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RESEARCH ARTICLE

Ruditapes philippinarum and *Ruditapes decussatus* under Hg environmental contamination

Cátia Velez[1,2] · Petrus Galvão[3] · Renan Longo[3] · Olaf Malm[3] · Amadeu M. V. M. Soares[1,2] · Etelvina Figueira[1,2] · Rosa Freitas[1,2]

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Abstract The native species *Ruditapes decussatus* and the invasive species *Ruditapes philippinarum* have an important ecological role and socio-economic value, from the Atlantic and Mediterranean to the Indo-Pacific region. In the aquatic environment, they are subjected to the presence of different contaminants, such as mercury (Hg) and its methylated form, methylmercury (MeHg). However, few studies have assessed the impacts of Hg on bivalves under environmental conditions, and little is known on bivalve oxidative stress patterns due to Hg contamination. Therefore, this study aims to assess the Hg contamination in sediments as well as the concentration of Hg and MeHg in *R. decussatus* and *R. philippinarum*, and to identify the detoxification strategies of both species living in sympatry, in an aquatic system with historical Hg contamination. The risk to human health due to the consumption of clams was also evaluated. The results obtained demonstrated that total Hg concentration found in sediments from the most contaminated area was higher than the maximum levels established by Sediment Quality Guidelines. This study further revealed that the total Hg and MeHg accumulation in both species was strongly correlated with the total Hg contamination of the sediments. Nonetheless, the THg concentration in both species was lower than maximum permissible limits (MPLs) of THg defined by international organizations. *R. decussatus* and *R. philippinarum* showed an increase in lipid peroxidation levels along with the increase of THg accumulation by clams. Nevertheless, for both species, no clear trend was obtained regarding the activity of antioxidant (superoxide dismutase, catalase) and biotransformation (glutathione *S*-transferase) enzymes and metallothioneins with the increase of THg in clams. Overall, the present work demonstrated that both species can be used as sentinel species of contamination and that the consumption of these clams does not constitute a risk for human health.

Keywords *Ruditapes decussatus* · *Ruditapes philippinarum* · Mercury · Methylmercury · Bivalves · Bioaccumulation · Oxidative stress · Maximum permissible limits (MPLs)

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✉ Rosa Freitas
rosafreitas@ua.pt

[1] Departamento de Biologia & CESAM, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

[2] CESAM—Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[3] Laboratório de Radioisótopos Eduardo Penna-Franca, Universidade Federal do Rio de Janeiro, 21941-900 Rio de Janeiro, RJ, Brazil

Introduction

Mercury (Hg) is one of the most toxic elements in the world (ATSDR 2014), even at low concentration (Zahir et al. 2005). The occurrence of Hg can be originated by natural sources, such as erosion and volcanic eruptions (Guilherme et al. 2008; Spada et al. 2012). However, Hg contamination usually results from human activities such as intense urbanization, industrial activity (chlor-alkali industry), abandoned Hg mines, and gold-mining prospection (Abreu et al. 2000; Donnici et al. 2012; Pereira et al. 2008; Randall and Chattopadhyay 2013), thus altering the natural cycle of this metal (Berlin et al. 2015).

In aquatic ecosystems, most of the Hg is found in the inorganic form (Hg) (Celo et al. 2006) adsorbed to sediments or present in suspended particulate matter, water column, and food sources (Spada et al. 2012). In sediments and water, the inorganic Hg can be converted into toxic organic forms, such

as methylmercury (MeHg) (Ullrich et al. 2001). This conversion may occur by sulfate/iron-reducing (Pak and Bartha 1998) and methanogenic archaea bacteria living in anoxic environments (Correia et al. 2013). Also, other organisms such as fungi have been reported to be able to methylate Hg (Rieder and Frey 2013). The methylation also depends on abiotic factors such as pH, salinity, and temperature, being favorable in acidic pH, low salinity, and high temperatures (Celo et al. 2006). However, this methylation can be inhibited when chloride ions (Randall and Chattopadhyay 2013), sulfur, organic matter, and inorganic particles interact with Hg.

Like other metals, the bioavailability of Hg and MeHg in aquatic ecosystems raises concerns, namely regarding marine bivalves due to their feeding behavior, sedentary lifestyle, wide distribution, and abundance (Hamza-Chaffai et al. 2000; Galvão et al. 2009). Due to their characteristics, bivalves, including clams, have been recommended as indicators of contamination for both seawater and sediments (Beedy 2001; Moschino et al. 2012; Hamza-Chaffai 2014). Recent studies by Hamza-Chaffai et al. (2014) pointed out the relevance of the clam *Ruditapes decussatus* as a biomonitor species of pollutants in coastal waters. Furthermore, previous studies by Ahmad et al. (2011a, b) showed that, besides their capacity to accumulate Hg, exposure to this metal can weaken bivalves' immune system and make them more susceptible to health problems. Due to their characteristics, bivalves can also provide relevant information on the associated human health risks due to Hg bioaccumulation and biomagnification along the food chains in aquatic ecosystems (Ahmad et al. 2012; Spada et al. 2012).

The Hg accumulation in marine organisms can induce physiological changes, which can lead to an increase in oxidative stress (Duarte et al. 2011). A recent study showed that Hg induced oxidative stress and, consequently, lipid and protein damage in the bivalve *Scrobicularia plana* from highly contaminated areas (Ahmad et al. 2011a). Ahmad et al. (2012) further demonstrated that in *S. plana*, under a moderate Hg contamination scenario, the intervention of the different antioxidant enzymes was harmonious. Verlecar et al. (2007) found that Hg exposure induced the formation of oxidative stress products, namely reactive oxygen species (ROS), in the mussel *Perna viridis*, which resulted in lipid peroxidation and the increase of the antioxidant defense system in order to prevent damage induced by ROS. Studies conducted by Géret et al. (2002) demonstrated that the oyster *Crassostrea gigas* and the mussel *Mytilus edulis* exposed to Hg, increased their metallothioneins concentrations in the gills, which was identified as a defense mechanism through chelation of metals to their ionic form (Bebianno and Serafim 1998) and later to the formation of granules (Marigómez et al. 2002).

In order to better understand the effects of Hg and MeHg on marine bivalves, the present work used two of the most successfully commercialized edible clams in Portugal (Anacleto et al. 2013), the native species *Ruditapes decussatus* (Linnaeus, 1758) and the invasive species *Ruditapes philippinarum* (Adams and Reeve, 1850). Previous studies developed under laboratory conditions showed that both species respond differently to other metals (e.g. Cd) (Figueira et al. 2012).

Thus, the present study aimed to (a) quantify the concentrations of total mercury (THg) in the sediments and clams (*R. decussatus* and *R. philippinarum*), collected from different areas in the Ria de Aveiro, a coastal lagoon historically contaminated with Hg (Abreu et al. 2000); (b) compare sediment and organism contamination, evaluating the bioaccumulation factor (BAF) obtained at each sampling area; (c) quantify the concentration of MeHg in clams; (d) identify the detoxification strategies of both species when subjected to Hg contamination; and (e) assess the human health risks associated with clam consumption.

Materials and methods

Study area and sampling

The Ria de Aveiro (Fig. 1) is a shallow lagoon located in the northwest Atlantic coast of Portugal, covering an area between 66 and 83 km^2, at low and high spring tides, respectively. The average depth of the lagoon is about 1 m, except in navigation channels where dredging operations are frequently carried out. The channel connecting the lagoon to the ocean has a depth of about 20 m and other navigation channels are about 7 m deep (Dias et al. 2000). This lagoon comprises several channels such as S. Jacinto, Ílhavo, Mira, Ovar, and Murtosa, large areas of mud flats and salt marshes (Dias et al. 2000).

Between 1950 and 1994, the Ria de Aveiro lagoon received highly contaminated effluent discharges from a Hg cell chlor-alkali plant located in the Estarreja industrial complex (Ramalhosa et al. 2005; Castro et al. 2006; Pereira et al. 2008). Due to these discharges, the Hg dispersed throughout the lagoon and accumulated mainly in the Laranjo Bay due to its semi-enclosed characteristics. In the last decades, the Hg discharge diminished considerably and is nowadays under the regulatory levels (50 μg L^{-1}, the limit value established for discharges from chlor-alkali electrolysis industries, in accordance with Directive 82/176/EEC 1982) (Marques et al. 2011). Nevertheless, in some areas of this lagoon, including the Laranjo Bay, the Hg concentration in the surface sediments is still higher than pre-industrial levels (0.05 mg kg^{-1}) (Cardoso et al. 2013; Ahmad et al. 2012). These discharges induced an environmental contamination of Hg inside the lagoon and consequently a high Hg accumulation in organisms (Abreu et al. 2000; Castro et al. 2009).

In the present study, three study areas were selected in the Ria de Aveiro (cf. Fig. 1) to represent an Hg gradient. The selection of these areas was based on the Hg concentration

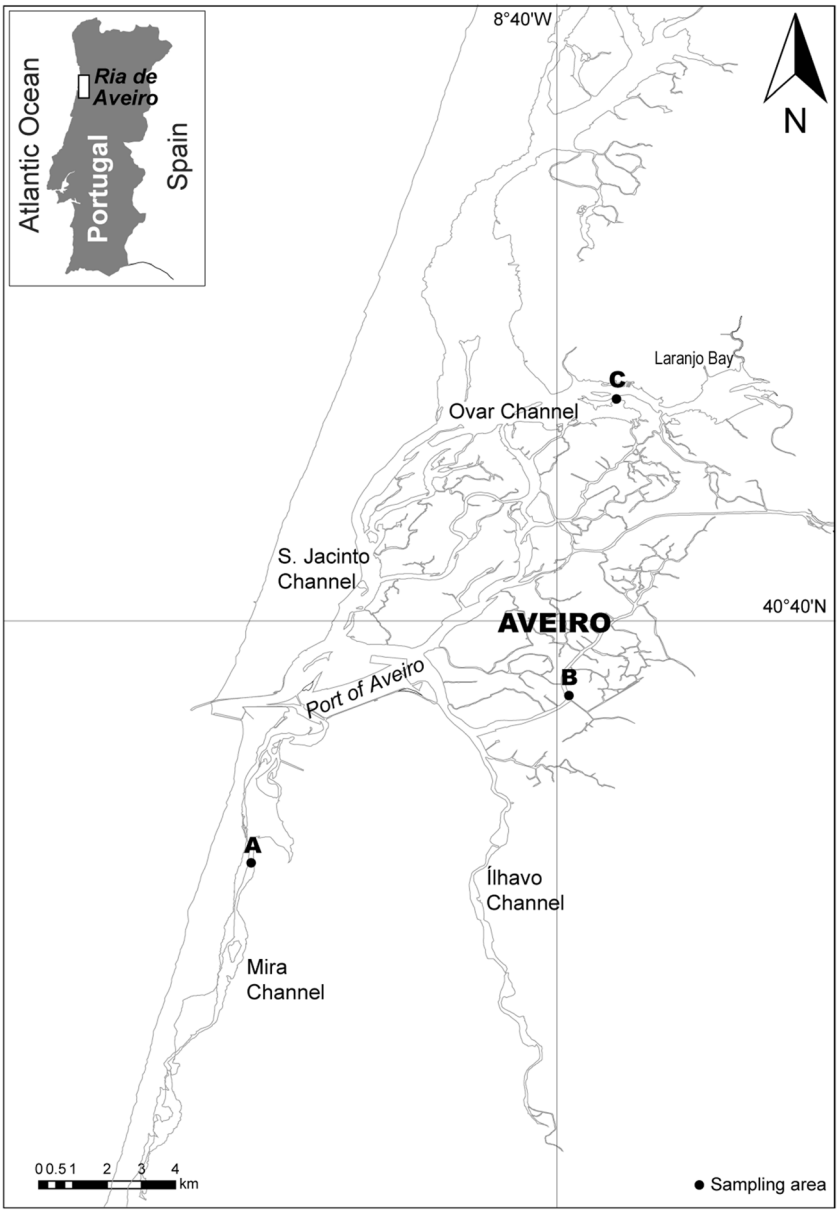

Fig. 1 Map of the sampling areas (*A*–*C*) within the Ria de Aveiro

previously documented for this system and on the presence of the clams *R. decussatus* and *R. philippinarum* (Castro et al. 2006; Pereira et al. 2008; Ahmad et al. 2012; Cardoso et al. 2013; Freitas et al. 2014; Velez et al. 2015). Clams and sediments were collected during high tide, in submerged areas. Area A was the farthest (18.87 km) and area C the closest (4.82 km) to the Laranjo Bay, the most Hg-contaminated area within the Ria de Aveiro. At each area (A, B, and C), three sites were selected (A_1, A_2, A_3, B_1, B_2, B_3; C_1, C_2, C_3) and three sediment samples and six specimens of each species were collected per site. The clams' weight variation, within each species, was 18–22 g in *R. decussatus* and 22–28 g in *R. philippinarum*.

At each site, the sediment samples were collected using a 30-cm-diameter corer and transported on ice (0 °C) to the laboratory. These samples were used for THg quantification and total organic matter content (TOM) determination. The physico-chemical characteristics (temperature, pH, and salinity) of the sediments were measured in the field, with specific probes (WTW, 330).

Laboratory analysis

Mercury quantification

Sediment Sediment samples were sieved and the particles <74 μm size dried at 35 °C to constant weight for the THg quantification. The percentage of fines content was determined, taking into account the total weight of sediment and the weight of fine particles per sample.

THg concentration present in sediments was determined according to Bastos et al. (1998). Sediment samples were weighted (0.1 g) and digested with the addition of 1 mL of Milli-Q water and 5 mL of HCl/HNO_3 (3:1 *v*/*v*) (TEDIA®—purity levels, 37 and 68 %, respectively), at 60 °C for 5 min in a water bath. Then, 5 mL of potassium permanganate 5 % (KMnO4—Merck®, purity level 99 %) was added and temperature set at 60 °C for 30 min. After cooling, samples were digested during 12 h. After that, 1 mL of hydroxylamine hydrochloride ($HNONH_3Cl$+NaCl 12 %; Merck®—both purity level 99 %) was added to each sample for neutralization and the samples were homogenized. The samples were filtered through a 0.45-μm (Whatman GF/C) glass filter and the final volume (12 mL) completed with high purity deionized water for all samples. The THg concentration was determined by cold vapor atomic absorption spectrophotometry (CV-AAS) using a Flow Injection Mercury System (FIMS-400; Perkin Elmer®, Waltham, MA, USA) with flow injection instrument (FIAS) and AS-90 autosampler. Software used was Powerful WinLab32 (Perkin Elmer®).

Reference material IAEA 405 was used (range recovery= 85.2–88.8 %). The results were expressed as milligrams per kilogram of total sediment dry weight (DW).

Clams THg concentration present in both species was determined according to Bastos et al. (1998), with some alterations. Samples were weighted, pulverized with liquid nitrogen, and divided into 0.4 g aliquots (fresh weight, FW). Afterwards, these samples were lyophilized (DW) and 0.05 g was digested with a solution of 1 mL of hydrogen peroxide (H_2O_2), 3 mL of sulfuric nitric acid mixture (H_2SO_4/HNO_3 1:1 *v*/*v*) (Tedia®—purity levels, 37 and 68 %, respectively) at 60 °C for 30 min in a water bath. Posteriorly, 5 mL of $KMnO_4$ 5 % (Merck®, purity level 99 %) solution was added, during 30 min at 60 °C, and digested overnight. The solution was neutralized with $HNONH_3Cl$+ NaCl 12 % (Merck®—both purity level 99 %) and the final volume (12 mL) was completed with Milli-Q water. The THg concentration was determined by cold vapor/AAS (FIMS-400; Perkin-Elmer), being $NaBH_4$ the reducing agent. The reference material IAEA-452 and NIST 2976 were analyzed (range recovery IAEA-452=85.1–100 % and Nist 2976=105.4–111.7 %). The results were expressed as milligrams per kilogram of fresh weight (FW).

MeHg concentration was quantified according to the EPA Method 1630. Samples were weighted, pulverized with liquid nitrogen, and divided into aliquots (FW). These samples were lyophilized (DW), weighted (0.03 g), and digested with KOH/methanol (25 %) (Sigma®, purity levels 85 and 99 %, respectively), during 6 h, at 60 °C. After 48 h, in 40-mL Teflon line borate glass bottles was added the digested samples and sodium acetate at pH 4.9 ethylated by sodium (Aldrich, purity level 97 %). The MeHg was measured with an automated analytical system (MERX, Brooks Rand). The standard reference material NIST 2976 was also analyzed (121–123 %). Results were expressed as milligrams per kilogram of FW. The percentage of MeHg was determined.

Bioaccumulation factor (BAF)

Following McGeer et al. (2003), the bioaccumulation factor (BAF) was determined by dividing the THg concentration in each species by the concentration of THg present in the sediment.

Total organic matter (TOM) content

The TOM content was determined according to Byers et al. (1978), measured as the percentage of weight loss in 1 g of dried sediment, after combustion at 450 °C, during 5 h.

Biochemical parameters

For biochemical analysis, clams were pulverized with liquid nitrogen and the extraction was performed with the specific buffer (1:2 *w*/*v*) for each determined biomarker. Samples were centrifuged (10,000×*g*) for 10 min at 4 °C and the supernatants stored at −20 °C.

The biochemical parameters analyzed were total protein and lipid peroxidation, GSHt, antioxidant enzymes activity (SOD and CAT), transformation enzymes activity (GSTs), and MTs. All the biochemical parameters were performed in duplicate.

Indicators of cellular damage Total protein content was determined by the Biuret method (Robinson and Hogden 1940). Bovine serum albumin (BSA) was used as standard (0–40 mg g^{-1}) (Calbiochem®, purity level 98 %). The colorimetric reaction was carried out at room temperature for 10 min at 30 °C and absorbance was measured at 540 nm. The results were expressed in milligrams per gram of fresh weight (FW).

Lipid peroxidation quantification was based on the reaction of lipid peroxidation (LPO) by-products, such as malondialdehyde (MDA), with 2-thiobarbituric acid (TBA), forming TBARS, according to the protocol described by Buege and Aust (1978). Samples were incubated at 96 °C during 25 min. The amount of TBARS, namely MDA, was quantified spectrophotometrically (wavelength of 532 nm) (1.56×105 M^{-1} cm^{-1} extinction coefficient) and results were expressed as nanomoles of MDA equivalents per gram of FW.

Total glutathione (GSHt) content was quantified according to Anderson (1985), taking into account the DTNB-glutathione reductase (GR) recycling assay. The standard curve was determined using oxidized glutathione (GSSG) standards (0–500 μmol L^{-1}) (Sigma®, purity level 98 %). The samples and standards were incubated for 5 min at room

temperature. Absorbance was measured at 412 nm and GSHt was expressed in micromoles per gram of FW.

Antioxidant and biotransformation enzymes (SOD, CAT, and GSTs) For SOD quantification, the method described by Beauchamp and Fridovich (1971) was followed, with slight modifications (Freitas et al. 2012). The standard curve was performed with SOD standards (0.25–60 U mL^{-1}) (Sigma®). SOD activity was measured spectrophotometrically (Amersham Biosciences, Ultrospec 2100 pro) at 560 nm. One unit of enzyme (U) corresponds to a reduction of 50 % of nitroblue tetrazolium (NBT). The results were expressed in units per gram FW.

CAT activity was determined by the reaction of the enzyme with methanol in the presence of H_2O_2 (Johansson and Borg 1988), following some modifications by Freitas et al. (2012). The standard curve was determined using formaldehyde standards (0–150 μM) (SIGMA®, purity levels 36.5–38 %). Samples were incubated during 20 min in a shaker, at room temperature. The formaldehyde formation with purpald (Sigma®, purity level 99 %) was measured at 540 nm. One unit (U) of enzyme is defined as the amount of enzyme that caused the formation of 1.0 nmol formaldehyde under the assay conditions. The results were expressed in units per gram FW.

GSTs activity was determined following the method described by Habig et al. (1974). GSTs catalyze the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione, forming a thioester. This formation can be followed by the absorbance increase at 340 nm during 5 min (intervals of 10 s). For the determination of GSTs activity, a time interval (5 min) was selected during linear enzyme activity. The enzymatic activity was expressed in units per gram of fresh tissue, where unit (U) represents the quantity of the enzyme that catalyzes the conversion of 1 μmol of substrate per minute. The activity of GSTs was determined using the extinction coefficient of 9.6 mM^{-1} cm^{-1} for CDNB. The results were expressed as units per gram FW.

Metal sequestration—metallothioneins (MTS) Proteins separation was done by SDS-PAGE, carried out in 4–20 % polyacrylamide (Mini-PROTEAN TGX—Bio-Rad, Mini-PROTEAN®) according to the procedure described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250 (Sigma®, purity level 98 %) and screened in a Densitometer apparatus (Bio-Rad—Model GS 710). Molecular mass and relative protein amount corresponding to each band were compared with a protein standard (NZY Colour Protein Marker II—NZY Tech Genes and Enzymes). Concentration of proteins was calculated using Quantity One program software (Bio-Rad®, version 4.6.6). After separation of proteins, each band was cut, and the extraction was done according to Milnerowicz and Bizoń (2010). Confirmation of MTs was done through quantification of thiol groups, according to Moron et al. (1979).

Data analyses

The Hg contamination in sediments, organisms' Hg bioaccumulation, BAF values, biochemical parameters, and the environmental parameters (pH, salinity, temperature, fines content, and TOM) were submitted to hypothesis testing using the PERMANOVA routine (permutational multivariate analysis of variance) by PRIMERv6® (Plymouth Marine Laboratory) (Anderson et al. 2008), following the calculation of Euclidean distance matrices among samples. A two-way hierarchical design, with sites nested in areas and these as the main fixed factor, was followed in this analysis. The *t* statistic in the pair-wise comparisons was evaluated in terms of significance among different areas and species. Values lower than 0.05 were considered as significantly different.

The null hypotheses tested were as follows: (a) for sediment contamination, no significant differences exist between areas; (b) for THg and MeHg accumulation in species, no significant differences exist between areas and species; (c) for BAF, no significant differences exist between species and areas; and (d) for each biochemical parameter, no significant differences exist between areas and species.

The results obtained were expressed as distinct letters for *R. decussatus* (A–C) and for *R. philippinarum* (a–c) to indicate differences between areas. To indicate differences between species, "*" was used.

The Spearman correlation was calculated by PRIMERv6® (Plymouth Marine Laboratory) (Anderson et al. 2008), evaluating the correlation between THg concentration present in sediment and in clams. The Spearman correlation value was classified as strong ($0.89>r>0.70$) and very strong ($1.00>r>0.90$).

Taking into consideration the bioaccumulation levels of both species, in the most and the least contaminated areas, the amount of clams that a 70-kg adult needs to consume in 1 week to exceed provisional tolerable weekly intake (PTWI) was determined. This value was obtained by dividing the PTWI of a 70-kg adult by the concentration of THg or MeHg in clams.

Results

Environmental parameters

The environmental parameters, such as temperature, pH, and salinity, are summarized in Table 1. Temperature among sampling areas ranged from 19.4 to 22.5 °C, with significant differences between all areas. The pH and salinity varied significantly among areas from 6.7 to 8.5 and from 28 to 33, respectively. The results on the sediment fines content showed that the area B presented the highest (52.2 %) and area A the lowest (0.4 %) percentage, showing significant differences among areas. Concerning the TOM content (cf. Table 1), the

Table 1 Environmental parameters of the sampling site: temperature (°C), pH, salinity, fines content (percentage of total sediment dry weight), total organic matter (TOM, %), total Hg in sediments (THg, mg kg^{-1} DW), bioaccumulation factor (BAF) in *Ruditapes decussatus* and *Ruditapes philippinarum*, and distance to Laranjo Bay (km), mean values (±standard deviation)

Areas	Temperature	pH	Salinity	Fines	TOM	THg	BAF *R.decussatus*	BAF *R.philippinarum*	Distance to Laranjo Bay (km)
A	19.4±0.13a	7.16±0.06a	28±0.42a	0.43±0.12a	0.71±0.22a	0.04±0.01a	0.40±0.04a	0.37±0.07a	4.82
B	22.5±0.40b	6.86±0.07b	32±0.65b	52.15±24.72b	4.62±2.97b	0.06±0.03a	0.48±0.13a	0.48±0.16a	10.60
C	21.7±0.57c	8.53±0.30c	32±0.26b	15.42±14.22c	5.29±4.67b	0.24±0.06b	0.23±0.08b	0.24±0.11b	18.87

Significant differences ($p \leq 0.05$) for each area are presented with letters (a–b)

results showed an increase among areas, with the highest value being found in area C (5.3 %) and the lowest value in area A (0.7 %), with significant differences observed between area A and the remaining areas.

Hg concentration in sediments and clams

The concentration of THg in sediments is presented in Fig. 2a and Table 1. The results showed a spatial gradient along the sampling areas with sediments from area A presenting the lowest THg concentration (0.04±0.01 mg kg^{-1} DW), while sediments from area C showed the highest THg levels (0.24± 0.06 mg kg^{-1} DW) (cf. Table 1 and Fig. 2a). The results demonstrated that significant differences were found between area C and the remaining areas, while no significant differences were found between areas A and B (cf. Table 1 and Fig. 2a).

Regarding the concentration of THg and MeHg present in clams (Fig. 2b), the results obtained showed a gradient, with the highest concentration of THg and MeHg found, in both species, at area C and the lowest concentrations at area A. For each area, no significant differences were found between species both for THg and MeHg concentrations. Furthermore, the results obtained showed a strong correlation (>0.85) between the THg in sediments and the THg in both species.

The BAF values (cf. Table 1) for both species in all areas ranged between 0.23 and 0.48, indicating low THg accumulation by both species in all the study areas. The BAF values obtained for clams collected at area C were significantly lower than BAF values showed by clams from areas A and B.

The percentage of MeHg present in *R. decussatus* showed no significant differences among areas (Fig. 2c), while in *R. philippinarum* significant differences were found between clams from areas B and C (cf. Fig. 2c). When comparing the MeHg percentage present in clams at each area, no significant differences were found between species (cf. Fig. 2c).

Biochemical parameters

Indicators of cellular damage

The protein content is shown in Fig. 3. The results obtained revealed that *R. decussatus* collected in the most contaminated area (area C) presented the highest protein content (33 ±7 mg g^{-1} FW), with significant differences in relation to clams from the remaining areas (25±6 mg g^{-1} FW, area A and 28±6 mg g^{-1} FW, area B). In *R. philippinarum*, the protein content was lower in area B (28±6 mg g^{-1} FW), with significant differences when compared to the less contaminated area (area A, 37±5 mg g^{-1} FW). At each area, when comparing the protein content in both species, significant differences between species were only found at the less contaminated area (area A), with *R. philippinarum* showing the highest protein content.

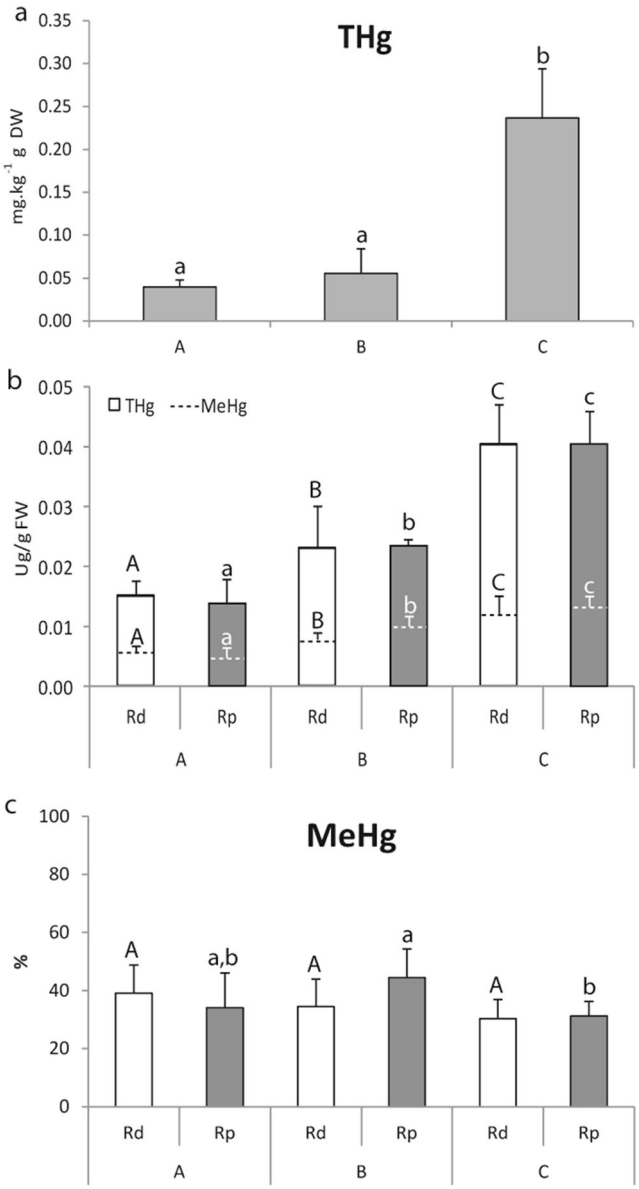

Fig. 2 **a** Total mercury (THg) concentration in fine particles of sediments (mg kg^{-1} dry weight, DW); **b** THg and methylmercury (MeHg) in *Ruditapes decussatus* (Rd) and *Ruditapes philippinarum* (Rp) (mg kg^{-1} of fresh weight, FW); **c** percentage of MeHg in *R. decussatus* (Rd) and *R. philippinarum* (Rp), mean values (±standard deviation) along the three sampling areas (*A*, *B*, *C*). Different letters (*A–B*, *a–b*) represent significant differences ($p \leq 0.05$) among areas for *R. decussatus* and *R. philippinarum*, respectively

Regarding the LPO results (Fig. 4a), for *R. decussatus*, the highest LPO values were recorded in clams with higher THg accumulation (area C, 26±3 nmol g^{-1} FW), while the lowest LPO values were found in clams with lower THg concentration (area A, 17±4 nmol g^{-1} FW). A similar pattern was found for *R. philippinarum*, which presented a significantly higher LPO in area C (47±4 nmol g^{-1}) when compared to the remaining areas (A and B). When comparing both species, the LPO levels were significantly higher in *R. philippinarum* than in *R. decussatus*, in all the studied areas.

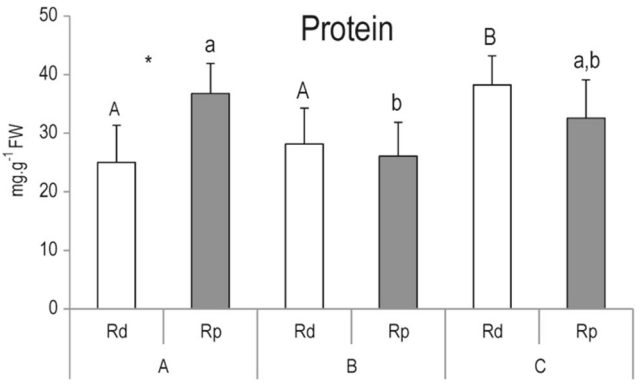

Fig. 3 Total protein content (mg g^{-1} FW), mean values (±standard deviation), in *Ruditapes decussatus* (Rd) and *Ruditapes philippinarum* (Rp) along the three sampling areas (*A*, *B*, *C*). Different letters (*A–B*, *a–b*) represent significant differences ($p \leq 0.05$) among areas for *R. decussatus* and *R. philippinarum*, respectively

When analyzing the GSHt content (Fig. 4b) in each species, no significant differences were found between areas. When comparing the GSHt content of both species at each sampling area, the results showed significant differences between *R. decussatus* and *R. philippinarum* in area A.

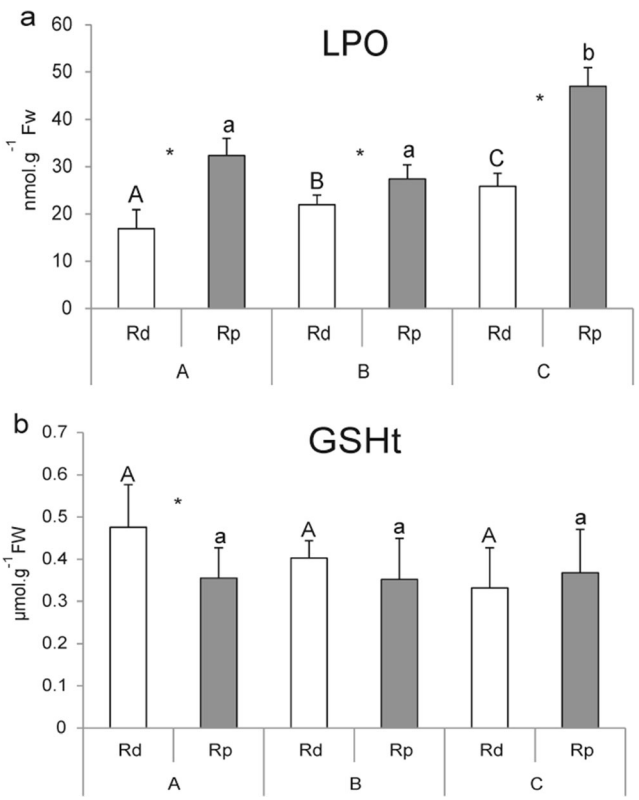

Fig. 4 Indicators of oxidative stress [*LPO* lipid peroxidation, nmol g^{-1} FW (**a**), *GSHt* total glutathione, µmol g^{-1} FW (**b**)], mean values (±standard deviation), in *Ruditapes decussatus* (Rd) and *Ruditapes philippinarum* (Rp) along the three sampling areas (*A*, *B*, *C*). Different letters (*A–B*, *a–b*) represent significant differences ($p \leq 0.05$) among areas for *R. decussatus* and *R. philippinarum*, respectively

Antioxidant and biotransformation enzymes (SOD, CAT, and GSTs)

The activity of the antioxidant enzymes, SOD and CAT, are presented in Fig. 5a and b, respectively. Concerning the SOD activity (cf. Fig. 5a), the results showed that in *R. decussatus* the lowest values were found in area B (5±1 U g^{-1} FW), while the highest activity was found in area C (6±1 U g^{-1} FW). For *R. philippinarum*, the activity of SOD was lowest in area A (4±1 U g^{-1} FW). The results also showed that for both species, no significant differences were found among areas. Furthermore, at each area, significant differences were found between species.

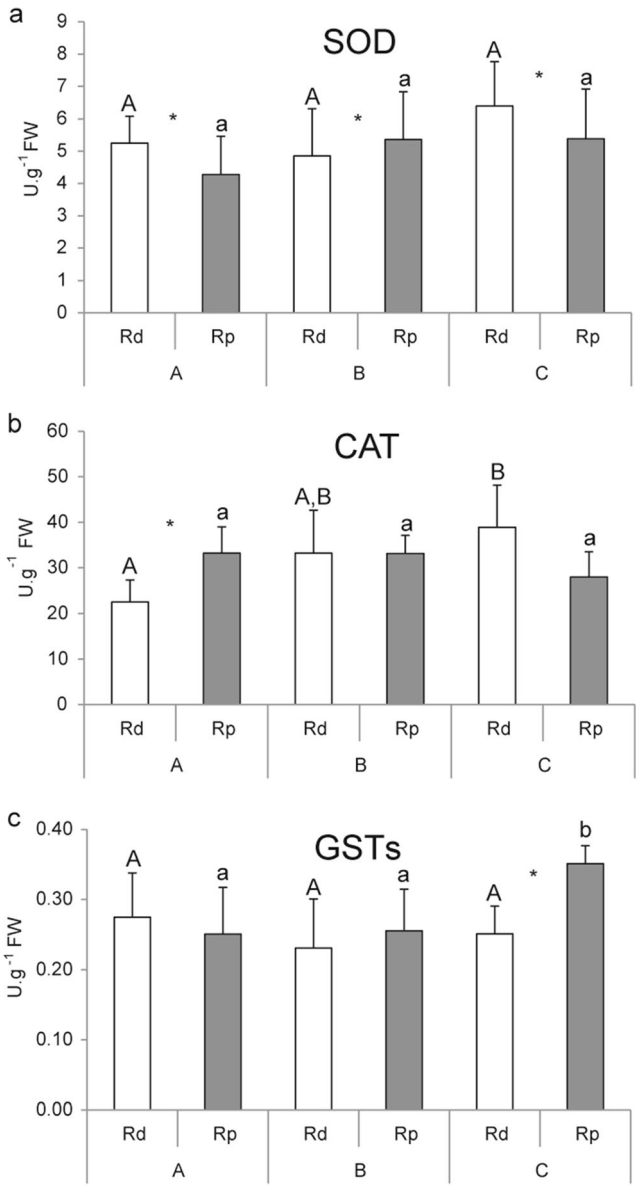

Fig. 5 Antioxidant enzymes (*SOD* superoxide dismutase, *CAT* catalase, *GST* glutathione *S*-transferase, U g^{-1} FW) in *Ruditapes decussatus* (Rd) and *Ruditapes philippinarum* (Rp) along the three sampling areas (*A*, *B*, *C*), mean values (±standard deviation). Different letters (*A–B*, *a–b*) represent significant differences ($p \leq 0.05$) among areas for *R. decussatus* and *R. philippinarum*, respectively

The activity of CAT (cf. Fig. 5b) in *R. decussatus* increased along with the increase of THg accumulated by clams (area A, 23±5 U g^{-1} FW to area C, 39±9 U g^{-1} FW), with significant differences found between areas A and C. For *R. philippinarum*, no significant differences among areas were detected. When comparing both species, significant differences were only identified in area A.

The activity of GSTs for both clams is shown in Fig. 5c. The results showed that no significant differences were found in *R. decussatus* from different areas. Nevertheless, for *R. philippinarum*, a significant increase was observed in area C. When comparing both species, significant differences were found between *R. philippinarum* and *R. decussatus* from the most contaminated area (C).

MTs

The MTs synthesis in *R. decussatus* and *R. philippinarum* presented no significant differences among areas (Fig. 6). However, *R. philippinarum* showed higher MTs levels than *R. decussatus* in all areas, with significant differences between species in area B.

Evaluation of human risk consumption

In all the study areas, the THg concentration found in *R. decussatus* and *R. philippinarum* did not exceed the maximum regulatory levels defined by international organizations (USFDA, United States Food and Drug Administration; EFSA, European Food Safe Authorities; FSANZ, Food Standards Australia and New Zealand) for THg.

The results here presented revealed that the amount of clams that a 70-kg adult needs to consume in 1 week to exceed PTWI was 8.75 kg for species collected from the most

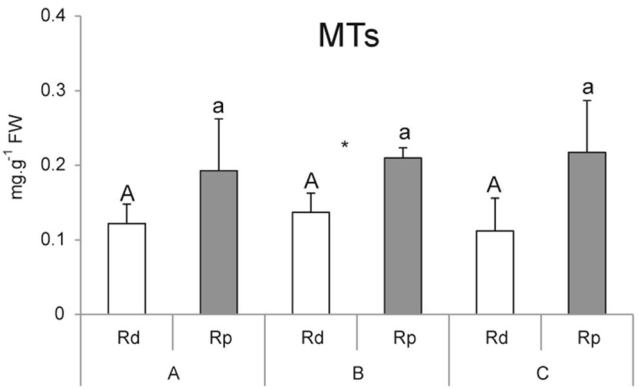

Fig. 6 Metallothioneins (MTs content, mg g^{-1} FW), mean values (±standard deviation), in *Ruditapes decussatus* (Rd) and *Ruditapes philippinarum* (Rp) along the three sampling areas (*A*, *B*, *C*). Different letters (*A–B*, *a–b*) represent significant differences ($p \leq 0.05$) among areas for *R. decussatus* and *R. philippinarum*, respectively

contaminated area (Table 2). For clams collected in the less contaminated area, the amount of clams that a 70-kg adult needs to consume in 1 week to exceed the PTWI was 17.5 and 35.0 kg for *R. decussatus* and *R. philippinarum*, respectively (cf. Table 2). Considering the MeHg, a 70-kg adult needs to consume 19.3 kg of *R. decussatus* and 17.7 kg of *R. philippinarum* from the most contaminated area, in 1 week, to exceed PTWI (cf. Table 2). From the lowest contaminated area, an adult (70 kg) needs to consume in 1 week 38.5 kg of *R. decussatus* and 46.3 kg of *R. philippinarum* to exceed the PTWI for MeHg (cf. Table 2).

Discussion

Even after 20 years of the last effluent discharge, the concentration of THg found in the sediments of the Laranjo Bay shows the impact of the chlor-alkali plant located in the lagoon (Cardoso et al. 2013). Due to this, the Ria de Aveiro has been studied by many authors (Ramalhosa et al. 2005; Ahmad et al. 2012; Cardoso et al. 2013; Coelho et al. 2014). However, this is the first study involving THg and MeHg bioaccumulation and oxidative stress patterns caused by THg contamination in *R. decussatus* and *R. philippinarum* species living in sympatry. Due to the ecological and socio-economic importance of these species, this study is extremely important since it can provide more relevant information on the potential negative impact of THg on marine ecosystems and, consequently, the risks for human health associated with the consumption of these clams.

THg concentration in sediments and clams

The results of the present study showed that in the area closer to the contamination source (Laranjo Bay), the THg concentration in sediments was higher (0.24±0.06 mg kg^{-1} DW) than in the other two areas (area A 0.04±0.01 and area B 0.06±0.03 mg kg^{-1} DW). These results are in agreement with previous studies which demonstrated a clear Hg spatial gradient in the Ria de Aveiro, being the highest concentration of THg found in areas closer to the Laranjo Bay (Nunes et al. 2008; Cardoso et al. 2013; Velez et al. 2015) and the lowest concentrations in areas located away from the discharge, suggesting a close relationship between the distance to the source and the THg concentration found in sediments.

In areas near the Laranjo Bay, according to Ahmad et al. (2012), the THg concentrations found in sediments between 2003 and 2008 ranged between 51 and 20 mg kg^{-1} DW. Later, Cardoso et al. (2013) revealed that THg concentrations decreased to 1 mg kg^{-1} DW in 2010. Our work, conducted in

Table 2 Minimum and maximum concentration of Hg and MeHg in *R. decussatus* and *R. philippinarum*, from the Ria de Aveiro, and the amount of clams that a 70-kg adult needs to consume to exceed PTWI per week

	Hg (mg kg^{-1})	MeHg (μg kg^{-1})	Ref
Maximum level			
EFSA	0.5		EC 2006
USFA	1		USFDA 2001
FSANZ	0.5		Vannoort and Thomson 2005
Ipolyi et al. (2004)		44	
THg concentration in *R. decussatus* (this study)			
Minimum values	0.02	6	
Maximum values	0.04	12	
THg concentration in *R. philippinarum* (this study)			
Minimum values	0.01	5	
Maximum values	0.04	13	
PTWI (mg kg^{-1} $week^{-1}$)			
JECFA	0.005	3.3	FAO/WHO 2006
FSANZ	0.005		FAO/WHO 2011
Amount of *R. decussatus* consumed/week, to exceed PTWI (kg) for Hg (this study)			
Minimum values	17.5	38.5	
Maximum values	8.75	19.3	
Amount of *R. philippinarum* consumed/week, to exceed PTWI (kg) for Hg (this study)			
Minimum values	35.0	46.2	
Maximum values	8.75	17.8	

The concentrations of Hg found in both clams were compared with maximum levels from international organizations standards (JECFA, Joint FAO/WHO Expert Committee on Food Additives; USFDA, United States Food and Drug Administration; EFSA, European Food Safe Authorities; FSANZ, Food Standards Australia and New Zealand)

2014, also demonstrated that THg concentration in sediments near the Laranjo Bay is decreasing. Recent studies, conducted by Válega et al. (2008), further demonstrated that sediments from this area presented no other organic Hg species rather than MeHg, which was in a very low percentage (0.5 %). Also, according to Ramalhosa et al. (2011), a low percentage of MeHg (0.1 %) was detected in sediments close to the Hg contamination source, in a concentration of 13.2 μg kg^{-1} (DW). Therefore, according to both studies and the results obtained in the present work, the MeHg % estimated to be in sediments near the Laranjo Bay was 0.24 μg kg^{-1} DW (0.1 % of MeHg) and 1.18 μg kg^{-1} DW (0.5 % of MeHg).

In the Mira channel, located 18.8 km from the Laranjo Bay, THg concentration found in sediments between 1997 and 1998 was 0.5 mg kg^{-1} of DW (Abreu et al. 2000), decreasing between 2003 and 2008 (0.3±0.1 to 0.08±0.03 mg kg^{-1} DW, Ahmad et al. 2011a, 2012). When comparing these data with results from the present study (area A, located at the Mira channel), it is possible to conclude that the THg concentration in the sediments from this channel is also decreasing (0.04± 0.01 mg kg^{-1} DW), suggesting the ecosystems recovery.

The present work further revealed that although the concentrations of THg in sediments from the two less contaminated areas (areas A and B) were lower than the standard values defined by the Sediment Quality Guidelines, namely Threshold Effect Level (TEL, 0.13 mg kg^{-1}; MacDonald et al. 1996) and the Effects Range Low (ERL, 0.15 mg kg^{-1}; Long et al. 1995), the concentrations found at the most contaminated area (area C) were higher than these standards. Nevertheless, the THg concentrations found in this study were similar or lower than values found at Guanabara Basin, Brazil (0.1–3.22 mg kg^{-1} DW; Covelli et al. 2012), Pialassa Baiona and Ravenna lagoon, Italy (0.25–250 mg kg^{-1} DW; Trombini et al. 2003), Venice lagoon, Italy (0.10–1.22 mg kg^{-1} DW; Sfriso et al. 2008), and Santander Basin, Spain (0.02–0.8 mg kg^{-1} DW; Ramos-Gómez et al. 2011).

It is well known that the metal contamination present in sediments can act as an important route of exposure to aquatic organisms living in contact or within the sediments (Burton 2002). Due to this, the native species *R. decussatus* and the invasive species *R. philippinarum* have been used as sentinel organisms to assess the impact of pollution in different marine ecosystems (El-Nemr et al. 2012; Giani et al. 2012; Matozzo et al. 2012; Moschino et al. 2012; Sfriso et al. 2008; Wang et al. 2012). In the present study, THg and MeHg found in *R. decussatus* (0.02–0.04 and 0.006–0.012 mg kg^{-1} FW, respectively) and *R. philippinarum* (0.01–0.04 and 0.005–0.012 mg kg^{-1} FW, respectively) increased along the increase of THg in the sediments. A strong correlation (r>0.85) between THg concentration for both species and THg contamination in sediment was found, indicating that metal concentration found in clams reflects the environmental THg contamination. In agreement with other studies (Cravo et al. 2012; El-Nemr et al. 2012; Matozzo et al. 2012; Wang et al. 2012), the present work demonstrated that the linear relationship between THg concentration present in clams and sediments allows to identify *R. decussatus* and *R. philippinarum* as good sentinel species in different marine ecosystems. Coelho et al. (2006) and Ahmad et al. (2012) also demonstrated that *Scrobicularia plana* reflected the Hg contamination in sediments.

Our findings showed that, in each area, no significant differences were observed in terms of Hg concentration in clams, indicating that both species presented similar capacity to bioaccumulate this metal. Furthermore, the present study also demonstrated that sediment contamination had no influence on both species spatial distribution, since *R. decussatus* and *R. philippinarum* were present in all the areas, independently on Hg concentration. Also, Velez et al. (2015) demonstrated that the spatial distributions of these species were not dependent on site contamination levels since both clams were presented at low and high contaminated areas. These authors also showed that when these species coexisted, the native species showed higher density, suggesting that the introduced clam has not yet supplanted the native species by occupying entirely its ecological niche. The present work may therefore indicate that metal tolerance will not be the mechanism responsible for the replacement of the native species.

The present study showed that THg concentration found in *R. philippinarum* was similar or even lower than the concentrations found in this species from Marano and Grado lagoon, Italy (0.176–0.605 mg kg^{-1} FW) (Giani et al. 2012), the Venice Lagoon, Italy (0.179–2.03 mg kg^{-1} DW) (Sfriso et al. 2008), the Spain's Southern Atlantic coast (0.03–0.39 mg kg^{-1}) (Usero et al. 1997), and from the Chinese Bohai Sea (0.01–0.08 mg kg^{-1} FW) (Wang et al. 2005). Also, in the present study, the THg concentration found in *R. decussatus* was lower than values found in this species from Spain's Southern Atlantic coast (0.01–0.07 mg kg^{-1} DW) (Usero et al. 1997).

The results here presented revealed that in all areas the percentage of MeHg found in both clams was comparable (*R. decussatus*, 30–39 % and *R. philippinarum*, 31–44 %). Microbial and abiotic methylations of THg are the key for transformation from inorganic Hg to organic compounds, such as MeHg (Randall and Chattopadhyay 2013). MeHg have high toxicity, becoming available to bioaccumulation and biomagnification in high trophic levels (Kehrig et al. 2002; Schmitt et al. 2011; Seixas et al. 2013). Thus, our findings suggest a similar percentage of MeHg available to be accumulated which may be a result of similar methylation/demethylation processes in the sampling areas (Ullrich et al. 2001; Avramescu et al. 2011). Also, Spada et al. (2012) found similar percentage of MeHg in bivalves *Chlamys varia* (33–45 %) and *Mytilus galloprovincialis* (25–35 %) from the areas with different THg contamination.

Mercury bioavailability and bioaccumulation

The bioavailability of THg is influenced by environmental parameters, such as pH and organic matter content (Pan and Wang 2011). The present study revealed that the interaction between THg, pH, fines, and TOM content influenced the bioavailability of this metal. In fact, the results obtained showed that the most contaminated area was characterized by high fines and TOM content, suggesting that these two abiotic factors may have contributed to the low THg bioavailability and consequently low accumulation in clams (BAF< 0.25). Previous studies identified the organic matter and fine particles content as two of the major factors that influence THg uptake, being responsible for reduced mobility and toxicity of Hg (Horvat 1977; Berto et al. 2006; Schiff 2000; Eggleton and Thomas 2004; Yu et al. 2012). Driscoll et al. (1995) demonstrated that the complexation of Hg and TOM tended to limit its uptake in biota. Also, alkaline pH appears to decrease the bioavailability of Hg in sediments (Wright and Welbourn 2002) since these conditions may favor the THg binding to sulfides or inorganic components (Randall and Chattopadhyay (2013). Thus, the high pH value found at the most contaminated area may also have contributed to the lowest bioaccumulation showed by clams from this area. On the other hand, the sediments from less contaminated areas showed low fines content and low TOM content which have influenced the availability of THg in sediments favoring the accumulation of THg by clams (BAF>0.40).

Cell damage and oxidative stress

The present study demonstrated that both clam species presented an increase in LPO levels with the increase of THg bioaccumulation, suggesting that THg can induce ROS production and damage on membrane lipids. The LPO content was significantly higher in *R. philippinarum* than in *R. decussatus*, suggesting different metabolic and biochemical responses in the two species. A similar increase in LPO levels was also obtained for the bivalve *S. plana* (Ahmad et al. 2011a) exposed to THg concentration. A similar pattern has already been observed in *R. decussatus* and *R. philippinarum* after exposure to other metals such as Cu, Hg, Cd, and Pb (Roméo and Gnassia-Barelli 1997; Figueira et al. 2012; Freitas et al. 2014).

The protein content was only significantly induced in *R. decussatus* when this species accumulated high Hg concentrations (area C), with no significant differences among areas for *R. philippinarum*. These results suggest that both species may not change the protein content when under low to moderate Hg accumulation conditions. These results were in accordance with Ahmad et al. (2011a) that found no significant differences in hemolymph plasma protein in *S. plana* from areas with different THg concentration.

The present work also evaluated the effects of THg accumulation on the enzymes GSHt, SOD, CAT, and GST activities. In *R. philippinarum* and *R. decussatus* a similar GSHt, SOD, and CAT response was found in all the study areas, suggesting a balance in production of ROS and in detoxification processes to eliminate ROS present in the cytosol by SOD and CAT enzymes (Alves de Almeida et al. 2007; Regoli and Giuliani 2014). In agreement with the present study, the mussel *Mytella guyanensis* from polluted mangroves (Brazil) showed an increase of LPO in areas with higher metal contamination but no variation in GSHt and SOD activity (Torres et al. 2002). Zhang et al. (2010) demonstrated that when the clam *Chlamys farreri* was exposed to environmentally relevant concentrations of Hg, no antioxidant responses were induced. However, an opposite behavior was shown by different authors, revealing a positive relationship between CAT and SOD activity and metal contamination in *R. philippinarum* (Wang et al. 2012). Figueira et al. (2012) noted that in *Ruditapes decussatus* and *R. philippinarum* the activity of the antioxidant enzymes CAT and SOD tended to increase after exposure to increasing Cd concentrations. The mussel *M. galloprovincialis* presented significant differences in CAT and SOD between polluted and non-polluted areas (Box et al. 2007). Also, *Mytilus edulis chilensis* increased the CAT activity and levels of LPO with the increase of Cd, Cu, and Zn concentrations in tissues (Duarte et al. 2011).

Previous studies reported that high GSTs levels where found in *P. viridis*, *C. farreri*, and *R. philippinarum* (Jena et al. 2009; Zhang et al. 2010; Wang et al. 2012) from polluted areas when compared with the non-polluted areas. The present study showed that along the accumulation of THg concentration, *R. decussatus* maintained the GSTs levels, which may suggest a balance between ROS production and elimination due to the capacity of this enzyme to eliminate reactive compounds by forming conjugates with glutathione (Wang et al. 2012). However, in *R. philippinarum*, higher GSTs levels were found in the area with higher THg accumulation, suggesting that in this species the increase in the GST may be the only defense response activated to prevent oxidative stress, while the activities of antioxidant enzymes are lowered. Wang et al. (2011) showed that with Cd contamination, *R. philippinarum* increased the activity of GSTs. Jena et al. (2009) and Zhang et al. (2010) also reported higher levels of GSTs in *P. viridis* and *C. farreri*, respectively, from polluted areas when compared with the control areas. According to Fernández et al. (2010), the GST activity and metal concentration (Pb, Hg, and Cd) in *M. galloprovincialis* presented a significantly positive correlation, suggesting that GSTs increase their activity in response ROS generation increase due to a high bioavailability of these metals in the marine environment. Nevertheless, Ramos-Gómez et al. (2011) did not find for *R. philippinarum* a significant response of GSTs activity to contaminated sediment exposure.

MTs have been used extensively in aquatic organisms as molecular biomarkers of metal species, especially due to their metal-chelating capacity, representing a specific response of the organisms to pollution by metals (Rajalakshmi and Mohandas 2008; Figueira et al. 2012). This protein decreases the toxicity of the metals in the cell through detoxification mechanisms (Valavanidis et al. 2006). In this study, MTs levels presented in both species were generally quite similar throughout the study areas, although slightly higher in *R. philippinarum*. These results suggest that Hg could be bound to membranes (insoluble fraction) because if they were present in their free form in cytosol, they would be bound to MTs. This is supported by Figueira et al. (2011) and Freitas et al. (2012) that found in *Cerastoderma* the majority of Hg in the insoluble fraction and no change on MTs levels, along an increasing contamination gradient. Different MTs patterns were observed by Figueira et al. (2012) in *R. decussatus* and *R. philippinarum*. These authors demonstrated that when exposed to different Cd concentrations, *R. decussatus* accumulated higher Cd amounts with low oxidative stress than *R. philippinarum*. The low oxidative stress revealed by *R. decussatus* was explained by the higher capacity of this species to increase the expression of MTs. Similar findings were observed in *R. decussatus* exposed to Pb (Freitas et al. 2014). Therefore, our results and previous studies can indicate that these two species may have a different tolerance capacity/mechanism towards different metals.

Evaluation of human risk consumption

Since Portugal is one of the top world seafood consumers, with an estimate seafood consumption of 58 kg/cap/year (FAO 2012), and clams are among the most relevant bivalve species for Portuguese consumers (Willemsen 2003; INE IP 2013; DGRM 2014), clam contamination should be an issue of special attention. Nevertheless, taking into account the maximum permissible limits (MPLs) of THg defined by international standards (0.5 mg kg^{-1} for EFSA, FSANZ and 1 mg kg^{-1} FW for USFDA), the concentrations found in this work for both clam species were lower than these regulatory guideline values. Furthermore, regarding PTWI values, our work revealed that both species represent a low risk of exceeding the PTWI for Hg and, therefore, represent a low risk to human health. When comparing to our results, Giani et al. (2012) showed that an adult (70 kg) needs to consume less *R. philippinarum* (357–420 g) from Marano and Grado lagoon during 1 week to exceed the PTWI. Mezghani-Chaari et al. (2011) demonstrated that the Hg contents of fish and seafood species (*Diplodus annularis*, *Sarpa salpa*, and *Sepia officinalis*) from the gulf of Gabes frequently exceeded the regulatory guideline value of 0.5 mg kg^{-1}, identifying fish and seafood consumption as a major contributor of Hg exposure in the surrounding human population.

The MeHg concentrations presented in *R. decussatus* and *R. philippinarum*, in all areas, were lower than the tolerable average residue level of MeHg (0.044 mg kg^{-1} wet weight) considered by Ipolyi et al. (2004).

Conclusion

The present study showed that the Ria de Aveiro is still recovering from the Hg discharges carried out between 1950 and 1994. Even after all these years, the THg concentration found in sediments revealed a spatial contamination gradient, with the highest concentration of THg found in areas closer to the discharge source. However, except for the most contaminated area, THg concentrations found were lower than TEL and ERL values.

The results obtained showed a strong correlation between THg concentration in *R. decussatus* and *R. philippinarum* clams and THg in the sediments, supporting the use of these species as sentinel species. Nevertheless, both species showed a low ability to bioaccumulate THg, as BAF values were always lower than 1.

These species presented lower THg concentrations than MPLs set by international standards organizations, therefore being safe to consume *R. decussatus* and *R. philippinarum*.

The similar percentages of MeHg in both clams from all areas are probably associated with a similar percentage of methylation of sulfate-reducing bacteria in sediments from the different study areas.

The present work further revealed that both species, but especially *R. philippinarum*, increased the LPO with the increase of THg in sediments, suggesting LPO as a good indicator of pollution. These results also demonstrated that, when in the same sampling area, although both species accumulated similar THg concentration, *R. philippinarum* is the most impacted species. The highest tolerance of *R. decussatus* will contribute to the maintenance of this species at the Ria de Aveiro, which is in agreement with Velez et al. (2015) that showed higher *R. decussatus* densities than *R. philippinarum* in areas where both species coexist.

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