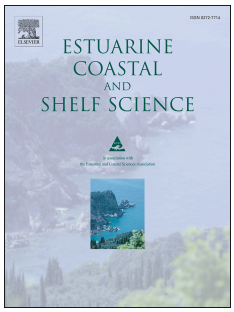


Bioaccumulation and biochemical patterns of *Ruditapes philippinarum* clams: Responses to seasonality and low contamination levels

Silvana Costa, Joel Lopes, Francesca Coppola, Simão Correia, Bruno Henriques, Carla Leite, Amadeu M.V.M. Soares, Jiang Zengjie, Eduarda Pereira, Stefania Chiesa, Rosa Freitas

PII: S0272-7714(20)30297-3

DOI: https://doi.org/10.1016/j.ecss.2020.106883

Reference: YECSS 106883

To appear in: *Estuarine, Coastal and Shelf Science*

Received Date: 24 March 2020

Revised Date: 21 May 2020

Accepted Date: 7 June 2020

Please cite this article as: Costa, S., Lopes, J., Coppola, F., Correia, Simã., Henriques, B., Leite, C., Soares, A.M.V.M., Zengjie, J., Pereira, E., Chiesa, S., Freitas, R., Bioaccumulation and biochemical patterns of *Ruditapes philippinarum* clams: Responses to seasonality and low contamination levels, *Estuarine, Coastal and Shelf Science* (2020), doi: https://doi.org/10.1016/j.ecss.2020.106883.

Bioaccumulation and biochemical patterns of *Ruditapes philippinarum* clams: responses to seasonality and low contamination levels.

Silvana Costa[a], Joel Lopes[a], Francesca Coppola[a], Simão Correia[a], Bruno Henriques[b], Carla Leite[a], Amadeu M.V.M. Soares[a], Jiang Zengjie[c], Eduarda Pereira[b], Stefania Chiesa[d], Rosa Freitas[a]

[a]Department of Biology & CESAM, University of Aveiro, Portugal

[b]Department of Chemistry & CESAM, University of Aveiro, Portugal

[c]Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, China

[d]Institute for Environmental Protection and Research (ISPRA), Rome, Italy

Corresponding author: Rosa Freitas

Address: Departamento de Biologia, Universidade de Aveiro

Campus Universitário de Santiago

3810-193 Aveiro, Portugal

e-mail address: rosafreitas@ua.pt

ABSTRACT

Shellfish farming and shellfish harvesting has been practiced for a long time in the Ria de Aveiro coastal lagoon (Portugal). Among commercial bivalves, Manila clam *Ruditapes philippinarum* represents one of the most important species inhabiting the Ria de Aveiro. Introduced in Portugal in 1984, naturalised *R. philippinarum* clam populations have been subjected to several pressures that may threaten this resource sustainable management: illegal fishing, harvesting in chemically polluted sites with impacts on human health, lack of control in terms of productivity with the risk of a progressive decline of the biomass. On behalf of the ASARISAFE project (with the title Safety and sustainable management of valuable clam product in Portugal and China) the environmental quality of Manila clam harvesting sites was evaluated, focusing on inorganic pollution, health status of clams in terms of bioaccumulation as well as biochemical performance. Seasonal sampling campaigns were conducted in six *R. philippinarum* harvesting areas evaluating inorganic pollution levels, in clam's tissues, sediment and water. Clams biochemical performance in terms of metabolism, energy reserves and oxidative stress was also assessed. The results obtained showed that mercury and arsenic (As) were the elements with the highest BAF (Bioaccumulation factor) values, but contamination levels in tissues and sediments varied among sampling areas and seasonal campaigns. The amount of clams consumed per week to exceed Provisional Tolerable Week Intake (PTWI, kg) was the lowest for As, revealing that less 0.05 kg of clams was enough to exceed PTWI. However, the results obtained further demonstrated that the clam's biochemical performance was not responding to tissues contamination levels but were closely related to seasons, with distinct metabolic capacity and oxidative stress levels among distinct sampling periods during the year.

Keywords: Clams; metal contamination; safety consumption; seasons; biochemical performance.

1. INTRODUCTION

Coastal ecosystems, including lagoons and estuaries, are complex systems with high primary production (McLusky, 1999). They have a role of paramount importance in providing several ecosystem services, often associated to the sustenance of vast biological resources (Lillebø et al., 2015). However, these ecosystems are often negatively impacted by natural shifts (Govender et al., 2011) and anthropogenic activity (Langston et al., 2010), including climate change related factors and pollution. Classical environmental monitoring and ecological health status evaluation through water and sediment chemical analysis associated to the evaluation of biological effects upon inhabiting biota are approaches commonly used (WFD, 2000/60/EC) in order to assess negative impacts derived from chemical exposure before it becomes relevant in superior levels of the biological organization (Picado et al., 2007). Environmental monitoring has been based on the effects induced in benthic organisms, by the evaluation of alterations at the community level (benthic community parameters), and more recently, on individual and cellular levels (physiological and biochemical markers), mainly to assess the impacts of pollutants but, more recently, to investigate alterations derived from climate change, especially related with extreme weather events. Cellular alterations are widely described in literature as a response to natural and anthropogenic stressors (Magalhães et al., 2018, Munari et al, 2018, Gonçalves et al., 2017, Velez et al., 2016a, Carregosa et al., 2014, Harley et al. 2006). In particular, cellular biomarkers have been used to assess the negative impacts of metals and metalloids (Coppola et al., 2018), temperature (Keller et al., 2004), salinity (Freitas et al., 2015, Moreira et al., 2016) and pH (Velez et al., 2016b). Within benthic macrofauna assemblages, clam species are identified as important bioindicators due to their high abundance and filter-feeding habits and socio-economic relevance (reviewed in Bebianno et al., 2004).

The Manila clam (*Ruditapes philippinarum*) is a native species from the Indo-Pacific region, introduced in Europe at the beginning of the 1970s for culture purposes (Flassch and Leborgne, 1992, Jensen et al., 2004), becoming a highly exploited resource (Pranovi et al., 2006, Dang et al., 2010). This species is commonly exploited in a wide variety of aquatic systems due to its fast adaption to new environmental scenarios, fast growth and high commercial value (Usero et al., 1997). More recently, *R. philippinarum* (Adams and Reeve, 1850) was introduced in Portugal, being currently one of the most widely used bivalve species to assess environmental quality (Costa et al., 2013, Martín-

Diaz et al., 2007, Shin et al., 2002). As an example, Costa et al. (2013) performed histopathological assays in *R. philippinarum* specimens, aiming to assess the environmental quality of the Portuguese south coast. Studies conducted by Moschino et al. (2012) also demonstrated the capacity of Manila clam as a bioindicator species, revealing the clam's responses to pollutants concentrations. Nevertheless, under environmental conditions when ecosystem pollution levels are low it is often difficult to determine whether effects are due to pollutants or natural environmental shifts closely linked with the organism's life cycle (Sheehan and Power, 1999, Hook et al., 2014), which can seriously compromise the interpretation of monitoring data. Thus, it is important to understand how the natural variations associated with seasonal changes such as salinity and temperature may impact the inhabiting fauna life cycle and, consequently, can alter the organism's responses to pollutants.

Therefore, the general aim of the present study was to evaluate the capacity of *R. philippinarum* as bioindicator species in a low contaminated coastal system along four distinct seasons, testing the hypothesis that pollution levels may hide the effects induced by seasons on the clam's natural biochemical performance. For this, the biochemical performance of *R. philippinarum* specimens, collected from six different areas along the Ria de Aveiro (Portugal), characterized by different metal(oid)s concentrations, was assessed during four seasons (spring, summer, fall, winter). The risk for human health derived from clam's consumption was also evaluated.

2. METHODOLOGY

2.1. SITE DESCRIPTION

The present study was conducted at the Ria de Aveiro (Figure 1), a shallow, vertically homogeneous, coastal lagoon located on the northwest coast of Portugal. This aquatic system is 45 km long and 10 km wide, comprising a total surface area of 83 km^2 at high tide, with 17 km^2 of intertidal flats emerging at low tide (Dias et al., 2000). In addition, this aquatic system is characterized by narrow channels and by large areas of mud flats and salt marshes (Picado et al., 2009).

Sampling was conducted in six different areas selected along the lagoon: Torreira (T - 40°45' 43.0'' N, 8°41' 56.7''W), Sporting (S - 40°40' 15 .2'' N, 8°38' 45.9''W), São Jacinto (SJ - 40°42' 24.1'' N, 8° 41' 50.6''W), Ílhavo (I - 40° 36' 59.3'' N, 8 °40' 51.3''W), Murtosa (M - 40° 43' 25.8'' N, 8° 3 9' 33.8''W) and Cale do Ouro (CO - 40°42' 02.9'' N, 8 °41' 09.3''W) (Figure 1).

2.2. SAMPLING PROCEDURE

In each studied area, three sampling sites were selected and seasonally sampled (Winter, Spring, Summer and Autumn), from late 2017 to early 2019.

From each sampling site, eighteen specimens of *R. philippinarum* with similar size were collected (length: 4.6±0.64; width: 3.7±0.26). The whole soft tissue of three individuals was used for elements quantification, while the other fifteen individuals were used for biochemical analyses.

Additionally, at each sampling site, pH, salinity, dissolved oxygen (DO), redox potential (Eh) and temperature were measured in the sediment-water interface using a handheld multiparametric probe. Sediment samples were collected for sediment grain-size analysis, total organic matter (TOM) determination and elements (chromium, Cr; nickel, Ni; copper, Cu; lead, Pb; cadmium, Cd; mercury, Hg; arsenic, As) quantification.

Organisms and sediment samples for TOM determination and elements quantification were stored and transported on ice (approx. 0 ℃) to the laboratory and afterwards preserved at −20 ℃ until analyses.

2.3. LABORATORY PROCEDURES

2.3.1. ENVIRONMENTAL PARAMETERS

Sediment grain-size was carried out following the procedure described by Quintino et al. (1989). Silt and clay fraction (fine particles, with diameter < 0.063 mm) were wet sieved, whereas sand (0.063 – 2.000 mm) and gravel (particles with diameter > 2.000 mm) fractions were dry sieved through a tower of sieves spaced at 1 phi (Φ) (Φ = $-\log_2$ the particle diameter (mm)). Data were used to calculate the median grain-size value, P50, expressed in Φ units. Median grain-size and the percentage of fines content were used to classify the sediment, according to the Wentworth scale: very fine sand (median from 3 to 4 Φ); fine sand (2–3 Φ); medium sand (1–2 Φ); coarse sand (0–1 Φ); very coarse sand (−1 to 0 Φ). All sediment grain-size fractions were expressed as a percentage of the whole sediment dry weight (DW). The final classification adopted the description 'clean', 'silty' or 'very silty' when the silt and clay fraction ranged from 0% to 5%, from 5% to 25% and from 25% to 50% of the total sediment DW, respectively (Doeglas, 1968). Samples with more than 50% fines content were classified as mud.

Total organic matter content (TOM) was determined according to Byers et al. (1978) as loss on ignition at 450 ℃ (with minimal risk of volatiz ing inorganic carbon) during 5 h.

2.3.2. ELEMENTS DETERMINATION

The concentration of mercury in water was determined by cold vapor atomic fluorescence spectroscopy (CV-AFS), with a PSA Millennium Merlin 10.036 analytical instrument, equipped with a detector PSA model 10.003. Stannous chloride (2% in 10% HCl) was used as reductant, and six standard solutions of Hg ranging between 2.5 and 60 ng/L, prepared by dilution of a commercial stock solution ($Hg(NO_3)_2$, 1000 ± 2 mg/L) in HNO_3 (2% v/v), were used to obtain the calibration curve. The limit of quantification of the method was assumed as the lowest calibration standard, and a relative standard deviation among replicates <5% was considered.

In sediment and organisms, mercury was directly quantified in freeze-dried samples (2-20 mg) by thermal decomposition atomic absorption spectrometry with gold amalgamation (LECO model AMA-254), as described by Costley et al. (2000). Detection and quantification limits were 0.01 ng Hg and 0.03 ng Hg, respectively. Each sample was analysed at least in triplicate with an acceptable relative standard deviation among replicates <10%. Blanks were run between sample analyses, and Certified Reference Materials TORT-2 (Lobster hepatopancreas; 0.27±0.06 mg/kg of total Hg) and

MESS-3 (Marine Sediment, 0.091± 0.009 mg/kg of total Hg) were analysed several times daily. All percentages of recovery were within the range of 90-110%.

The concentrations of Cu, As, Cd, Pb, Ni and Cr in water were measured by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) at the Central Analysis Laboratory of the University of Aveiro. All samples were previously diluted 15x in HNO_3 (2 %, v/v) to avoid interferences (due to the matrix) associated with salinity. The limits of quantification in water samples for the studied elements were assumed as the lowest calibration standards: 2 µg/L (As, Cu), 1 µg/L (Ni), 0.5 µg/L (Cr), 0.2 µg/L (Pb) and 0.1 µg/L (Cd).

In sediments and clams, the concentrations of Cu, As, Cd, Pb, Ni and Cr were also analysed by ICP-MS, after microwave assisted acid digestion, using a microwave system CEM MARS 5, model 240/50. For quantification in sediments, 200 mg of homogenized air-dried sample was digested with 3 mL of HNO_3 (69%) and 6 mL of HF (40%) in Teflon vessel during 5 min with a ramping heating until 175 ℃, followed by 5 min at constant temperature of 175 ℃. The samples were then evaporated near to dryness at 175 ℃ in a plate heater, followed by re-dissolution with 1.5 mL HCl (1:1 V/V) and 1 mL HNO_3 (69%), and finally transferred into 25 mL polyethylene flasks with the volume made up with ultrapure water. For quantification of the clam's soft tissues, 200 mg (previously frozen-dried) was transferred to Teflon bombs with 1 mL HNO_3 65% (v/v) (Suprapur, Merck), 2 mL H_2O_2 and 1 mL mili-Q H_2O. Samples were left 15 min in the microwave with increasing temperature up to 180 ºC, which was maintained for 3 min. After cooling, samples were collected in polyethylene flasks, made up to a final volume of 25 mL with ultrapure water and stored at room temperature until quantification. The quality control was assured by running procedural blanks (reaction vessels without sample) and certified reference materials TORT-2 (for clams) and MESS-3 (for sediments) in parallel with samples. All blanks were below the quantification limit and the element recoveries in reference materials were always within the acceptable range of 80 to 120%.

2.3.3. BIOCHEMICAL PARAMETERS

After sampling, the clams were frozen, pulverized individually with liquid nitrogen and divided in 0.3 g fresh weight (FW) aliquots. Biochemical analyses were repeated in duplicate for each sample and biomarker. Extractions were performed using a 1:2 (w/v) proportion of specific buffers such as 20% (w/v) trichloroacetic acid (TCA) buffer to perform lipid peroxidation (LPO). Reduced (GSH) and

oxidized (GSSG) glutathione parameters were carried out using a KPE buffer with 0.1% (v/v) Triton X-100 and 0.6% (w/v) sulfosalicylic acid. Potassium phosphate (50 mM, pH=7), 1mM EDTA, 1% (v/v) Triton X-100, 1mM DTT was used to perform superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), S-glutathione transferase (GST's), protein (PROT), glycogen (GLY) and Acetylcholinesterase (ATChI-ChE) tests. To assess electron transport system (ETS) activity, samples were extracted using a 0.1 M Tris-HCl pH 8.5 with 15% (w/v) PVP, 153 µM magnesium sulfate ($MgSO_4$) and 0.2% (v/v) Triton X-100 buffer.

2.3.3.1. Indicators of cellular damage and redox balance

LPO was measured by quantifying malondialdehyde (MDA) according to the method described by Ohkawa et al. (1979) and the respective modifications referred in Carregosa et al. (2014). Absorbance was read at 535 nm (ε = 156 mM^{-1} cm^{-1}). LPO levels were calculated using Lambert-Beer Law and expressed in nmol of MDA formed per g of FW.

GSH and GSSG glutathione contents were determined according to Rahman et al. (2006) using GSSG as standards. Absorbance of GSH and GSSG was read at 412 nm. GSH and GSSG were expressed in µmol per g of FW. Reduced to oxidised glutathione ratio (GSH/GSSG) was calculated dividing GSH content by 2 x the amount of GSSG (adimensional).

2.3.3.2. Enzymatic defences

SOD activity was determined based on Beauchamp and Fridovich (1971) method. SOD standards (0.25 – 60 U/ml) were used to perform calibration curve and SOD activity was measured spectrophotometrically at 560 nm. Activity was expressed in units of enzyme (U) per g of FW. One U corresponds to the conversion of 1 µmol per min.

CAT activity was quantified according to Johansson and Borg (1988). The assay was carried out using formaldehyde standards and the absorbance was measured at 540 nm. The results were expressed in U per g of FW. One U is defined as the amount of enzyme that caused the formation of 1.0 nmol of formaldehyde, per min.

Activity of GPx was quantified following Paglia and Valentine (1967). The absorbance was measured at 340 nm and determined using ε = 6.22 $mmol^{-1}cm^{-1}$ and the results were expressed as U

per g of FW. One unit of enzyme (U) represents the number of enzymes that caused the formation of 1.0 μmol nicotinamide adenine dinucleotide phosphate (NADPH) per min.

GSTs was determined following Habig et al. (1974). The absorbance was determined at 340 nm using an extinction coefficient of 9.6 mM^{-1} cm^{-1}, expressed in U per g of FW. One unit of enzyme (U) corresponds to the amount of enzyme that caused the formation of 1 μmol of dinitrophenyl thioether per min.

2.3.3.3. Metabolic capacity and energy reserves

ETS activity was measured based on King and Packard (1975) and modifications performed by De Coen and Janssen (1997) and the absorbance was read at 490 nm using Ɛ = 15,900 $M^{-1}cm^{-1}$, expressed in nmol/min per g of FW.

PROT content was determined according to Robinson and Hogden (1940) and was carried out using bovine serum albumin (BSA) standards. The absorbance was read at 540 nm. Results were expressed in mgμ of PROT per g FW.

Following the procedure described by Dubois et al (1956), GLY was quantified by the phenol–sulfuric acid method using glucose standards. The absorbance was measured at 492 nm. Results were expressed in mg of GLY per g of FW.

2.3.3.4. Neurotoxicity

Acetylthiocholine iodide (ATChI, 5 mM) substrates were used for the determination of Acetylcholinesterase (ATChI-ChE) following the methods of Ellman et al. (1961) and modifications by Mennillo et al. (2017). The absorbance was measured at 412 nm and determined using ε = 13600 $nmol^{-1}cm^{-1}$. The results were measured in nmol per min per g of FW and express the formation of the dianion of 5-thio-2-nitrobenzoic acid (TNB) per unit time (minute).

2.4. DATA ANALYSIS

Bioaccumulation factor (BAF) was determined dividing the total concentration of a given element in the organism tissue (DW) by the concentration of that element in the sediment (DW) (McGeer et al., 2003). The data matrix with the BAF per site and season [BAF x sampling area x

season] was normalised and the Euclidean similarity calculated between sampling areas. A Principal Coordinates Ordination analysis (PCO) was used to visualize differences among areas. The abiotic data highly correlated ($r > 0.75$) were represented as superimposed vectors in the graph.

Data on sediment characteristics (contamination and physico-chemical properties) and species contamination were submitted to hypothesis testing using permutation multivariate analysis of variance with the PERMANOVA + add-on in PRIMER v6 (Anderson et al., 2008). The pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance. When the main test revealed statistical significant differences ($p \leq 0.05$), pairwise comparisons were performed. The t-statistic in the pair-wise comparisons was evaluated in terms of significance among different conditions. The main null hypotheses tested were: a) considering clams bioaccumulation, for each area, no significant differences existed among different seasons; b) considering clams bioaccumulation, for each season, no significant differences existed among different areas.

The data matrix including biomarkers and environmental data per site was normalised and the Euclidean distance calculated among centroids (i.e. the mean position of all the points representing a given sampling site for each one of the 4 seasons). Afterwards, the Euclidean similarity matrix was analysed using the PERMANOVA + add-on in PRIMER-E v.6 (Anderson et al., 2008) following unrestricted permutation of the raw data (9999 permutations) and the calculation of type III sums of squares. The main null hypotheses were: 1) considering clams biochemical responses, for each area, no significant differences existed among different seasons; 2) considering clams biochemical responses, for each season, no significant differences existed among different areas.

Afterwards, the matrix containing biomarkers and metal(oids) concentrations per sampling area and season was used to perform another Principal Coordinates Ordination (PCO) analysis. In the PCO graph, the variables presenting a correlation higher than 75 % with samples ordination were represented as superimposed vectors.

3. RESULTS

3.1 ENVIRONMENTAL PARAMETERS

In the present study, the obtained results showed that salinity and water temperature were higher in the Summer compared to the coldest seasons, Winter and Autumn, which presented the lowest values respectively. In warmer seasons, area T presented the highest temperature values, while areas S and CO were the coldest ones. Nonetheless, the lowest water temperature value of this study was recorded during Autumn in area M. Regarding salinity, the highest value was obtained in area T, during summer sampling, but CO presented the highest values in the remaining seasons. Area S showed the lowest values of salinity. Additionally, the highest values of pH and DO were also registered in Summer with areas M and T. On the other hand, area S, on average, presented the lowest pH values and area CO the lowest DO. Eh values were higher during Autumn in area M, while the lowest values were in Summer in area CO. Nevertheless, both highest and lowest single values were obtained in Autumn in areas T and CO, respectively (Table 1 mean values per season, Table 1 Supplementary material for full data).

Concerning sediment data, Summer displayed higher median grain-size and percentage of fines. On the other hand, sediment mean grain-size was lower in Winter, whereas the lowest fines content was found in Spring. Comparing sampling areas, higher grain-size values and fines percentage were found in area CO and the lowest values observed in area SJ. Nonetheless, the majority of the sediments were classified as fine to very fine sand, as the areas presented Φ values between 2 and 4, with general proportions of fines greater than 10 %. Although values were similar most seasons, the percentage of organic matter content (TOM) was higher during Winter and lower during Spring. Area S presented the highest TOM, while area SJ showed the lowest content of organic matter (Table 1, Table 1 Supplementary material).

3.2. ELEMENTS CONCENTRATION

Comparing seasons, Summer presented the highest elements concentrations in the water column for As, Ni and Cr compared to the 3 other seasons that for the majority of the elements presented concentrations lower than the LOQ (Table 2 mean values per season, Table 2 Supplementary material for full data). In terms of sampling areas, area CO was, in general, the area that presenting the highest levels of elements' concentration regardless the season. Nonetheless,

area M and area SJ presented the highest elements concentration in Autumn and Winter, respectively (Table 2 Supplementary material).

Considering elements concentrations in sediments, Summer and Autumn were the seasons that for all areas presented higher values, with Cr being the element with higher concentration for the majority of the sampling areas (mean values equal to 26.2 µg/ g dw and 27.9 µg/ g dw for Summer and Autumn, respectively) (Table 2). Comparing areas, sediments with higher elements concentrations were found mainly in area CO in Summer and Autumn, while higher elements concentrations were observed in area S in spring and area SJ in winter (Table 2 Supplementary material).

The highest element concentration for clam's tissues were recorded during Winter season. Elements concentration in clams showed higher As concentration compared to the remaining elements (Winter mean: 72.3 µg/ g dw) (Table 2). Among areas, the highest concentrations were observed in area I (148 µg/ g dw), where As was the element with the highest concentration (Table 2 Supplementary material).

Winter was the season that recorded higher BAF values namely for As (13.5), which was the element more bioaccumulated in comparison with the remaining metal(loids) (Table 2). Area I presented the highest BAF levels for most of the elements (Table 2 Supplementary material).

The Principal Coordinates Ordination (PCO) regarding BAF values demonstrated that the axis 1 explained approximately 49 % of the total variation, separating most of the area M samplings (except Winter sampling) and the Summer sampling of area I, on the positive side of the axis, from areas T, S and SJ regardless the season, on the negative side. Nonetheless, no abiotic factor showed a strong correlation with this axis. On the other hand, the axis 2, that described 21 % of total variation, divided areas M and T, on the positive side of the axis, from area I except summer sapling, on the negative side. Salinity presented a positive correlation with this axis (Figure 2).

3.3. DIETARY RISK ASSESSMENT

The concentrations of most of the elements quantified in clam's tissues were below the EFSA (European Food Safety Authority), USFDA (U.S. Food and Drug Administration) and FSANZ (Food Standards Australia New Zealand) maximum levels (Table 3 Supplementary material), except for As,

which exceeded safety limits. Overall, for the sampling sites under this study, As was the element of most concern in terms of human health. The obtained data showed that As values ranged between 0.033 kg and 0.351 kg to exceed PTWI (1.05 mg 70 kg^{-1} $week^{-1}$) (Table 3). When comparing sampling areas, results showed higher human health risks for areas S and I, regardless the season (Table 3).

3.4. BIOCHEMICAL PARAMETERS

When analysing each sampling period (season) independently some patterns are highlighted: i) summer was the season that presented the highest values for LPO and GSH, while presenting the lowest values for ETS, GSTs and SOD. Autumn registered the highest values for SOD and CAT with the lowest values for AChE. Spring showed the highest values of ETS, GLY, GSTs and GSSG, while presenting the lowest values for PROT and GSH/GSSG. Winter was the season with higher levels of PROT, GPx, and AChE, while the lowest for GLY and CAT; ii) regardless the season, it was possible to identify a particular area for each set of biomarkers with the highest values for LPO, ETS and GLY in area T; PROT, GSTs and SOD in area S; area M for GPx and CAT; area I for AChE, GSH, GSSG and GSH/GSSG (Table 3).

The PCO axis 1 explained 40.1% of the total variation of data separating Winter (in the negative side) from the remaining seasons (in the positive side). Axis 2 described 24.9% of the total variation, separating Summer (in the negative side) from Spring (in the positive side). The results obtained clearly demonstrated that sampling areas grouped together according to season, with different areas from the same season clustering together. GPx, AChE and PROT presented a strong correlation ($r > 0.75$) with PCO axis 1 negative side, with higher values associated to clams collected in Winter, regardless of the sampling area. On the other hand, CAT, GSSG and Ni content presented high correlation ($r > 0.75$) with PCO axis 1 positive side, with higher values associated with clams collected in Autumn. ETS, GSTs and GLY showed a strong correlation ($r > 0.75$) with the positive side of PCO axis 2, with higher values associated to clams collected during Spring in all the areas. LPO and GSH content presented a strong negative correlation with PCO axis 2, with higher values associated with clams from all areas collected during Summer (Figure 3).

4. DISCUSSION

4.1. ELEMENTS DETERMINATION

In the present study the results obtained showed higher water contamination during Summer, with area CO being the one with the highest values. The seasonal effect upon element concentration in water column was highlighted in this study suggesting higher metal(loids) water levels led to an additional concern about the elements' bioavailability, particularly during Summer. Generally, higher metal(loids) concentrations in water are related to environmental parameters such as temperature and DO as a result of greater dissolution of metals (Waldichuk, 1985).

Overall, the results obtained showed that the sampling areas represent low polluted to uncontaminated areas, with the concentration of elements in the sediments similar to values found in unpolluted areas (Chiesa et al., 2018; Velez et al., 2015; Freitas et al., 2012). In the sediments, higher concentrations of elements were obtained during Summer and Autumn and the most polluted area for these sampling periods was CO. Sediment becomes an important sink for metals that originally contaminate the water. Changes in the physicochemical parameters of water alter the bioavailability of the metals (Simpson and Batley 2003). The complex processes which influence the metal concentrations in the sediment are mainly pH, temperature, salinity, dissolved oxygen and organic matter content (Simpson et al. 2003), resulting in complex chemical reactivity and interactions between the solid and the solution phases of the metals (Guieu and Martin 2002, Peng et al. 2009). As example, Gati et al. (2016) assessed the sediment contamination with metals in the Danube Delta and how environmental shifts during one year can impact elements contamination. The authors showed that the deposition process was more intense at higher pH and temperature conditions.

Despite sediments showed to be an important source of metals, Bat et al. (2013) showed that sediment can reduce metals toxicity to mussels. Concerning clams' elements concentrations, the results obtained showed that Winter was the season with higher metal(loids) concentration where the most polluted area was I. The element of main concern was As. Velez et al. (2015) also assessed metals and As contamination on native and invasive clams from several areas of Ria de Aveiro (Portugal). The authors recognized this ecosystem as a low contaminated despite the concern associated to high concentrations of As. Despite higher metal(loids) concentrations in the water column during hotter seasons, clam's tissues showed higher accumulation during colder periods. This might be related with the low pH verified in the most affected sites (S and I). pH affects both solubility

of metal hydroxide minerals and adsorption-desorption processes. The solubility of metal hydroxide minerals increases along with the acidification leading to more dissolved metals that might become available for incorporation in biological processes. Ionic metal species are also commonly the most toxic forms to aquatic organisms (Salomons, 1995) which may explain different biochemical responses for different seasons. Riba et al. (2003) studied the effect of both pH and salinity upon water and sediment interactions by assessing biological effects on *R. philippinarum* organisms. The authors concluded that at low values of both variables (pH=6.5 and S=10), the biological effects were the highest, and it was related with free ion occurrence. Thus, this hypothesis might help to establish a pattern that varies not only with the temperature but also with other physico-chemical parameters.

The concentrations of As, Cd and Hg presented higher BAF values, with higher concentrations in the organisms (BAF>1) than in sediments, while for the remaining elements the concentrations were higher in the sediments than in the organisms (BAF<1). The toxicity of an element is not only dependent on the total amount accumulated but on its' partition as well. Elements in solution interfere with macromolecules with metabolically important functions, such as enzymes, transporters or DNA and therefore are more toxic than insoluble elements (Valko et al., 2005; Pytharopoulou et al., 2008; Zhang et al.,2010). Elements such as Cd and As, that are accumulated in higher proportions in the soluble fraction, are potentially more toxic than the others. These results are in agreement with the study conducted by Freitas et al. (2012) that performed an environmental study for *R. philippinarum* in the same ecosystem.

4.2. DIETARY RISK ASSESSMENT

Clams are one of the main shellfish resources in the world, with 3.5 million tonnes produced in 2010 (FAO, 2011). The results presented in this work show that the risk of dietary exposure to inorganic elements from clam's consumption occurs predominantly in Spring time. However, for all the seasons, data showed a distinct hazard associated to As, especially during Spring at I area. In this area, data showed that an adult (70 kg) is in health risk danger by consuming 0.033 kg of clams, in one week. When comparing element concentrations of clams with previous studies also from an area in Ria de Aveiro with similar element concentrations (Figueira and Freitas et al., 2013), it is possible to observe the same pattern for the same elements. However, the metal(loids) concentrations increased when comparing the obtained Spring results with this study conducted by Figueira and Freitas et al.

(2013) (carried out in March) which may suggest a decrease of the ecosystem quality in the past years. When comparing with other systems worldwide, consumers of clams from this coastal system have a similar or lower risk of exceeding the PTWI for Cd, As, Pb, and Hg (Hamza-Chaffai et al., 2000, Kucuksezgin et al., 2010, l et al., 2012). In agreement with the study conducted by Figueira and Freitas (2013) the results obtained also evidence that, even at low-contamination areas, the maximum levels for some elements can easily be achieved, prohibiting marketing and preventing clams culture for commercial purposes in many areas. However, it is well known that other ecosystems (more polluted) have higher hazard standards such as China, in which the Environmental Quality Standards (EQSs) for Cd, Cu, and Zn are 0.35, 3.02, 51.4 μg/g dw in clams, respectively (Lu et al., 2019). Liu et al. (2017) conducted an environmental assessment along Laizhou Bay, China where Cd (53.19 mg/kg DW) and Hg (9.18 mg/kg DW) were the metals with higher tissues' concentrations. In comparison with the present study it is important to highlight that all the results are within the health risk levels and clams from Ria de Aveiro constitute a low source of metal(loids) through diet. Moreover, the hazard character of each element is well marked regardless the contamination level of the ecosystem, once that Liu et al. (2017) also concluded that As was the element of higher human health concern by hazard quotient assessment.

It is of paramount interest to carry out more environmental assessment and quality monitoring to understand how the potentially hazardous elements will impact human health. Despite the fact that this general concern is already well established, the dietary risk assessment is yet neglected favouring bioaccumulation, water and sediment concentration evaluations.

4.3. BIOCHEMICAL PARAMETERS

The present study reveals biochemical responses of *R. philippinarum* when subjected to environmental stressors, analysing the relationship between pollutants levels in clams' tissues and biochemical responses. When exposed to stress factors, organisms may be subjected to oxidative damage resulting from increased concentration of Reactive Oxygen Species (ROS). Lipid peroxidation (LPO) and protein oxidation are well known effects of cellular oxidative damage, provoked by peroxidation of membrane lipids and proteins, respectively. In order to eliminate ROS and prevent cellular damages, bivalves may be capable of activating their antioxidant defences, namely by increasing their antioxidant enzymes.

In the present study the abiotic factors had an important role once the cellular damage increased along temperature, salinity and DO. Moreover, during colder seasons organisms were able to activate defence mechanisms in order to successfully prevent oxidative stress. Also higher detoxification rates seemed to respond to lower pH and Eh. In view to this, higher metabolic rates were verified which may explain the increase of the element's bioaccumulation. The importance and impact not only of environmental factors but also pollutants upon cellular fitness is already described in literature (Andrade et al., 2019; Coppola 2017, 2018; De Marchi et al.,2017; Velez et al., 2016a,b,c; Carregosa et al., 2014; Verlecar et al., 2007; Heise et al., 2003).

The results obtained further demonstrated that clams biochemical performance was closely related with seasons and not with areas elements concentrations, highlighting that pollution levels in all the studied areas were low and seasons induced higher impacts than pollutants on clams' biochemical responses. The present findings are in accordance with to a previously study by Guo et al. (2017), who detected the impact of contaminants in Qingdao coastal area of China in scallop *Chlamys farreri* during the year. In particular, the results obtained showed that during Summer clams presented higher LPO levels, which may indicate cellular damage, while it was demonstrated that higher neurotoxicity was observed during Winter. Higher LPO levels and GSH/GSSG ratio during Summer may result from ROS overproduction due to thermal stress but also due to increased mitochondrial respiratory activity as well observed in the Spring period. To prevent the accumulation of these molecules organisms produce and/or activate antioxidant enzymes. However, during summer, these defence mechanisms did not show well marked responses to detoxify ROS caused by thermal stress, leading to the occurrence of cellular damage (Solan and Whiteley, 2016). Also, Velez et al. (2017) verified higher LPO levels when exposed *R. decussatus* and *R. philippinarum* to warming conditions (21 ºC) for 28 days. Furthermore, during Autumn the highest SOD and CAT activities were observed. These results may indicate that temperatures from 17 to 19 ºC influence the antioxidant enzymes activations to prevent the cellular damage. Moreover, during Winter (lower temperatures), the GPx, GRed as well SOD and CAT activities showed to be able to prevent the membranes integrity, reduce the GSH/GSSG ratio and AChE showed a well-marked increase. Also De Marchi et al. (2017, 2018) studied the impact of pH and salinity combined with multi-walled carbon nanotubes and did not observe significant differences for AChE activities when exposed *R. philippinarum* organisms to low pH and salinity alone. Thus, the present study highlights the importance of

temperature and elements interactions. The water temperature rise is well documented in literature as an important environmental stressor alone or combined with other pollutants upon bivalves (Andrade et al., 2019; Coppola 2017, 2018; Verlecar et al., 2007; Heise et al.,2003), causing several effects assessed through different biomarkers, namely upon clams (Dubousquet et al.,2016; Anacleto et al., 2014; Abele et al., 2002). However, it is of paramount interest to assess and control environmental conditions in order to understand how several pollutants mixed in the water column interact among each other and with the inhabitant organisms. This purpose will allow adequate laws establishment and ensure the consumer's safety in terms of health risk.

5. CONCLUSIONS

It is of major importance to highlight that element partitioning is nowadays neglected by shellfish marketing. The impact that this has on the elements' bioavailability is very important to the consumers once that depends on the digestive capacity of each person (Rainbow and Smith, 2010; Metian et al., 2009). Bivalve species that have higher proportion of elements in solution generally constitutes higher risk to consumers than species that accumulate insoluble forms of metal(loids). The present study further highlights the importance of identifying the potential interfering factors and their impacts on the biomarker signals observed in wild populations. Biomarkers can thus, be significantly affected not only by anthropogenic or natural stressors but also by the combined action of both. Moreover, the optimal season for carrying out biomarker field studies or regular monitoring is of utmost relevance and should be investigated prior to including biomarkers in monitoring programs.

In conclusion, the present study emphasizes that benthic communities may provide more reliable information relatively to environmental fluctuations. Biomarkers can be used as complementary tools, however special attention is needed to choose appropriate bioindicator species, season as well as suitable battery of markers depending on nature of possible contaminants. Thus, this may lead to an increased ability to discriminate natural effects from others making biomarkers reliable in risk assessment studies.

6. ACKNOWLEDGMENTS

Rosa Freitas was funded by national funds (OE), though Fundação para a Ciência e a Tecnologia (FCT), in the scope of the framework contract foreseen in the numbers 4, 5 and 6 of the

article 23 of the Decree-Law 57/2016 of August 29, changed by Law 57/2017 of July 19. This work was also financially supported by the project ASARISAFE – Safety and sustainable management of valuable clam product in Portugal and China funded by the National Funds through the Portuguese Science Foundation (FCT). Silvana Costa and Carla Leite benefited from a research fellowship funded by the Portuguese Science Foundation (FCT) through the research project ASARISAFE (NSFC/0001/2016). Francesca Coppola benefited from a PhD grants (SFRH/BD/118582/2016) given by FCT, supported by FSE and Programa Operacional Capital Humano (POCH) e da União Europeia. Simão Correia benefited from a research fellowship funded by INTERREG-ALTANTIC program through the research project COCKLES (EAPA_458/2016 COCKLES Co-Operation for Restoring CocKle SheLlfisheries & its Ecosystem-Services in the Atlantic Area). Thanks are due to FCT/MCTES for the financial support to CESAM (UIDB/50017/2020+UIDP/50017/2020), through national funds. Authors would also like to thank Elena Lynch for English editing.

7. REFERENCES

Abele, D., Heise, K., Pörtner, H. O., & Puntarulo, S. (2002). Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. Journal of Experimental Biology, 205(13), 1831-1841.

Adams, A., Reeve, L., & Adams, A. (1850). *Mollusca*, Part III. The zoology of the voyage of HMS Samarang, 1843-1846.

Andrade, M., De Marchi, L., Soares, A. M., Rocha, R. J., Figueira, E., & Freitas, R. (2019). Are the effects induced by increased temperature enhanced in *Mytilus galloprovincialis* submitted to air exposure? Science of the total environment, 647, 431-440.

Allam, B., Paillard, C., Howard, A., & Le Pennec, M. (2000). Isolation of the pathogen Vibrio tapetis and defense parameters in brown ring diseased Manila clams *Ruditapes philippinarum* cultivated in England. Diseases of Aquatic Organisms, 41(2), 105-113.

Almeida, J. C., Cardoso, C. E., Tavares, D. S., Freitas, R., Trindade, T., Vale, C., & Pereira, E. (2019). Chromium removal from contaminated waters using nanomaterials–a review. Trends in Analytical Chemistry, 118, 277-291.

539 Anacleto, P., Maulvault, A. L., Lopes, V. M., Repolho, T., Diniz, M., Nunes, M. L., Marques, A., Rosa,
540 R. (2014). Ecophysiology of native and alien-invasive clams in an ocean warming context.
541 Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 175, 28-
542 37.
543 Anderson, M., Gorley, R. N., Clarke, R. K. (2008). Permanova+ for Primer: Guide to Software and
544 Statistical Methods. Primer-E Limited.
545 Banni, M., Bouraoui, Z., Ghedira, J., Clearandeau, C., Jebali, J., Boussetta, H. (2009). Seasonal
546 variation of oxidative stress biomarkers in clams *Ruditapes decussatussa*-pled from Tunisian
547 coastal areas. Environmental Monitoring Assessment 155, 119–128.
548 Bat, L., Üstün, F., Baki, O. G., Şahin, F. (2013). Effects of some heavy metals on the sizes of the
549 Mediterranean mussel (*Mytilus galloprovincialis* Lamarck, 1819). Fresenius Environmental
550 Bulletin, 22, 1933-1938.
551 Beauchamp, C., Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay
552 applicable to acrylamide gels. Analytical Biochemistry, 44, 276-287
553 Bebianno, M. J., Geret, F., Hoarau, P., Serafim, M. A., Coelho, M. R., Gnassia-Barelli, M., & Romeo,
554 M. (2004). Biomarkers in *Ruditapes decussatus*: a potential bioindicator species. Biomarkers,
555 9(4-5), 305-330.
556 Byers, S. C., Mills, E. L., & Stewart, P. L. (1978). A comparison of methods of determining organic
557 carbon in marine sediments, with suggestions for a standard method. Hydrobiologia, 58(1), 43-
558 47.
559 Carregosa, V., Velez, C., Soares, A. M., Figueira, E., & Freitas, R. (2014). Physiological and
560 biochemical responses of three Veneridae clams exposed to salinity changes. Comparative
561 Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 177, 1-9.
562 Chiesa, L. M., Ceriani, F., Caligara, M., Di Candia, D., Malandra, R., Panseri, S., & Arioli, F. (2018).
563 Mussels and clams from the italian fish market. is there a human exposition risk to metals and
564 arsenic? Chemosphere, 194, 644-649.
565 Colin, N., Porte, C., Fernandes, D., Barata, C., Padrós, F., Carrassón, M., Monroy, M., Rocabayera,
566 O. C., De Sostoa, A., Piña, B., & Maceda-Veiga, A. (2016). Ecological relevance of biomarkers
567 in monitoring studies of macro-invertebrates and fish in Mediterranean rivers. Science of the
568 Total Environment, 540, 307-323.

569 Coppola, F., Almeida, Â., Henriques, B., Soares, A. M., Figueira, E., Pereira, E., & Freitas, R. (2018).
570 Biochemical responses and accumulation patterns of *Mytilus galloprovincialis* exposed to
571 thermal stress and Arsenic contamination. Ecotoxicology and environmental safety, 147, 954-
572 962.
573 Coppola, F., Almeida, Â., Henriques, B., Soares, A. M., Figueira, E., Pereira, E., & Freitas, R. (2017).
574 Biochemical impacts of Hg in *Mytilus galloprovincialis* under present and predicted warming
575 scenarios. Science of the Total Environment, 601, 1129-1138.
576 Costa, P. M., Carreira, S., Costa, M. H., & Caeiro, S. (2013). Development of histopathological indices
577 in a commercial marine bivalve (*Ruditapes decussatus*) to determine environmental quality.
578 Aquatic Toxicology, 126, 442-454.
579 Costley, C. T., Mossop, K. F., Dean, J. R., Garden, L. M., Marshall, J., & Carroll, J. (2000).
580 Determination of mercury in environmental and biological samples using pyrolysis atomic
581 absorption spectrometry with gold amalgamation. Analytica Chimica Acta, 405(1-2), 179-183.
582 Dang, C., de Montaudouin, X., Gam, M., Paroissin, C., Bru, N., & Caill-Milly, N. (2010). The Manila
583 clam population in Arcachon Bay (SW France): can it be kept sustainable? Journal of Sea
584 Research, 63(2), 108-118.
585 De Coen, W.M., Janssen, C.R. (1997). The use of biomarkers in Daphnia magna toxicity testing. IV.
586 Cellular energy allocation: a new methodology to assess the energy budget of toxicant-stressed
587 Daphnia populations. Journal of Aquatic Ecosystem Stress and Recovery, 6, 43–55.
588 Delgado, M., & Pérez-Camacho, A. (2007). Comparative study of gonadal development of *Ruditapes*
589 *philippinarum* (Adams and Reeve) and *Ruditapes decussatus* (L.) (Mollusca: Bivalvia):
590 Influence of temperature. Scientia Marina, 71(3), 471-484.
591 Doeglas, D.J. (1968). Grain size indices, classification and environment. Sedimentology, 10, 8-82.
592 Elliott, J. M. (1977). Some methods for the statistical analysis of samples of benthic invertebrates.
593 Scientific Publications Freshwater Biological Association, 25, 155.
594 Ellman, G. L., Courtney, K. D., Andres Jr, V., & Featherstone, R. M. (1961). A new and rapid
595 colorimetric determination of acetylcholinesterase activity. Biochemical pharmacology, 7(2), 88-
596 95.
597 FAO (2011) Review of the state of world marine fishery resources. FAO Fisheries and Aquaculture
598 Technical Paper No. 569 Rome.

599 Figueira, E., & Freitas, R. (2013). Consumption of *Ruditapes philippinarum* and *Ruditapes*
600 *decussatus*: comparison of element accumulation and health risk. Environ. Sci. Pollut. R.,
601 20(8), 5682-5691.
602 Figueira, E., Cardoso, P., & Freitas, R. (2012). *Ruditapes decussatus* and *Ruditapes philippinarum*
603 exposed to cadmium: toxicological effects and bioaccumulation patterns. Comparative
604 Biochemistry and Physiology Part C: Toxicology & Pharmacology, 156(2), 80-86.
605 Flassch, J. P., & Leborgne, Y. (1992). Introduction in Europe, from 1972 to 1980, of the Japanese
606 Manila clam (*Tapes philippinarum*) and the effects on aquaculture production and natural
607 settlement. In ICES marine science symposia. Copenhagen [ICES MAR. SCI. SYMP.]. 1992.
608 Freitas, R., Pires, A., Velez, C., Almeida, Â., Wrona, F. J., Soares, A. M., & Figueira, E. (2015). The
609 effects of salinity changes on the Polychaete *Diopatra neapolitana*: impacts on regenerative
610 capacity and biochemical markers. Aquatic Toxicology, 163, 167-176.
611 Freitas, R., Pinto, L. R., Sampaio, M., Costa, A., Silva, M., Rodrigues, A. M., Quintino, V., & Figueira,
612 E. (2012). Effects of depuration on the element concentration in bivalves: Comparison between
613 sympatric *Ruditapes decussatus* and *Ruditapes philippinarum*. Estuarine Coastal and Shelf
614 Science, 110, 43-53.
615 Gati, G., Pop, C., Brudaşcă, F., Gurzău, A. E., & Spînu, M. (2016). The ecological risk of heavy
616 metals in sediment from the Danube Delta. Ecotoxicology, 25(4), 688-696.
617 Gonçalves, A. M. M., Barroso, D. V., Serafim, T. L., Verdelhos, T., Marques, J. C., & Gonçalves, F.
618 (2017). The biochemical response of two commercial bivalve species to exposure to strong
619 salinity changes illustrated by selected biomarkers. Ecological Indicators, 77, 59-66.
620 Govender, N., Smit, A. J., & Perissinotto, R. (2011). Trophic functioning of the St. Lucia estuarine lake
621 during a drought phase assessed using stable isotopes. Estuarine, Coastal and Shelf Science,
622 93(2), 87-97.
623 Guieu, C., & Martin, J. M. (2002). The level and fate of metals in the Danube River plume. Estuarine,
624 Coastal and Shelf Science, 54(3), 501-512.
625 Guo, R., Pan, L., Ji, R., (2017). A multi-biomarker approach in scallop *Chlamys farreri* to assess the
626 impact of contaminants in Qingdao coastal area of China. Ecotoxicology and Environmental
627 Safety. 142, 399–409.

628 Habig, W.H., Pabst, M.J., Jakoby, W.B. (1974). Glutathione S-transferases. The first enzymatic step
629 in mercapturic acid formation. Journal of Biological Chemistry, 249, 7130-7139.
630 Hamza-Chaffai, A., Amiard, J. C., Pellerin, J., Joux, L., & Berthet, B. (2000). The potential use of
631 metallothionein in the clam *Ruditapes decussatus* as a biomarker of in situ metal exposure.
632 Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and
633 Endocrinology, 127(2), 185-197.
634 Harley, C. D., Randall Hughes, A., Hultgren, K. M., Miner, B. G., Sorte, C. J., Thornber, C. S.,
635 Rodriguez, L. F., Tomanek, L., & Williams, S. L. (2006). The impacts of climate change in
636 coastal marine systems. Ecology letters, 9(2), 228-241.
637 Heise, K., Puntarulo, S., Pörtner, H. O., & Abele, D. (2003). Production of reactive oxygen species by
638 isolated mitochondria of the Antarctic bivalve Laternulaelliptica (King and Broderip) under heat
639 stress. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 134(1),
640 79-90.
641 Hook, S.E., Gallagher, E.P., Batley, G.E. (2014). The role of biomarkers in the assessment of aquatic
642 ecosystem health. Integrated Environmental Assessment and Management, 10, 327–341.
643 Jensen, A. C., Humphreys, J., Caldow, R. W. G., Grisley, C., & Dyrynda, P. E. J. (2004).
644 Naturalization of the Manila clam (*Tapes philippinarum*), an alien species, and establishment of
645 a clam fishery within Poole Harbour, Dorset. Journal of the Marine Biological Association of the
646 United Kingdom, 84(5), 1069-1073.
647 Johansson LH, Borg, L.A.H. (1988) A spectrophotometric method for determination of Catalase
648 activity in small tissue samples. Analytical Biochemistry, 174, 331-336.
649 Keller, M., Sommer, A. M., Pörtner, H. O., & Abele, D. (2004). Seasonality of energetic functioning
650 and production of reactive oxygen species by lugworm (*Arenicola marina*) mitochondria
651 exposed to acute temperature changes. Journal of experimental biology, 207(14), 2529-2538.
652 King, F. D., & Packard, T. T. (1975). Respiration and the activity of the respiratory electron transport
653 system in marine zooplankton 1. Limnology and Oceanography, 20(5), 849-854.
654 Kucuksezgin, F., Kacar, A., Kucuksezgin, G., & Uluturhan, E. (2010). Monitoring metal contamination
655 levels and fecal pollution in clam (*Tapes decussatus*) collected from Izmir Bay (Turkey).
656 Environmental monitoring and assessment, 162(1-4), 407-415.

Langston, W. J., Pope, N. D., Jonas, P. J. C., Nikitic, C., Field, M. D. R., Dowell, B., Shillabeer, N., Swarbrick, R.H. & Brown, A. R. (2010). Contaminants in fine sediments and their consequences for biota of the Severn Estuary. Marine Pollution Bulletin, 61(1-3), 68-82.

Lillebø, A. I., Ameixa, O. M. C. C., Sousa, L. P., Sousa, A. I., Soares, J. A., Dolbeth, M., & Alves, F. L. (2015). The physio-geographical background and ecology of Ria de Aveiro. coastal lagoons in europe, 21.

Liu, J., Cao, L., Dou, S. (2017). Bioaccumulation of heavy metals and health risk assessment in three benthic bivalves along the coast of Laizhou Bay, China. Marine pollution bulletin, 117(1-2), 98-110.

Lu, G., Zhu, A., Fang, H., Dong, Y., Wang, W. X. (2019). Establishing baseline trace metals in marine bivalves in China and worldwide: Meta-analysis and modeling approach. Science of the Total Environment, 669, 746-753.

Magalhães, L., de Montaudouin, X., Figueira, E., & Freitas, R. (2018). Trematode infection modulates cockles biochemical response to climate change. Science of the Total Environment, 637, 30-40.

De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini, F., Morelli, A., Soares, A.M.V.M., Freitas, R. (2018). Effects of multi-walled carbon nanotube materials on *Ruditapes philippinarum* under climate change: the case of salinity shifts. Aquatic Toxicology, 199, 199-211.

De Marchi, L., Neto, V., Pretti, C., Figueira, E., Chiellini, F., Morelli, A., Soares, A.M.V.M., Freitas, R. (2017). The impacts of seawater acidification on *Ruditapes philippinarum* sensitivity to carbon nanoparticles. Environmental Science: Nano, 4(8), 1692-1704.

Dias, J.M., Lopes, J.F., & Dekeyser, I. (2000). Tidal propagation in Ria de Aveiro lagoon, Portugal. Physics and Chemistry of the Earth Part B-Hydrology Oceans and Atmosphere, 25, 369-374.

Dubousquet, V., Gros, E., Berteaux-Lecellier, V., Viguier, B., Raharivelomanana, P., Bertrand, C., & Lecellier, G. J. (2016). Changes in fatty acid composition in the giant clam Tridacna maxima in response to thermal stress. Biology open, 5(10), 1400-1407.

Martín-Díaz, M. L., Blasco, J., Sales, D., & DelValls, T. A. (2007). Biomarkers study for sediment quality assessment in Spanish ports using the crab *Carcinus maenas* and the clam *Ruditapes philippinarum*. Archives of Environmental Contamination and Toxicology, 53(1), 66-76.

686 Matozzo, V., Chinellato, A., Munari, M., Bressan, M., & Marin, M. G. (2013). Can the combination of
687 decreased pH and increased temperature values induce oxidative stress in the clam *Chamelea*
688 *gallina* and the mussel *Mytilus galloprovincialis*? Marine pollution bulletin, 72(1), 34-40.
689 McLusky, D. S. (1999). Estuarine benthic ecology: A European perspective. Australian journal of
690 ecology, 24(4), 302-311.
691 McGeer, J. C., Brix, K. V., Skeaff, J. M., DeForest, D., Brigham, S. I., Adams, W.J., & Green, A.
692 (2003). Inverse relationship between bioconcentration factor and exposure concentration for
693 metals: implications for hazard assessment of metals in the aquatic environment.
694 Environmental Toxicology and Chemistry, 22, 1017–1037.
695 Mennillo, E., Casu, V., Tardelli, F., De Marchi, L., Freitas, R., & Pretti, C. (2017). Suitability of
696 cholinesterase of polychaete *Diopatra neapolitana* as biomarker of exposure to pesticides: In
697 vitro characterization. Comparative Biochemistry and Physiology Part C: Toxicology &
698 Pharmacology, 191, 152-159.
699 Metian, M., Charbonnier, L., Oberhänsli, F., Bustamante, P., Jeffree, R., Amiard, J. C., & Warnau, M.
700 (2009). Assessment of metal, metalloid, and radionuclide bioaccessibility from mussels to
701 human consumers, using centrifugation and simulated digestion methods coupled with
702 radiotracer techniques. Ecotoxicology and Environmental Safety, 72(5), 1499-1502.
703 Moreira, A., Figueira, E., Soares, A.M.V.M., & Freitas, R. (2016). Salinity influences the biochemical
704 response of Crassostrea angulate to Arsenic. Environmental Pollution (Barking, Essex: 1987)
705 214, 756–766.
706 Moschino, V., Delaney, E., & Da Ros, L. (2012). Assessing the significance of *Ruditapes*
707 *philippinarum* as a sentinel for sediment pollution: bioaccumulation and biomarker responses.
708 Environmental pollution, 171, 52-60.
709 Munari, M., Matozzo, V., Gagné, F., Chemello, G., Riedl, V., Finos, L., Pastore, P., Badocco, D., &
710 Marin, M. G. (2018). Does exposure to reduced pH and diclofenac induce oxidative stress in
711 marine bivalves? A comparative study with the mussel *Mytilus galloprovincialis* and the clam
712 *Ruditapes philippinarum*. Environmental pollution, 240, 925-937.
713 Ohkawa, H., Ohishi, N., & Yagi, Y. (1979). Assay of lipid peroxides in animal tissue by thiobarbituric
714 acid reaction. Analytical Biochemistry, 95, 351e358.

715 Paglia, D. E., & Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of
716 erythrocyte glutathione peroxidase. The Journal of laboratory and clinical medicine, 70(1), 158-
717 169.
718 Peng, J. F., Song, Y. H., Yuan, P., Cui, X. Y., & Qiu, G. L. (2009). The remediation of heavy metals
719 contaminated sediment. Journal of hazardous materials, 161(2-3), 633-640.
720 Picado, A., Dias, J. M., & Fortunato, A. B. (2009). Effect of flooding the salt pans in the Ria de Aveiro.
721 Journal of Coastal Research, 1395-1399.
722 Picado, A., Bebianno, M. J., Costa, M. H., Ferreira, A., & Vale, C. (2007). Biomarkers: a strategic tool
723 in the assessment of environmental quality of coastal waters. Hydrobiologia, 587(1), 79.
724 Pranovi, F., Franceschini, G., Casale, M., Zucchetta, M., Torricelli, P., & Giovanardi, O. (2006). An
725 ecological imbalance induced by a non-native species: the Manila clam in the Venice Lagoon.
726 Biological Invasions, 8(4), 595-609.
727 Pytharopoulou, S., Sazaklib, E., Grintzalisc, K., Georgiouc, C., Leotsinidis, M., & Kalpaxisa, D. (2008).
728 Translational responses of *Mytilus galloprovincialis* to environmental pollution: integrating the
729 responses to oxidative stress and other biomarker responses into a general stress index.
730 Aquatic Toxicology 89:18–27
731 Quintino, V., Rodrigues, A.M., & Gentil, F. (1989). Assessment of macrozoobenthic communities in
732 the lagoon of Óbidos, western of Portugal. Scientia Marina, 53, 645-654.
733 Rahman, M.A., Hasegawa, H., & Lim., R.P. (2012). Bioaccumulation, biotransformation and trophic
734 transfer of arsenic in the aquatic food chain. Environmental Research, 116, 118-135.
735 Riba, E. García-Luque, Blasco, J., & DelValls, T.A. (2003). Bioavailability of heavy metals bound to
736 estuarine sediments as a function of pH and salinity values. Chemical Speciation &
737 Bioavailability, 15:4, 101-114.
738 Rainbow, P. S., & Smith, B. D. (2010). Trophic transfer of trace metals: subcellular
739 compartmentalisation in bivalve prey and comparative assimilation efficiencies of two
740 invertebrate predators. Journal of Experimental Marine Biology and Ecology, 390(2), 143-148.
741 Robinson, H.W., & Hogden, C.G. (1940). The biuret reaction in the determination of serum proteins.
742 The Journal of Biological Chemistry 135, 707-725.

743 Rodrigues, A. M., Quintino, V., Sampaio, L., Freitas, R., & Neves, R. (2011). Benthic biodiversity
744 patterns in Ria de Aveiro, Western Portugal: Environmental-biological relationships. Estuarine,
745 Coastal and Shelf Science, 95(2-3), 338-348.
746 Salomons, W., 1995, Environmental impact of metals derived from mining activities: Processes,
747 predictions, prevention: Journal of Geochemical Exploration, v. 52, p. 5-23.
748 Sheehan, D., & Power, A. (1999). Effects of seasonality on xenobiotic and antioxidant defence
749 mechanisms of bivalve molluscs. Comparative Biochemistry and Physiology Part C:
750 Pharmacology, Toxicology and Endocrinology, 123(3), 193-199.
751 Shin, P. K. S., Ng, A. W. M., & Cheung, R. Y. H. (2002). Burrowing responses of the short-neck clam
752 *Ruditapes philippinarum* to sediment contaminants. Marine pollution bulletin, 45(1-12), 133-
753 139.
754 Simpson, S. L., & Batley, G. E. (2003). Disturbances to metal partitioning during toxicity testing of iron
755 (II)-rich estuarine pore waters and whole sediments. Environmental Toxicology and Chemistry:
756 An International Journal, 22(2), 424-432.
757 Smedley, P. L., & Kinniburgh, D. G. (2002). A review of the source, behaviour and distribution of
758 arsenic in natural waters. Applied geochemistry, 17(5), 517-568.
759 Sulejman, S. (2012). Heavy metals in soil, sediments, mussels, and water from Butrinti Lagoon
760 (Albania).
761 Usero, J., Gonzalez-Regalado, E., & Gracia, I. (1997). Trace metals in the bivalve molluscs *Ruditapes*
762 *decussatus* and *Ruditapes philippinarum* from the Atlantic Coast of Southern Spain.
763 Environment International, 23(3), 291-298.
764 Waldichuk, M. (1985). Biological availability of metals to marine organisms. Marine Pollution Bulletin,
765 16(1), 7-11.
766 Wang, Z., Yan, C., Vulpe, C. D., Yan, Y., & Chi, Q. (2012). Incorporation of in situ exposure and
767 biomarkers response in clams *Ruditapes philippinarum* for assessment of metal pollution in
768 coastal areas from the Maluan Bay of China. Marine pollution bulletin, 64(1), 90-98.
769 WFD 2000/60/EC (2000) Directive 2000/60/EC of the European Parliament and of the council of 23
770 October 2000 establishing a framework for Community action in the field of water policy, pp. 72.
771 Valko M, Morris, H., & Cronin, M. (2005) Metals, toxicity and oxidative stress. Current Medicinal
772 Chemistry, 12:1161–1208.

773 Velez, C., Figueira, E., Soares, A. M., & Freitas, R. (2017). Effects of seawater temperature increase
774 on economically relevant native and introduced clam species. Marine environmental research,
775 123, 62-70.
776 Velez, C., Figueira, E., Soares, A. M., & Freitas, R. (2016a). Native and introduced clams biochemical
777 responses to salinity and pH changes. Science of the Total Environment, 566, 260-268.
778 Velez, C., Figueira, E., Soares, A. M., & Freitas, R. (2016b). The impacts of As accumulation under
779 different pH levels: Comparing *Ruditapes decussatus* and *Ruditapes philippinarum* biochemical
780 performance. Environmental research, 151, 653-662.
781 Velez, C., Figueira, E., Soares, A., & Freitas, R. (2015). Spatial distribution and bioaccumulation
782 patterns in three clam populations from a low contaminated ecosystem. Estuarine, Coastal and
783 Shelf Science, 155, 114-125.
784 Verlecar, X. N., Jena, K. B., & Chainy, G. B. N. (2007). Biochemical markers of oxidative stress in
785 *Perna viridis* exposed to mercury and temperature. Chemico-biological interactions, 167(3),
786 219-226.
787 Solan, M., & Whiteley, N. (2016). Stressors in the Marine Environment: Physiological and ecological
788 responses; societal implications. Oxford University Press.
789 Zhang, Y., Songa, Y., Yuana, H., Xua, Y., Hec, Z., & Duana, L. (2010). Biomarker responses in the
790 bivalve (*Chlamys farreri*) to exposure of the environmentally relevant concentrations of lead,
791 mercury, copper. Environmental Toxicology and Pharmacology, 30:19–25
792

Figure captions

Figure 1. Sampling areas: T - Torreira; M - Murtosa; SJ - São Jacinto; CO - Cale do Ouro; S - Sporting; I - Ílhavo.

Figure 2. Centroid ordination diagram (PCO) based on water and sediment physico-chemical parameters and values for the bioaccumulation factor (BAF), measured for all the sampling areas along four seasons. Pearson correlation vectors are superimposed as supplementary variables ($r > 0.7$).

Figure 3. Centroid ordination diagram (PCO) based on biochemical data and clams metal(loids) concentrations, measured for all the sampling areas along four seasons. Pearson correlation vectors are superimposed as supplementary variables ($r > 0.8$).

Table 1. Environmental characterization for each season (mean values ±standard deviation), in terms of water parameters (salinity, temperature (TEMP. / °C), pH, dissolved oxygen (DO / µg/L) and redox potential (Eh / mV)) and sediment parameters (median value (Φ), fines (%), total organic matter content (TOM / %)).

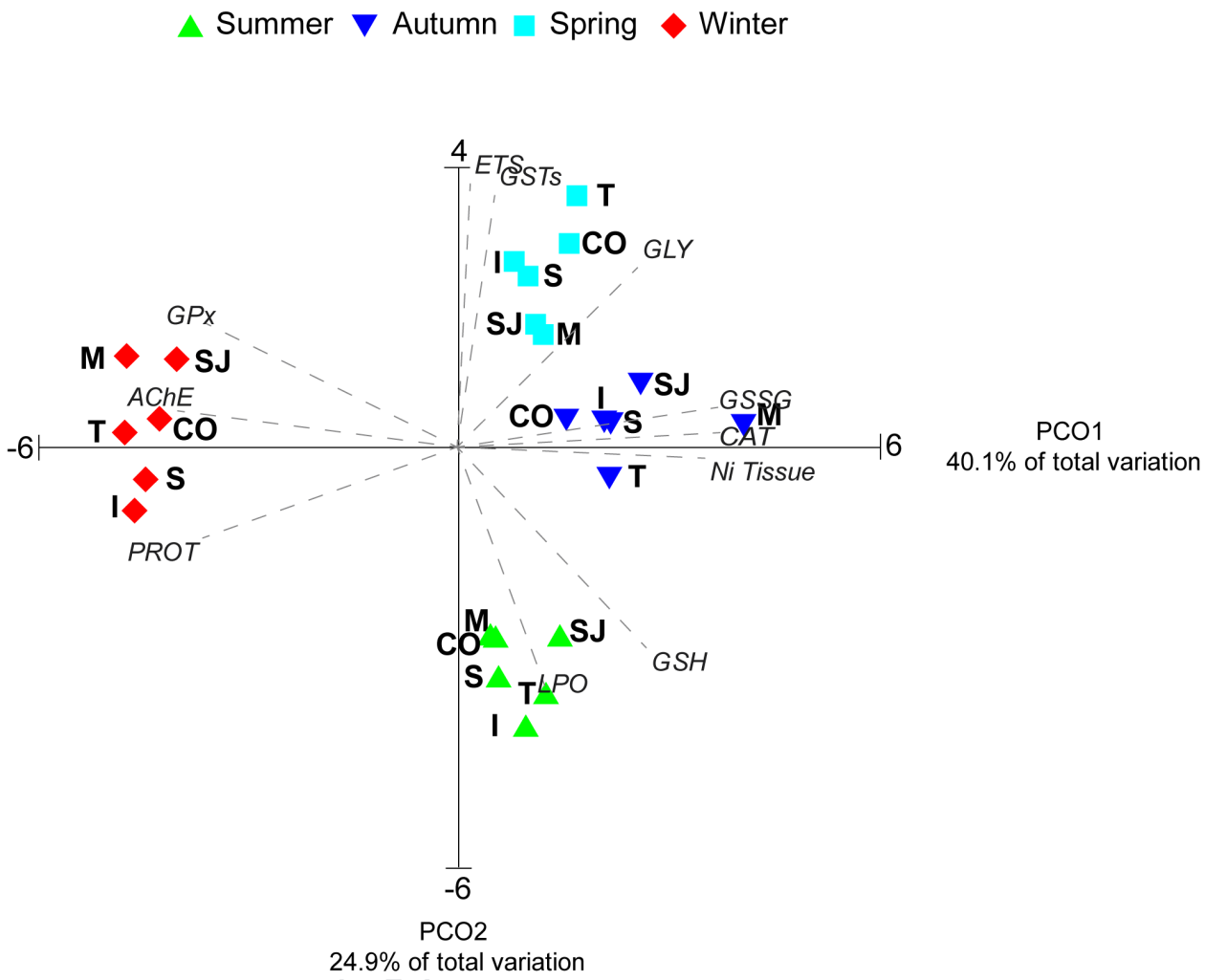
SEASON	TEMP	pH	SALINITY	DO	Eh	Φ	FINES	TOM
SUMMER	20.0 ± 1.8	8.1 ± 0.3	35.1 ± 0.8	9.3 ± 3.4	120 ± 27	2.7 ± 0.9	38.3 ± 29.8	4.54 ± 2.30
AUTUMN	10.1 ± 0.7	8.0 ± 0.1	34.4 ± 0.9	7.5 ± 0.3	187 ± 72	2.6 ± 0.7	23.1 ± 13.7	4.25 ± 1.76
SPRING	19.1 ± 1.4	7.8 ± 0.1	25.3 ± 4.7	7.7 ± 1.0	148 ± 53	2.4 ± 0.4	15.7 ± 9.3	4.15 ± 1.93
WINTER	11.5 ± 1.2	7.8 ± 0.2	23.9 ± 7.4	8.7 ± 0.2	141 ± 10	2.4 ± 0.3	27.0 ± 18.4	4.63 ± 2.80

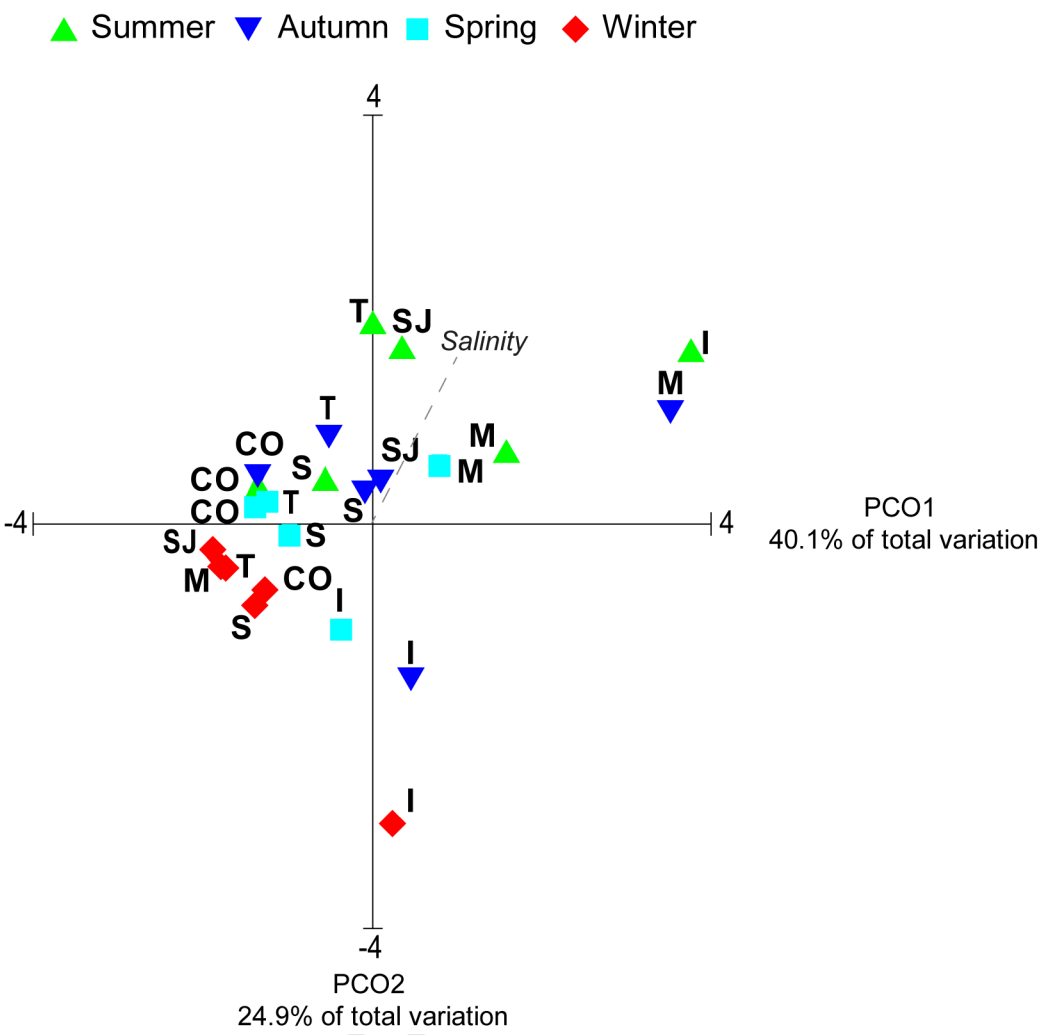
Table 2. Elements concentration (Cu, As, Cd, Pb, Hg, Ni and Cr) in Water (µg/L), sediments (µg/g, dry weight) and clams' tissue (µg/g, dry weight) and the bioaccumulation factor (BAF: ratio between element concentration in the tissue and in the sediment) for each season (mean value ± standard deviation (SD)). Quantification limit (QL) in water samples per element in µg/ L (Cu, 2; As, 2; Cd, 0.1; Pb, 0.2; Hg, 0.0025; Ni, 1; Cr, 0.5). Values below this limit represented as <QL.

Season	Cu				As				Cd				Pb				Hg				Ni				Cr			
	Wat.	Sed.	Tis.	BAF	Wat.	Sed.	Tis.	BAF	Wat.	Sed.	Tis.	BAF	Wat.	Sed.	Tis.	BAF	Wat.	Sed.	Tis.	BAF	Wat.	Sed.	Tis.	BAF	Wat.	Sed.	Tis.	BAF
SUMMER	40.7 ±9.7	11.7 ±6.6	6.3 ±0.9	0.9 ±0.7	37.8 ±6.2	11.9 ±4.8	50.0 ±30.9	6.2 ±7.2	<QL	0.3 ±0.1	0.6 ±0.1	3.3 ±2.0	<QL	25.2 ±10.7	3.0 ±1.5	0.16 ±0.11	18.2 ±18.8	0.12 ±0.11	0.2 ±0.1	3.5 ±2.2	25.7 ±2.5	11.3 ±6.5	6.38 ±1.3	1.1 ±1.1	14.0 ±4.0	26.2 ±15.2	2.6 ±0.3	0.2 ±0.1
AUTUMN	45.8 ±3.7	12.2 ±6.3	6.1 ±1.1	0.8 ±0.5	<QL	13.0 ±4.3	39.4 ±27.6	4.4 ±2.4	<QL	0.3 ±0.1	0.8 ±0.1	3.4 ±1.8	4.1 ±0.3	25.5 ±6.7	1.3 ±0.4	0.06 ±0.01	30.2 ±18.4	0.11 ±0.09	0.2 ±0.1	8.7 ±15.5	29.3 ±0.0	12.5 ±5.8	10.0 ±2.8	1.27 ±1.0	8.3 ±0.7	27.9 ±12.9	8.7 ±8.1	0.1 ±0.2
SPRING	46.0 ±0.0	7.7 ±4.2	6.3 ±1.0	2.2 ±3.1	<QL	6.8 ±3.1	53.6 ±54.0	10.6 ±10.6	<QL	0.3 ±0.1	0.4 ±0.1	2.1 ±1.9	3.8 ±0.3	24.1 ±8.8	1.2 ±0.6	0.06 ±0.04	9.9 ±6.5	0.10 ±0.07	0.2 ±0.1	6.9 ±11.1	<QL	7.80 ±4.2	6.49 ±1.3	1.45 ±1.1	<QL	19.9 ±9.2	3.4 ±1.1	0.1 ±0.1
WINTER	<QL	9.4 ±4.0	7.6 ±0.8	1.1 ±0.9	<QL	9.4 ±4.0	72.3 ±46.3	13.5 ±18.8	<QL		0.7 ±0.2		<QL		0.5 ±0.1		53.8 ±55.0	0.07 ±0.04	0.3 ±0.1	5.3 ±1.5	<QL	14.0 ±7.6	2.98 ±0.8	0.32 ±0.3	<QL	27.6 ±13.8	0.9 ±0.5	0.1 ±0.1

Table 3. Biochemical parameters results for each sampling site (T, Torreira; S, Sporting; SJ, São Jacinto; I, Ílhavo; M, Murtosa; CO, Cale do Ouro) and season (Summer, Autumn, Spring and Winter). The highest values per season are with **bold** and highest levels per year are marked with a *. Results expressed in the following values: LPO in nmol MDA/g FW; ETS in nmol/ min/g FW; PROT and GLY in mg/ g FW; SOD, CAT, GPx and GRed in U/g FW; GSH and GSSG in µmol/g FW; AChE in µmol/ min/ g FW (FW: fresh weight).

		BIOCHEMICAL PARAMETERS											
SITE		LPO	ETS	PROT	GLY	GPx	GSTs	SOD	CAT	GSH	GSSG	GSH/GSSG	AChE
SUMMER	T	**27.0***	9.15	**25.2**	2.14	4.59×10^{-3}	7.66×10^{-3}	7.86×10^{-2}	**19.9**	5.94×10^{-2}	5.56×10^{-2}	1.13	**5.53×10^{-1}**
	S	24.2	11.5	24.0	1.91	8.41×10^{-3}	4.54×10^{-3}	8.13×10^{-2}	14.6	6.06×10^{-2}	5.61×10^{-2}	1.10	4.63×10^{-1}
	SJ	15.2	7.84	24.5	**5.31**	1.85×10^{-2}	**8.02×10^{-3}**	8.70×10^{-2}	21.4	6.24×10^{-2}	6.10×10^{-2}	1.06	6.01×10^{-1}
	I	25.4	**12.5**	22.4	1.59	**1.86×10^{-2}**	7.42×10^{-3}	**9.84**$\times10^{-2}$	21.6	**1.03×10^{-1}***	5.38×10^{-2}	**2.25**	4.36×10^{-1}
	M	18.7	8.95	21.4	1.59	6.15×10^{-3}	7.62×10^{-3}	7.97×10^{-2}	17.5	6.94×10^{-2}	**6.74×10^{-2}**	1.05	5.37×10^{-1}
	CO	19.5	10.7	22.4	1.59	8.93×10^{-3}	5.01×10^{-3}	7.74×10^{-2}	9.57	7.10×10^{-2}	6.02×10^{-2}	1.20	4.87×10^{-1}
AUTUMN	T	**16.3**	55.0	19.2	8.14	1.02×10^{-2}	9.84×10^{-2}	1.44	15.8	4.07×10^{-2}	5.48×10^{-2}	**7.65×10^{-1}**	2.38×10^{-1}
	S	12,8	55.1	**25.1**	**11.3**	6.69×10^{-3}	**2.12×10^{-1}**	**3.69***	26.7	3.81×10^{-2}	5.40×10^{-2}	7.10×10^{-1}	**3.44×10^{-1}**
	SJ	9.14	49.1	15.1	9.14	9.29×10^{-3}	1.19×10^{-1}	1.84	22.6	3.62×10^{-2}	**6.60×10^{-2}**	5.63×10^{-1}	2.21×10^{-1}
	I	13.8	54.7	19.1	7.88	5.95×10^{-2}	1.52×10^{-1}	2.52	21.9	3.99×10^{-2}	5.38×10^{-2}	7.50×10^{-1}	3.38×10^{-1}
	M	14.5	**70.0**	15.0	6.02	**1.17×10^{-2}**	1.61×10^{-1}	3.29	**31.8***	**4.08×10^{-2}**	6.37×10^{-2}	7.01×10^{-1}	2.85×10^{-1}
	CO	8.62	38.7	16.7	3.83	9.93×10^{-3}	1.36×10^{-1}	3.43	29.4	3.65×10^{-2}	5.49×10^{-2}	6.74×10^{-1}	2.43×10^{-1}
SPRING	T	7.55	**95.7***	14.9	**15.9***	8.68×10^{-2}	5.81×10^{-1}	4.12×10^{-1}	19.6	2.52×10^{-2}	9.38×10^{-2}	1.41×10^{-1}	7.54×10^{-1}
	S	8.07	82.1	10.4	3.89	8.10×10^{-2}	**7.18×10^{-1}***	3.98×10^{-1}	21.1	2.39×10^{-2}	7.83×10^{-2}	1.57×10^{-1}	4.36×10^{-1}
	SJ	**14.7**	71.4	17.4	7.07	9.18×10^{-2}	4.26×10^{-1}	1.34×10^{-1}	17.7	2.21×10^{-2}	8.58×10^{-2}	1.44×10^{-1}	5.60×10^{-1}
	I	10.4	95.3	16.4	7.94	6.61×10^{-2}	6.65×10^{-1}	3.80×10^{-1}	**25.9**	**2.68×10^{-2}**	**1.08×10^{-1}**	1.27×10^{-1}	**1.07**
	M	14.2	82.7	**20.9**	6.44	**1.14×10^{-1}**	4.42×10^{-1}	**1.91**	17.8	2.28×10^{-2}	6.73×10^{-2}	1.55×10^{-1}	4.02×10^{-1}
	CO	12.3	87.3	16.1	11.0	8.92×10^{-2}	5.88×10^{-1}	1.43	16.3	2.47×10^{-2}	7.81×10^{-2}	**1.69×10^{-1}**	7.22×10^{-1}
WINTER	T	9.15	**70.4**	38.1	1.77	2.70×10^{-1}	6.22×10^{-2}	1.91×10^{-1}	7.53	9.56×10^{-3}	4.09×10^{-3}	1.40	4.39
	S	11.5	63.8	**38.9***	1.69	2.48×10^{-1}	**7.99×10^{-2}**	1.92×10^{-1}	7.15	1.06×10^{-2}	7.74×10^{-3}	**2.30***	2.60
	SJ	7.84	27.2	33.1	1.87	2.67×10^{-1}	7.86×10^{-2}	1.91×10^{-1}	4.81	3.50×10^{-3}	**1.39×10^{-2}***	1.94×10^{-1}	2.95
	I	**12.5**	35.1	37.2	1.70	2.65×10^{-1}	7.57×10^{-2}	1.91×10^{-1}	**7.78**	**2.15×10^{-2}**	8.92×10^{-3}	1.43	4.20
	M	8.95	51.2	36.0	1.55	**3.14×10^{-1}***	7.20×10^{-2}	1.92×10^{-1}	6.70	9.17×10^{-3}	6.52×10^{-3}	7.68×10^{-1}	**4.60***
	CO	10.7	38.3	34.4	**2.63**	2.68×10^{-1}	6.24×10^{-2}	**1.93×10^{-1}**	4.74	7.47×10^{-3}	9.90×10^{-3}	3.85×10^{-1}	3.61


Summer
Autumn
Spring
Winter
4
-6
6
-6
ETS
GSTs
GLY
GPx
AChE
PROT
GSSG
CAT
Ni Tissue
GSH
LPO
T
CO
I
S
SJ
M
PCO1
40.1% of total variation
PCO2
24.9% of total variation


Summer
Autumn
Spring
Winter
4
-4
4
-4
Salinity
PCO1
40.1% of total variation
PCO2
24.9% of total variation
T
SJ
I
M
T
CO
S
SJ
M
M
CO
T
CO
S
S
SJ
T
M
CO
I
S
I
I

• Seasonal changes overlaps pollution levels effects on clam's biochemical machinery.

• Cu, Cr and As are the elements with higher concentrations in sediments, water and tissues, respectively.

• As is the element of most concern in terms of human health, with values as lower as 0.05 kg to exceed PTWI

• Higher LPO levels and GSH/GSSG ratio during Summer and Spring;

• Membranes' integrity prevention during Winter and Autumn due to antioxidant defences.

Conflict of Interest

The Authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affi liations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: