/j.jclepro.2016.03.005
43. Costa MJ, Vasconcelos R, Costa JL, Cabral HN (2007) River flow influence on the fish community of the Tagus estuary (Portugal). Hydrobiologia 587:113–123. https://doi.org/10.1007/s10750-007-0690-x
44. Chainho P, Chaves ML, Costa JL, Costa MJ, Dauer DM (2008) Use of multimetric indices to classify estuaries with different hydromorphological characteristics and different levels of human pressure. Mar Pollut Bull 56:1128–1137. https://doi.org/10.1016/j.marpolbul.2008.03.018
45. Cabral S, Carvalho F, Gaspar M et al (2019) Non-indigenous species in soft-sediments: are some estuaries more invaded than others? Ecol Indic 110:105640. https://doi.org/10.1016/j.ecolind.2019.105640
46. Caeiro S, Goovaerts P, Painho M, Costa MH (2003) Delineation of estuarine management areas using multivariate geostatistics: the case of Sado estuary. Environ Sci Technol 37:4052–4059. https://doi.org/10.1021/es0262075
47. Carreira S, Costa PM, Martins M, Lobo J, Costa MH, Caeiro S (2013) Ecotoxicological Heterogeneity in transitional coastal habitats assessed through the integration of biomarkers and sediment-contamination profiles: a case study using a commercial clam. Arch Environ Contam Toxicol 64:97–109. https://doi.org/10.1007/s00244-012-9812-1
48. Caeiro S, Costa MH, DelValls A, Repolho T, Gonçalves M, Mosca A, Coimbra AP, Ramos TB, Painho M (2009) Ecological risk assessment of sediment management areas: application to Sado Estuary, Portugal. Ecotoxicology 18:1165–1175. https://doi.org/10.1007/s10646-009-0372-8
49. Gonçalves C, Brogueira MJ, Nogueira M (2015) Tidal and spatial variability of nitrous oxide (N2O) in Sado estuary (Portugal). Estuar Coast Shelf Sci 167:466–474. https://doi.org/10.1016/j.ecss.2015.10.028
50. Lillebø AI, Coelho PJ, Pato P, Válega M, Margalho R, Reis M, Raposo J, Pereira E, Duarte AC, Pardal MA (2011) Assessment of mercury in water, sediments and biota of a Southern European estuary (Sado estuary, Portugal). Water Air Soil Pollut 214:667–680. https://doi.org/10.1007/s11270-010-0457-2
51. Chainho P (2014) ICES WGITMO Report 2014. In: Despacho n.º 15264/2013; Portaria n.º 14/2014
52. Caçador I, Caetano M, Duarte B, Vale C (2009) Stock and losses of trace metals from salt marsh plants. Mar Environ Res 67:75–82. https://doi.org/10.1016/j.marenvres.2008.11.004
53. Viet T, Khanh N, Thinh D, et al (2018) Trace metals analysis of marine benthos Certified Reference Materials by inductively coupled plasma mass spectrometry following hot plate heating digestion method. J Mil Sci Technol
54. Lê S, Josse J, Rennes A, Husson F (2008) FactoMineR: an R Package for multivariate analysis
55. R Core Team (2012) R: A language and environment for statistical computing
56. Anderson MJ, Gorley RN, Clarke KR (2008) PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods. In: Plymouth, UK
57. Clarke K, And RW-A approach to statistical analysis, 2001 U (2001) Change in marine communities. researchgate.net 2nd editio:
58. Kindt R, Coe R (2005) Tree Diversity Analysis. A manual and software for common statistical methods and biodiversity studies. 10.13140/RG.2.1.1993.7684
59. Oksanen J (2008) Vegan: an introduction to ordination
60. Du Laing G, Rinklebe J, Vandecasteele B et al (2009) Trace metal behaviour in estuarine and riverine floodplain soils and sediments: a review. Sci Total Environ 407:3972–3985. https://doi.org/10.1016/J.SCITOTENV.2008.07.025
61. Mohamed AS, Saad, Dajem B et al (2020) Freshwater Clam as a potential bioindicator for silver/saponin nanocomposites toxicity. Bull Environ Contam Toxicol 105:827–834. https://doi.org/10.1007/s00128-020-03038-x
62. Oaten JFP, Hudson MD, Jensen AC, Williams ID (2017) Seasonal effects to metallothionein responses to metal exposure in a naturalised population of Ruditapes philippinarum in a semi-enclosed estuarine environment. Sci Total Environ 575:1279–1290. https://doi.org/10.1016/j.scitotenv.2016.09.202
63. Fortunato AB, Oliveira A, Baptista AM (1999) On the effect of tidal flats on the hydrodynamics of the Tagus estuary. Oceanol Acta 22:31–44. https://doi.org/10.1016/S0399-1784(99)80030-9
64. Du Laing G (2012) Redox metal processes and controls in estuaries. Elsevier Inc.
65. Caeiro S, Costa MH, Ramos TB, Fernandes F, Silveira N, Coimbra A, Medeiros G, Painho M (2005) Assessing heavy metal contamination in Sado estuary sediment: an index analysis approach. Ecol Indic 5:151–169. https://doi.org/10.1016/j.ecolind.2005.02.001
66. Vinagre C, Cabral HN, Caçador I (2008) Influence of halophytes and metal contamination on salt marsh macro-benthic communities. Estuar Coast Shelf Sci 76:715–722. https://doi.org/10.1016/j.ecss.2007.08.001
67. Rodrigues JRP (2017) Bioturbação de uma espécie de amêijoa nativa e uma invasora em simpatria: implicações nas funções de um ecossistema estuarino. Universidade de Aveiro
68. Zhao L, Yang F, Yan X, Huo Z, Zhang G (2012) Heavy Metal Concentrations in Surface Sediments and Manila Clams (Ruditapes philippinarum) from the Dalian Coast, China after the Dalian Port Oil Spill. Biol Trace Elem Res 149:241–247. https://doi.org/10.1007/s12011-012-9412-y
69. Aide MT, Aide C (2012) Rare earth elements: their importance in understanding soil genesis. Int Sch Res Netw ISRN Soil Sci 2012:11–11. https://doi.org/10.5402/2012/783876