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The use of *Cerastoderma glaucum* as a sentinel and bioindicator species: Take-home message

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ABSTRACT

Bivalves are frequently used to assess environmental contamination, and are often considered good sentinel and/or bioindicator species. For that reason the bioaccumulation and toxicity induced by metals and As in the cockle *Cerastoderma glaucum*, collected from areas with different contamination levels along the Óbidos lagoon (Portugal), were used to evaluate the use of this species as sentinel and/or bioindicator. The results showed that areas in the middle of the lagoon presented lower metals and As concentrations, lower total organic matter content and lower percentage of fine particles than areas in the Bom Sucesso arm. In all areas Cr, Pb and Cu were the most abundant elements, while Ni, As, Cd and Hg were less abundant. Results also showed a moderate correlation between total elements concentrations found in *C. glaucum* and in sediment, and thus caution should be taken when considering this species as a good sentinel species. The present study also revealed that, in general, *C. glaucum* from areas in the middle of the lagoon accumulated higher concentrations of metals and As (Biota-Sediment Accumulation Factor >1) than cockles from the most polluted areas located in the Bom Sucesso arm. However, in all areas, the majority of metals (Cu, Cr, Pb) were found in cockles insoluble fraction which may explain low cellular damage and reduced oxidative stress responses observed. Therefore, our results may further alert for caution when identifying *C. glaucum* as a good bioindicator species. Thus, our findings highlight the fact that studies should be cautious when selecting species for environmental monitoring, since good sentinels or bioindicators in highly polluted systems may not act in the same way in low or moderately contaminated areas. Furthermore, our study warns for the misclassification of cockles in different ecosystems.

1. Introduction

Coastal systems have been constantly threatened by pollution, due to the increase of urbanization, agriculture and industrial activities (Green-Ruiz and Páez-Osuna, 2001; Poulos et al., 2000). Considering this, it is well established that sediment act as a sink for a variety of contaminants, such as metals and metalloids (Buruaem et al., 2012; Hoffman et al., 2002), affecting benthic organisms (Dauvin, 2008). Environmental impact assessment studies have relied on monitoring benthic community parameters (e.g. species richness and abundance), measuring the concentrations of selected contaminants in sediments, water and organisms, and assessing induced toxicity in organisms (Box et al., 2007; Calabretta and Oviatt, 2008; Cheggour et al., 2005; Machreki-Ajmi and Hamza-Chaffai, 2006). Benthic communities typically consist of a variety of species that exhibit a wide range of physiological stress tolerances, feeding modes, and trophic interactions, making them good biological indicators to evaluate the impact of environmental contamination (Calabretta and Oviatt, 2008; Cheggour et al., 2005; Moschino et al., 2012). Despite their importance, studies conducted at the community level are time consuming and require a lot of expertise for species identification. For this reason the use of different species as sentinel organisms has become a common practice (e.g. Ferreira et al., 2009; Lima et al., 2008; Moschino et al., 2012). In this particular case the concentration of contaminants is measured in the organisms, and then used to assess environmental contamination. However, measuring the concentration of contaminants in organisms does not give information about impacts on

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their health status. Therefore, recent studies regarding environmental pollution assessment have relied on the use of bioindicator species, defined as species or group of species that readily reflect the abiotic or biotic state of an environment, revealing the impact of environmental changes on a population, community or ecosystem (Hamza-Chaffai, 2014; Holt and Miller, 2011). In the last years, different biochemical markers have been used in marine species to predict potential detrimental effects induced by different contaminants on organisms (Bergayou et al., 2009; Hamza-Chaffai, 2014; Valavanidis et al., 2006). This approach is suitable for early and sensitive detection of exposure to chemicals, since the measurement of toxicity at the cellular level constitutes the initial interaction between chemicals and biological systems (Monserrat et al., 2007). For this reason, the assessment of biochemical alterations imposed by a contaminant into the aquatic environment has become a common practice. Measuring the same biomarkers in the same species but in different sites gives us information about the pollution status and provides for a better comprehension of organisms responses (Chandurvelan et al., 2015). Recent studies have demonstrated that benthic organisms, namely bivalves, are successfully used as sentinel and/or bioindicator species to monitor coastal areas (Chandurvelan et al., 2015; Hamza-Chaffai, 2014; Karray et al., 2015; Torres et al., 2002). These organisms are chosen due to their ability to accumulate contaminants usually from water and food, reflecting the bio-available fraction (Chandurvelan et al., 2015). In addition, their relative immobility, wide distribution among different aquatic habitats, abundance, persistence, and ease of collection, make them good long-term indicators of environmental contamination (Hamza-Chaffai, 2014). In fact, several studies regarding environmental pollution and biomarkers have focused on bivalves such as the clams *Ruditapes philippinarum* (Adams & Reeve, 1850) (Chora et al., 2009; Moschino et al., 2012), *Chamelea gallina* (Linnaeus, 1758) (Monari et al., 2011), *Ruditapes decussatus* (Linnaeus, 1758) (Bebianno et al., 2004; Chora et al., 2009; Hamza-Chaffai et al., 2003; Smaoui-Damak et al., 2004; Velez et al., 2015a,b), the mussels *Mytella guyanensis* (Lamarck, 1819) (Torres et al., 2002), *Mytilus galloprovincialis* (Lamark, 1819), *Mytilus edulis* (Linnaeus, 1758) (Smaoui-Damak et al., 2004) and *Perna viridis* (Linnaeus, 1758) (Yusof et al., 2004), and the cockle *Cerastoderma edule* (Linnaeus, 1758) (Cheggour et al., 2001; Freitas et al., 2012; Nilin et al., 2012). Few studies are however known using the cockle *Cerastoderma glaucum* (Bruguière, 1789) (e.g. González-Fernández et al., 2015; Ladhar-Chaabouni et al., 2009; Machreki-Ajmi et al., 2008, 2011; Machreki-Ajmi and Hamza-Chaffai, 2006, 2008; Hamza-Chaffai, 2014). This species is a sedentary suspension and deposit feeder (Caspers, 1981) with a wide spatial distribution (WoRMS, 2015), which allows the use of this species as a good candidate as a sentinel (Karray et al., 2015; Szefer et al., 1999) and bioindicator (Machreki-Ajmi and Hamza-Chaffai, 2006; Machreki-Ajmi et al., 2008) species, reflecting environmental pollution levels and impacts. Biochemical alterations induced in this species by contaminants have been focused on a limited number of biomarkers (acetylcholinesterase activity, lipid peroxidation and metallothioneins levels), on few aquatic systems (Tunisian coast and Arcachon Bay, France) and particularly, in low contaminated areas (González-Fernández et al., 2015; Hamza-Chaffai, 2014; Ladhar-Chaabouni et al., 2009; Machreki-Ajmi et al., 2008; Machreki-Ajmi and Hamza-Chaffai, 2006, 2008; Paul-Pont et al., 2010). Nevertheless, due to *C. glaucum* ecological and economic importance (Abdallah et al., 2011; Kandeel et al., 2013), and wide spatial distribution (WoRMS, 2015), it is of prime relevance to evaluate this species performance when under different contamination scenarios.

Thus, the present study aimed to characterize metals and As bioaccumulation and cellular partitioning, and the overall biochemical responses of *C. glaucum* under different environmental conditions. For that purpose, a multi biomarker approach was used, including the measurement of lipid peroxidation (LPO), total protein content, reduced (GSH) and oxidized (GSSG) glutathione content, glutathione *S*-transferases (GSTs), superoxide dismutase (SOD) and catalase (CAT) activities and metallothioneins (MTs). This approach used different biomarkers to reflect the effects of different contaminants in *C. glaucum* collected from different areas along the Óbidos lagoon (Portugal). To ensure the correct identification of the species, often misidentified as *C. edule*, morphological and genetic analyses were performed.

2. Material and methods

2.1. Study area

The Óbidos lagoon is a small and shallow coastal system permanently connected to the sea, located in the Atlantic West Coast of Portugal (Oliveira et al., 2006). This lagoon covers an area of approximately 7 km^2 and with an average depth of approximately 1 m. The Óbidos lagoon is divided in two arms: Barrosa and Bom Sucesso arms. The Barrosa arm receives agriculture and urban effluents from Caldas da Rainha city, resulting in an area with high nutrients availability, previously classified as eutrophic (Carvalho et al., 2011; Pereira et al., 2009a). This arm is mostly contaminated by metals and metalloids, and the major source of these contaminants is related to wastewater discharges in Cal River (Oliveira et al., 2006; Pereira et al., 2008). The Bom Sucesso arm receives a smaller freshwater flow (Vala do Ameal) with better water quality than the Cal River (Carvalho et al., 2011). According to previous studies this lagoon has been classified as a moderately contaminated system (Pereira et al., 2009b,c; Carvalho et al., 2011).

2.2. Sampling procedure

Cerastoderma sp. specimens were collected in 6 different areas (named A to F) of the Óbidos lagoon (Fig. 1), representing different contamination levels and physico-chemical characteristics. In each area three sites were selected and in each site all cockles present in a 11.25 cm^2 rectangle (45 cm × 25 cm) were collected. At each area, three sediment replicates were collected (one per site) for sediment grain size analysis, total organic matter (TOM) content determination, and quantification of elements concentration (chromium (Cr), nickel (Ni), copper (Cu), lead (Pb), cadmium (Cd), mercury (Hg), and arsenic (As)).

The environmental variables pH, salinity and temperature were measured with specific probes at each sampling site. After sampling, specimens and sediment were transported on ice (0 °C) to the laboratory.

2.3. Laboratory analysis

2.3.1. Sediment grain size and organic matter content determination

Sediment grain-size was analyzed by wet and dry sieving following the procedure described by Quintino et al. (1989). The median value, P_{50}, was calculated and expressed in phi (ϕ) units, corresponding to the diameter that has half the grains finer and half coarser (dry weight). The median and the percent content of fines were used to classify the sediment, according to the Wentworth scale: very fine sand (median between 3–4ϕ); fine sand (2–3ϕ); medium sand (1–2ϕ); coarse sand (0–1ϕ); very coarse sand (−1 to 0ϕ). The silt and clay fraction was obtained by wet sieving through a 0.063 mm mesh screen and classified as “clean“, “silty” or “very silty” according to fine fraction range (0–5%, 5–25% and 25–50%, respectively) of the total sediment, dry weight (Doeglas, 1968). Samples with more than 50% fines content were classified as mud.

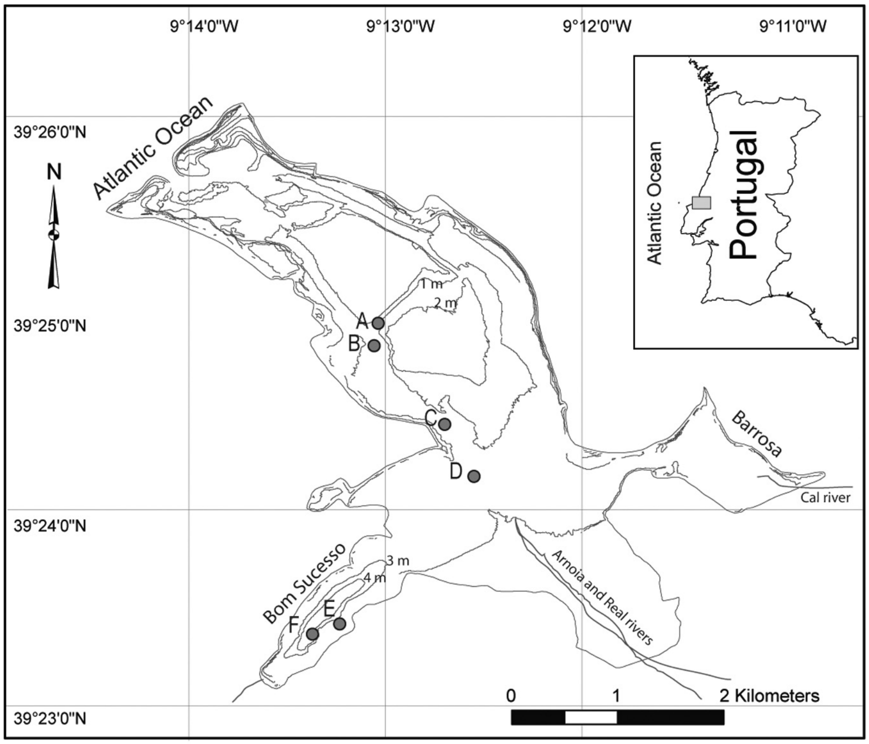

Fig. 1. Schematic map of the sampling areas (A to F) in the Óbidos lagoon.

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

2.3.2. Metals and As quantification in sediment and cockles

The concentration of 7 elements (Cr, Ni, Cu, Pb, Cd, Hg, and As) was quantified in individuals soluble and insoluble fractions and bulk sediment.

For sediment (bulk samples), 2 g of homogenized sediment was digested overnight at 115 °C with 10 mL of concentrated $1HNO_3$:3HCl in Teflon bombs (sealed chambers). After cooling, the final volume was made up to 50 mL with high purity deionized water for all samples. Once isolated, samples were preserved at 4 °C until quantification of metals and As concentrations in sediment.

For soluble and insoluble fractions, individuals were thawed and homogenized with liquid nitrogen using a mortar and a pestle, and subjected to subcellular fractioning by centrifugation at 1450 *g*, for 15 min at 4 °C. Fractioning resulted in the isolation of two distinct subcellular fractions. According to Wallace and Luoma (2003), the soluble fraction is defined as the elements soluble in cytosol in their free form or bound to cytoplasmic molecules (including metallothionein-like proteins), while the insoluble fraction includes the elements in the organelles (metal-rich granules, MRG) and cellular debris. Both fractions were digested overnight at 115 °C with 2 mL of concentrated $1HNO_3$:3HCl in Teflon bombs (sealed chambers). To prevent the loss of metals by volatilization, chambers were only open when completely cooled. The final volume was made up to 15 mL with high purity deionized water for all samples.

Element quantification was done by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry), in a certified laboratory at the University of Aveiro. Regarding quality controls, the calibration of the apparatus was made with successive dilutions of multi-element standard ICP 71A from IV (Inorganic Venture, Christiansburg, VA, USA). The fitness of the calibration curve was checked with CRM NIST 1643e. The procedure was verified with standard certificated reference materials (CRM): MESS-3 (for sediment) and TORT-2 (for cockles soft tissue), both from NRCC (National Research Council of Canada). The values obtained for all the CRM analyses ranged from 90% to 110% of the concentration defined for these materials. All samples below this accuracy level were rejected and the analysis repeated to ensure that quantification of elements was precisely done.

The concentration of elements in cockles was expressed in mg per kg fresh weight (FW) for comparison with the maximum regulatory levels (USFDA, United States Food and Drug Administration; EFSA, European Food Safe Authorities; FSANZ, Food Standards Australia and New Zealand) which are expressed in mg/kg fresh weight (FW). The concentration of elements in cockles was further converted into mg/kg dry weight (DW) following Ponsero et al. (2009) for better comparison with literature data.

The concentration of elements in sediment was expressed in mg per kg of DW because sediment quality guidelines (threshold effect level, TEL; effects range low, ERL; Macdonald et al., 1996) are expressed in mg/kg DW. For this, sediments were dried during 48 h at 25 °C.

The Biota-Sediment Accumulation Factor (BSAF) was determined according to Cheng et al. (2013), dividing a given metal or As concentration found in cockles tissue (dry weight) by the concentration of the same element in the sediment (dry weight).

2.3.3. Organisms morphological, genetic and biochemical characterization

In the laboratory, 20 specimens from each area were used for morphological characterization. For genetic analyses, a subsample of 5 individuals from each area were preserved in refrigerated ethanol 96 °C and then stored at −80 °C to prevent DNA degradation. After morphological inspection, to ensure species correct identification, whole soft tissues of 6 individuals from each site were used for metal and As quantification and also for biochemical analysis.

2.3.3.1. Morphological characterization of Cerastoderma *spp.*
C. glaucum and *C. edule* are frequently misidentified, since *C. glaucum* is commonly identified as *C. edule*. For this reason, morphological characterization of *Cerastoderma* spp., specimens was conducted to distinguish both species. The following morphological features were examined according to Machado and Costa (1994) recommendation: shell shape and color, type of calcareous scales, valve profile, ligament visibility, ventral and posterior valve junctions, periostracum covering, and internal spots.

2.3.3.2. Genetic analyses.

DNA extraction. To assure the correct identification of the specimens, molecular analysis were conducted. Total genomic DNA was extracted with DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Purified DNA was aliquoted in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and subsequently stored at −20 °C.

PCR amplification of COI gene fragments. Partial regions of the mitochondrial cytochrome c oxidase subunit I (*COI*) (∼700 bp) gene were amplified by PCR using the universal primers: LCO 1490 (5′-GGTCAACAAATCATA AAGATATTGG-3′) and HCO 2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994). PCR reactions were performed in a final volume of 25 μL containing 10–100 ng of genomic DNA, 1 μM of each primer, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP (Nzytech) and 0.65 U Taq DNA polymerase (Nzytech). Amplification occurred with the following thermal cycling parameters: an initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 65 °C for 30 s and extension at 72 °C for 1 min and final extension at 72 °C for 7 min (Tarnowska et al., 2010).

Amplification products were visualized, after agarose gel electrophoresis and ethidium bromide staining, to confirm the amplicons sizes.

DNA sequencing and analysis. Nucleotide sequencing of each PCR-amplified fragment on both orientations and from two independent reactions were commercially performed (STAB Vida, Portugal).

The obtained sequences were compared with those available in genomic databases using Blast and multiple alignments of sense and antisense sequences, conducted with MEGA v6 (Tamura et al., 2013) with CLUSTALW, using the default alignment settings and subsequently manually edited by eye using BioEdit version 7.0.0 (Hall, 1999). Gap positions and regions that could not be aligned unambiguously were excluded from the analysis. Concerning phylogenetic analysis, the experimental sequences were aligned with those of *C. glaucum* downloaded in GenBank (Accession Numbers: AY226908; FJ555387; FJ555445; JQ319627) and *C. edule* (Accession Numbers: EU523671; JQ319620; KJ659801). The species *Tridacna squamosa* (Lamarck, 1819) (Accession Number: JN392025) was used as outgroup belonging to the same Cardioidea Superfamily.

Phylogenetic analyses of *COI* gene fragments were conducted with the software MEGA v6 (Tamura et al., 2013) by applying Maximum Likelihood estimating standard error by a bootstrap procedure (1000 replicates).

2.3.3.3. Oxidative stress biomarkers.
Cockles whole soft tissue (0.5 g) was used for each biochemical parameter and from each individual. Samples were extracted with a specific buffer for each biomarker and centrifuged for 15 min at 10 000 *g* at 4 °C. Supernatants were stored at −80 °C or immediately used to determine total soluble protein, lipid peroxidation (LPO), glutathione S-transferases (GSTs), reduced (GSH) and oxidized glutathione (GSSG), superoxide dismutase (SOD), catalase (CAT) and metallothioneins (MTs).

Total soluble protein content was determined according to Robinson and Hogden (1940), following the Biuret method, using bovine serum albumin (BSA) standards (0–40 mg/g) and measuring the absorbance at 540 nm. The results were expressed in mg per g of fresh weight (FW).

LPO content was based on the quantification of malondialdehyde (MDA), with 2-thiobarbituric acid (TBA) (forming TBARS), according to the protocol described by Buege and Aust (1978), with adaptations described by Freitas et al. (2012). The samples were incubated at 96 °C for 25 min and transferred to an ice bath to stop the reaction. The absorbance was measured at 535 nm ($\varepsilon = 156\ mM^{-1}\ cm^{-1}$). Results were expressed in nmol of MDA formed per g of FW.

GSTs activity was determined following the method described by Habig et al. (1974) and adaptations described by Carregosa et al. (2014), measuring the absorbance at 340 nm ($E = 9.6\ mM^{-1}\ cm^{-1}$). The samples were incubated at room temperature. Results were expressed in U per g of FW, where U corresponds to the amount of enzyme that caused the formation of 1 μmol of thioether per min under the assay conditions.

GSH and GSSG content were determined according to Rahman et al. (2006). The absorbance was read at 420 nm. The samples were incubated at room temperature. Results were expressed in μmol per g of FW.

SOD activity was determined by the method described by Beauchamp and Fridovich (1971), with slight modifications (Freitas et al., 2012), using SOD as standard (0.25 to 60 U/g). Samples were incubated at room temperature. Results were expressed in U per g of FW, where U corresponds to a reduction of 50% of nitroblue tetrazolium (NBT).

CAT activity was determined according to Johansson and Borg (1988), with some modifications (Freitas et al., 2012). Formaldehyde (0–150 μM) standards were used. Samples were incubated at room temperature. Absorbance was read at 540 nm. Results were expressed in U per g FW, where U represents the formation of 1 nmol formaldehyde per min, under the assay conditions.

For MTs levels, proteins polypeptides were separated by SDS–PAGE, carried out in 4–20% of polyacrylamide (Mini PROTEAN TGX—Bio-Rad) according to the procedure described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250 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 Color Protein Marker II—NZY Tech Genes and Enzymes). The absorbance of proteins was calculated using Quantity One program software (Bio-Rad) and the protein concentration was measured according to the Robinson and Hogden (1940) method, using bovine serum albumin (BSA) as standard. Then each band of proteins was cut and the extraction done according to Milnerowicz and Bizoń (2010). The confirmation of MTs was done through quantification of thiol groups, according to Moron et al. (1979).

2.4. Data analyses

Environmental parameters (pH, salinity, temperature, fines and TOM content), metals and As concentrations in sediment and organisms, oxidative stress biomarkers and BSAF values were independently submitted to hypothesis testing using permutational multivariate analysis of variance with the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008). Thus, for each descriptor, a two-way hierarchical design was followed, with sites nested in areas and these as the main fixed factor. The *t*-statistic of pair-wise comparisons for each descriptor were evaluated in terms of significance among different areas. Values lower than 0.05 were considered as significantly different. The null hypotheses tested were: (a) for environmental parameters: no significant differences exist among areas; (b) for sediment contamination: no significant differences exist among areas; (c) for metals and As accumulation in organisms: no significant differences exist among areas; (d) for soluble and insoluble fraction: no significant differences exist among areas; (e) for each biochemical marker: no significant differences exist among areas; (f) for BSAF values: no significant differences exist among areas.

The significant differences obtained were identified with letters (a–d): distinct letters corresponded to significant differences among areas, while the same letter indicated no significant differences among areas.

The Spearman correlation was used to evaluate the correlation between elements concentration in sediments, environmental parameters, and elements concentration in cockles, cellular biomarkers and BSAF values. The Spearman rank was classified as weak (0.26–0.49), moderate (0.50–0.70), strong (0.70–0.89) and very strong (0.90–1.00) (Munro, 2005).

Taking into consideration the bioaccumulation levels found in cockles from all areas, the amount of soft tissues that an adult with 70 kg needs to consume in one 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 a given element in cockles.

3. Results

3.1. Physico-chemical characteristics of sampling areas

All sampling areas in the Óbidos lagoon were characterized by a temperature range of 22.0–26.3 °C and pH of 7.3–7.9, with no significant differences among areas. Salinity was significantly lower in area A (21.0) compared to the remaining areas (32–38.3). Sediment in areas located in the middle of the lagoon (areas A to D) were classified as clean and silty medium sand (median values from 1.66 to 2.24), with high percentage of sand (94.71–98.17%), low percentage of fine particles (1.83–5.29%) and low percentage of total organic matter (1.13–2.80%). The Bom Sucesso arm areas (E and F) were classified as mud, with low percentage of sand (10.60 to 33.42%), high percentage of fine particles (66.58 to 89.40%) and high TOM content (9.65 to 12.31%).

3.2. Metals and As concentrations in sediment and cockles

The total concentration of the elements analyzed in the sediment is presented in Fig. 2A. The results obtained showed that elements concentration was significantly lower in the areas located in the central part of the lagoon (areas A to D, 7.15–14.77 mg/kg DW) than in the areas located in the Bom Sucesso arm (areas E and F, 74.16–98.03 mg/kg DW) (cf. Fig. 2A). The concentration levels obtained in sediment were correlated with the physico-chemical parameters (pH, temperature, salinity, fine particles and TOM), showing a moderate correlation with the TOM content (0.66) and with the percentage of fine particles (0.56).

The results obtained further revealed that, among the analyzed elements in sediment, the most abundant were Cr, Pb and Cu (28–36%, 16–31% and 17–28%, respectively), while Ni, As, Cd and Hg were the least abundant elements (11–16%, 10–13%, less than 1% for Cd and Hg, respectively) (Fig. 2B). The concentration of

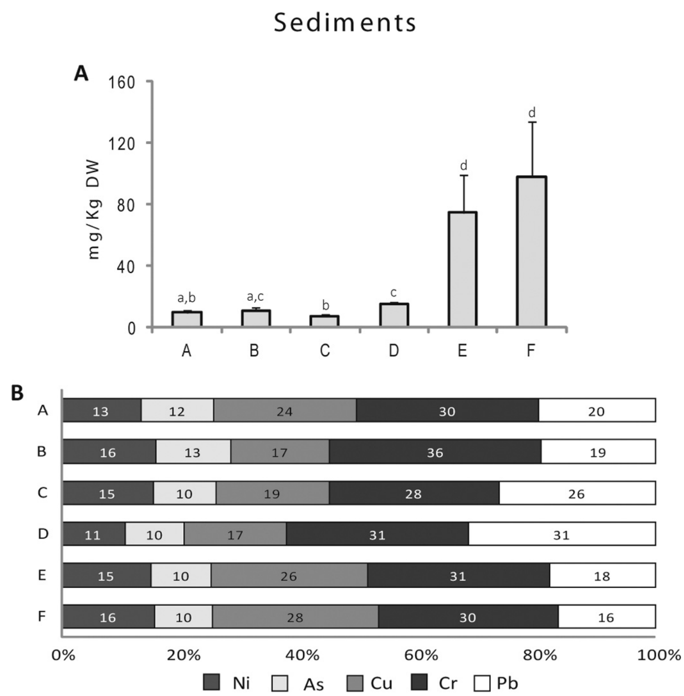

Fig. 2. (A) Total element concentration (mg/kg DW) and (B) percentage of each element (Ni, As, Cu, Cr, Pb) in sediment, along the 6 sampling areas (A to F). Significant differences ($p \leq 0.05$) among areas are represented with different letters (a–d).

Table 1
Element concentration (mg/kg dry weight) in sediment from the 6 sampling areas (A to F). Highlighted in gray are the concentrations higher than ERL and TEL values. For each element and for total element concentration, significant differences ($p \leq 0.05$) among areas are presented with distinct letters (a–d).

Areas	As	Pb	Hg	Cr	Ni	Cu	Cd	Total
A	1.18± 0.13^{a}	1.9 0± 0.1^{a}	<0.03^{a}	2.98 ± 0.63^{a,b}	1.30 ± 0.25^{a}	2.33 ± 0.54^{a,b,c}	<0.03^{a}	9.71 ± 1.63^{a,b}
B	1.37 ± 0.09^{a}	2.10 ± 0.10^{a}	<0.03^{a}	3.98 ± 1.08^{a,c}	1.79 ± 0.68^{a}	1.80 ± 0.04^{a}	<0.03^{a}	11.06±1.77^{a,c}
C	0.74 ± 0.10^{b}	1.90 ± 0.20^{a}	<0.03^{a}	2.03±0.24^{b}	1.13± 0.45^{a}	1.36±0.25^{b}	<0.03^{a}	7.15 ± 1.06^{b}
D	1.48 ± 0.16^{a}	4.70 ± 1.30^{b}	<0.03^{a}	4.48 ± 0.15^{c}	1.58 ± 0.17^{a}	2.53 ± 0.11^{c}	<0.03^{a}	14.77 ± 1.58^{c}
E	7.36 ± 1.99^{c}	15.86± 5.03^{c}	<0.03^{a}	29.46 ± 10.48^{d}	15.53 ±5.69^{b}	27.74 ± 11.63^{d}	0.05±0.03^{a,b}	74.16±25.08^{d}
F	9.36 ±2.99^{c}	13.07±4.09^{c}	<0.03^{a}	22.69 ± 7.22^{d}	11.26 ±3.96^{b}	19.71 ± 7.91^{d}	0.07±0.02^{b}	98.03 ±35.76^{d}
ERL	8.20	46.70	0.15	81.00	20.90	34.00	1.20	
ERM	70.00	218.00	0.71	370.00	51.60	270.00	9.60	
TEL	7.24	30.24	0.13	52.30	15.90	18.70	0.68	
PEL	41.60	112.00	0.70	160.00	42.80	108.00	4.21	

TEL, threshold effect level (Macdonald et al., 1996); PEL, probable effects levels (Macdonald et al., 1996); ERL, effects range low (Long and Morgan, 1990; Long et al., 1995); ERM, effects range median (Long and Morgan, 1990; Long et al., 1995).

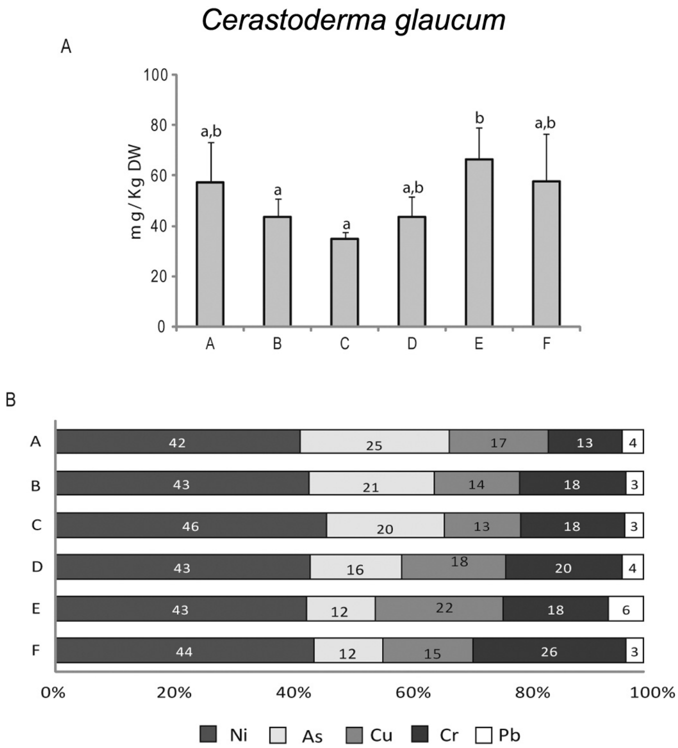

Fig. 3. (A) Total element concentration (mg/kg DW) and (B) percentage of each element (Ni, As, Cu, Cr. Pb) in *C. glaucum*, along the 6 sampling areas (A to F). Significant differences ($p \leq 0.05$) among areas are represented with different letters (a and b).

As (9.36 ± 2.99 mg/kg DW) and Cu (27.74 ± 11.63 mg/kg DW and 19.71 ± 7.91 mg/kg DW) found in Bom Sucesso areas (E and F) reached the sediment quality guideline levels, namely the values for the effects range low (ERL) (8.20 mg/kg DW for As) and the threshold effect level (TEL) (7.24 mg/kg DW for As and 18.70 mg/kg DW for Cu) (Table 1) (Long and Morgan, 1990; Macdonald et al., 1996).

Total elements concentration analyzed in cockles is present in Fig. 3A. In general, results showed that *C. glaucum* from areas A, E and F presented higher total elements concentration (57.48 ± 15.64 to 67.86 ± 12.80 mg/kg DW) than cockles from areas B, C and D (34.68 ± 3.03 to 43.48 ± 8.29 mg/kg DW). Nevertheless, no significant differences were found between areas, except between areas B, C and E (cf. Fig. 3A). Total element concentration found in *C. glaucum* showed a moderate correlation (0.54) with the total element concentration in sediment.

The BSAF values for *C. glaucum* revealed higher elements bioaccumulation (BSAF > 1) in cockles from areas located in the middle of the lagoon (Table 2) while organisms from areas with higher element concentrations (Areas E and F, 0.92 ± 0.17 to 0.60 ± 0.20) showed lower bioaccumulation capacity (BSAF < 1) (cf. Table 2).

The concentration and percentage of each element in *C. glaucum* are presented in Fig. 3B and Table 2, respectively. Ni was the most abundant element present in *C. glaucum* from all areas (16.00 ± 2.99 to 28.94 ± 10.43 mg/kg DW), also showing the highest percentage (42–46%) among the elements quantified (cf. Fig. 3B). Results obtained also showed that Pb, Hg and Cd (cf. Table 3) were the least abundant elements found in *C. glaucum* in all the areas (1.12 ± 0.20 to 4.05 ± 1.87 mg/kg DW for Pb and less than 0.17 mg/kg DW for Hg and Cd).

Elements concentration in *C. glaucum* were below the maximum regulatory levels defined by the international organizations (USFDA, United States Food and Drug Administration; EFSA, European Food Safe Authorities; FSANZ, Food Standards Australia and New Zealand), with the exception of As which was above the FSANZ values (1 mg/kg FW; 1.03 ± 0.16 to 1.78 ± 0.86 mg/kg FW, areas A, B, C, E). Regarding the amount of cockles soft tissue that a 70 kg adult needs to consume in one week to exceed the PTWI for As, results showed that it is necessary to consume only between 0.59 kg (area C) and 1.33 kg (area F) of cockle flesh.

The percentage of each element and the percentage of total element concentration quantified in cockles soluble and insoluble fractions are presented in Table 3. Overall, the results obtained for each element, showed that the majority of Ni and As (49.1 ± 17.5 to 54.4 ± 8.5% and 78.3 ± 7.6 to 60.1 ± 1.6%) were found in the soluble fraction, while the majority of Cu, Cr and Pb (54.0 ± 11.2 to 64.4 ± 7.8%, 52.7 ± 19.3 to 74.4 ± 13.5% and 63.6 ± 9.1 to 74.7 ± 4.7%, respectively) was found in the insoluble fraction (cf. Table 3). Furthermore, concerning the total concentration of the elements analyzed, the present findings revealed a similar percentage in the soluble and insoluble fractions (46.2 ± 6.2 to 54.5 ± 5.3% and 44.2 ± 6.9 to 53.8 ± 6.2%, respectively), with no significant differences among areas.

3.3. *Morphological characterization of* Cerastoderma *spp.*

The analyses conducted allowed to identify the specimens as *C. glaucum*. Among the collected specimens only one was identified as *C. edule*. The two species (*C. glaucum* and *C. edule*), frequently confounded, were distinguished mainly through valve profile, shape of valve junction, type of calcareous scales and shell color. In *C. glaucum*, the valve profile is characterized by protuberant ribs and the shape of valve junction is commonly straight, both ventrally and posteriorly, while *C. edule* is characterized by smooth to flat, non-protuberant ribs in the valves and crenulated valve junctions. The ligament was most of the times visible in *C. edule* shells but

Table 2
Element concentration (mg/kg dry weight) in *C. glaucum* and BSAF (Biota-Sediment Accumulation Factor) along the sampling areas (A to F). The most and least abundant element in cockles from each area is highlighted in dark and light gray, respectively.

Area	Ni	As	Cu	Cr	Pb	Hg	Cd	Total	BSAF
A	23.92±7.37^{a,b}	14.58±7.22^{a}	9.69±2.27^{a,c}	7.19±1.63^{a}	2.09±0.80^{a,b}	<0.17^{a}	<0.17^{a}	57.48±15.64^{a,b}	6.03±1.62^{a}
B	18.75±2.96^{a}	9.33±2.61^{a}	6.31±0.83^{a,b}	7.86±3.10^{a}	1.34±0.18^{a}	<0.17^{a}	<0.17^{a}	43.59±7.10^{a}	4.03±0.65^{a,b}
C	16.00±2.99^{a}	6.92±1.31^{a}	4.50±1.46^{b}	6.14±0.57^{a}	1.12±0.20^{a}	<0.17^{a}	<0.17^{a}	34.68±3.03^{a}	4.83±0.44^{a}
D	18.81±2.92^{a}	6.78±1.35^{a}	7.72±2.37^{a,b,c}	8.56±2.00^{a}	1.62±0.35^{a}	<0.17^{a}	<0.17^{a}	43.48±8.29a	3.01±0.56^{b}
E	28.94±10.43^{a,b}	8.00±2.32^{a}	14.78±4.89^{c}	12.08±5.20^{a}	4.05±1.87^{b}	<0.17^{a}	<0.17^{a}	67.86±12.80^{b}	0.92±0.17^{c}
F	25.58±2.39^{b}	6.83±2.29^{a}	8.97±05.98^{a,b,c}	15.06±9.47^{a}	1.76±0.41^{a}	<0.17^{a}	<0.17^{a}	57.34±18.40^{a,b}	0.60±0.20^{c}

Significant differences ($p \leq 0.05$) among areas for each total element concentration are presented with letters (a–c).

Table 3
Percentage of each element (Ni, As, Cu, Cr and Pb) and total element percentage found in the soluble and insoluble fractions of *Cerastoderma glaucum*.

	Ni		As		Cu		Cr		Pb		Total	
	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble
A	52.2±4.9[a]	47.8±4.9[a]	68.4±0.4[a]	31.6±3.4[a]	35.6±7.8[a]	64.4±7.8[a]	42.0±9.3[a]	58.0±9.3[a]	27.9±7.8[a]	72.1±7.8[a]	51.2±3.8[a]	48.8±3.8[a]
B	54.4±8.5[a]	45.6±8.5[a]	76.1±5.5[a]	23.9±5.5[a]	46.0±6.0[a]	54.0±11.2[a]	37.6±10.3[a]	62.4±10.3[a]	31.7±1.8[a]	68.3±1.8[a]	54.5±5.3[a]	45.5±5.3[a]
C	49.1±17.5[a]	50.9±17.5[a]	78.3±7.6[a]	21.7±6.5[a]	42.7±6.9[a]	57.3±6.9[a]	43.8±3.8[b]	56.2±3.8[a]	36.4±9.1[a]	63.6±9.1[a]	52.1±2.5[a]	44.2±6.9[a]
D	53.4±13.0[a]	46.6±13[a]	60.4±4.2[a,c]	33.6±4.2[c]	45.6±33.4[a]	54.4±18.4[a]	32.9±13.2[a,c]	67.1±13.2[a]	25.3±4.7[a]	74.7±4.7[a]	49.0±12.1[a]	51±12.1[a]
E	51.7±8.4[a]	48.3±8.4[a]	60.1±1.6[a,c]	39.9±1.9[c]	52.2±25.5[a]	47.8±25.5[a]	47.3±19.3[a,c]	52.7±19.3[a]	54.3±17.9[a]	45.7±17.9[a]	53.7±11.4[a]	46.3±11.4[a]
F	51.1±16.7[a]	48.9±13[a]	66.3±14.1[c]	33.7±14.1[c]	55.7±18.2[a]	44.3±18.2[a]	25.6±13.5[c]	74.4±13.5[a]	32.1±5.5[a]	67.9±5.5[a]	46.2±6.2[a]	53.8±6.2[a]

Significant differences ($p \leq 0.05$) among areas for each element concentration in the soluble and insoluble fractions are presented with letters (a–c). The most abundant element, in cockle from each of the areas, is highlighted in gray.

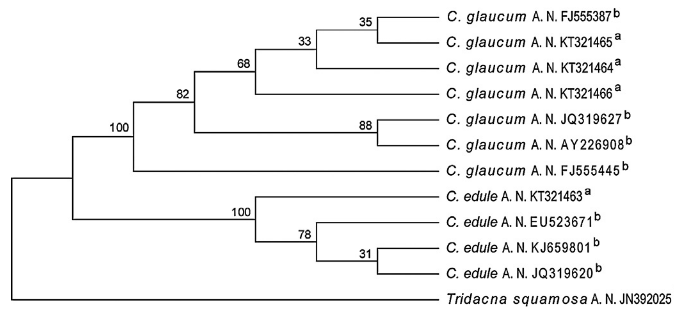

Fig. 4. Maximum Likelihood tree (ML) of *COI* sequences of *C. glaucum* and *C. edule* with bootstrapping values reported. Letter a refers to sequences from this study and letter b refers to sequences downloaded from GenBank.

concealed in *C. glaucum*, when shells were inspected laterally. The calcareous scales in *C. glaucum* were worn away or slightly thick, bent to the umbonal region while in *C. edule* they are less worn away, laminar and perpendicularly inserted in the valves. Both species exhibit a large periostracum and the shell color is glaucum-yellow or salt-pit green in *C. glaucum* and variable colored in *C. edule*. The shell shape and internal brown spot variability was similar for both species.

It is interesting to highlight that *C. edule* from the Óbidos lagoon was morphologically closer to *C. glaucum* than *C. edule* specimens from the Mira channel in the Ria de Aveiro (L. Sampaio, personal communication), namely in shell shape (less rounded) and ligament visibility (shorter length).

The weight of *C. glaucum* from the sampling areas varied between 6.2 ± 0.1 and 8.5 ± 2.6 g. The length and width values ranged between 3.3 ± 0.1 and 2.4 ± 0.2 cm, and between 2.7 ± 0.2 and 2.1 ± 0.2 cm, respectively.

3.4. Genetic analyses

A 551-bp *COI* fragment was successfully amplified and sequenced from 21 individuals of *C. glaucum* distributed among all sampled areas and 1 from *C. edule*, from area A. Nucleotide sequences from both species were deposited at EMBL GenBank database, under the accession numbers: KT321463 to KT321466.

In the case of *C. glaucum*, two substitutions were detected, corresponding to two transitions at positions 161 (adenine by guanine) and 302 (thymine by cytosine).

Maximum Likelihood analysis of *COI* sequences confirmed the separation of *Cerastoderma* spp. samples collected in the Óbidos lagoon into two distinct species (Fig. 4). The percentage of nucleotide divergence of the *COI* gene between *C. glaucum* and *C. edule* was 19% (nucleotide substitution). Moreover, translating DNA sequences into amino acids revealed no differences in the amino acid composition of the analyzed *COI* fragment between both species. The majority of the differences in nucleotides between both species occurred on the third position of the codon and therefore corresponded to silent alterations. The silent alterations are generally affected by the third position of the nucleotides in the codons, not changing the amino acid coding (Stryer, 1999).

3.5. Oxidative stress biomarkers

Results concerning oxidative stress biomarkers (LPO, protein content, CAT, SOD, GSTs, GSH, GSSG and MTs), measured in cockles collected from all areas, are presented in Fig. 5.

Protein content was higher in areas A and B (20.2 ± 6.19 and 22.3 to 9.28 mg/g FW, respectively) than in the remaining areas

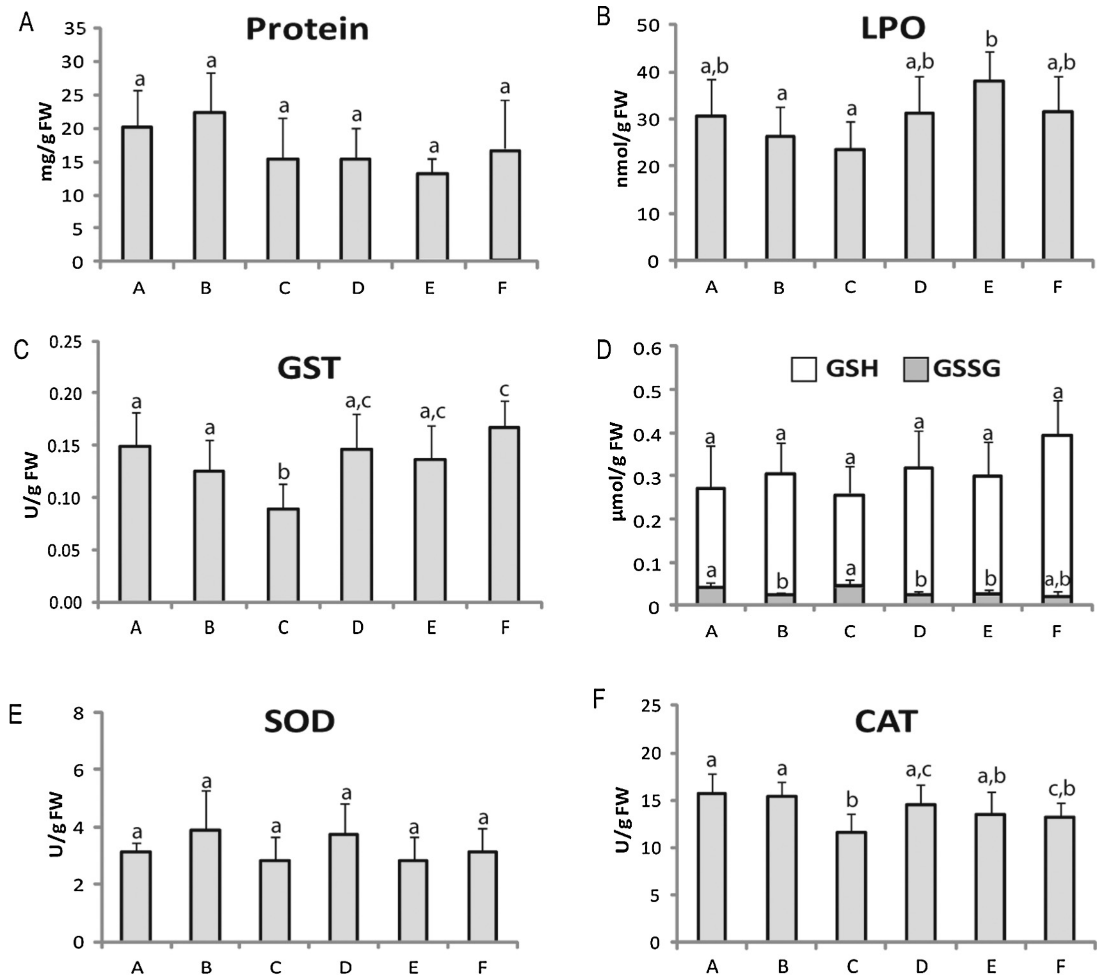

Fig. 5. (A) Total protein content, (B) lipid peroxidation (LPO), (C) GST, glutathione-*S*-transferase, (D) reduced glutathione (GSH) and oxidized glutathione (GSSG), (E) superoxide dismutase (SOD) and (F) catalase (CAT) in *C. glaucum*, along the 6 sampling areas (A to F). Significant differences ($p \leq 0.05$) among areas are represented with different letters (a–c).

(13.1 ± 4.3 to 15.3 to 4.1 mg/g FW) (Fig. 5A), but no significant differences were found among areas.

LPO was lower in areas B and C compared with values obtained for the remaining areas (Fig. 5B). Cockles with the highest total elements accumulation (Area E) presented the highest LPO levels (Figs. 3A and 5B). Significant differences were found between areas B, C and E.

GSTs activity was significantly lower in cockles from area C (area with lower elements concentration in cockles) when compared with the remaining areas (Fig. 5C).

GSH levels showed no significant differences among areas (Fig. 5D), while the GSSG levels was higher in cockles from area A and C (0.04 ± 0.02 U/g FW) than in the remaining areas (0.02 ± 0.01 to 0.03 ± 0.01 U/g FW) (Fig. 5D).

SOD and CAT activity showed no significant differences among areas, except for CAT activity in area C, where this enzyme presented significantly lower activity compared to areas A, B and D (Fig. 5E and F).

MTs levels were significantly higher in cockles from areas A to C than in cockles from areas D to F (Fig. 6). When correlating MTs levels with metals and As concentrations in cockles the results showed no correlation.

Overall, the correlation of biochemical parameters and BSAF values in cockles was weak (<0.49), except for BSAF values and MTs levels (0.76).

4. Discussion

The capability that bivalves have to accumulate substantial amounts of metals and metalloids from water and sediment has been described by several authors (Baudrimont et al., 1997; Chandurvelan et al., 2015; Cheggour et al., 2005; Velez et al., 2015a,b), as well as their use as bioindicators or sentinel species to evaluate environmental pollution (e.g. Chora et al., 2009; Moschino et al., 2012; Szefer et al., 1999; Yusof et al., 2004).

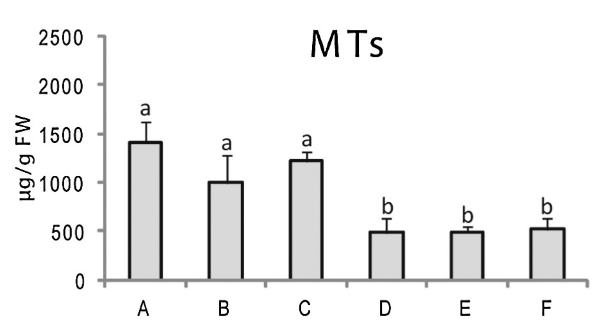

Fig. 6. Metallothioneins (MTs) levels in *C. glaucum*, along the 6 sampling areas (A to F). Significant differences ($p \leq 0.05$) among areas are represented with different letters (a and b).

Nevertheless, up to date, scarce information on the biochemical impacts induced by the accumulation of metals and As have been documented on *C. glaucum*, a species with important ecological and economic relevance (Abdallah et al., 2011; Kandeel et al., 2013) and a wide spatial distribution (WoRMS, 2015). *C. glaucum* is frequently miss-identified as *C. edule* and, for this reason, the present study established the correct taxonomy of this species through the identification of morphological characteristics and subsequent confirmation by genetic analyses.

4.1. Metals and As in sediments and cockles

Regarding total elements concentration analyzed in sediment, our results showed that areas in the middle of the Óbidos lagoon presented significantly lower contamination (areas A to D, 7.15–14.77 mg/kg DW) than areas located at the Bom Sucesso arm (areas E and F, 74.16–98.03 mg/kg DW). The total element concentrations observed in the present study were similar to concentrations found in a low contaminated system (Ria de Aveiro, Portugal) (4.31–52.15 mg/kg DW, Velez et al., 2015a), but lower than values found in sediments from other lagoons, namely the Venice lagoon (Italy) (67–1630 mg/kg DW, Sfriso et al., 2008; Moschino et al., 2012), the Saint Louis estuary (Senegal, Africa) (98–1563 mg/kg DW, Diop et al., 2015) and the Nador lagoon (Morocco) (69–995 mg/kg FW, Maanan et al., 2015).

When assessing each element separately, results showed that Cr, Pb and Cu were the most abundant elements while Ni, As, Cd and Hg were the least abundant. Carvalho et al. (2006) also reported Cr, Pb and Cu among the elements with higher concentrations in the Óbidos lagoon. According to Diop et al. (2015) Pb was also the most abundant element present in sediments from Saint Louis estuary (Africa), while the less abundant was Cd. Other authors also described that Cd was the less abundant element in sediment from Moroccan estuaries (North of Africa) (Cheggour et al., 2005; Maanan et al., 2015).

Comparing with the present study, higher concentrations of Pb, Hg, Cr, Cu and Cd (33.0, 0.1, 58.0, 44.0 and 0.28 mg/kg DW, respectively) were found by Trefry and Trocine (2011) in the Indian River Lagoon (India). Similar findings were reported by Duarte et al. (2011) for Pb (6.33–45 mg/kg, respectively) in sediments from Beagle Channel coast (Argentina). Also comparing with the present results, Cheggour et al. (2001) reported that Moroccan Atlantic lagoons presented higher Pb, Cu and Cd concentrations (22.42–33.0, 32.9–36.9 and 0.94–3.67 mg/kg DW, respectively). Cadmium concentrations (0.20–3.05 mg/kg DW) in sediments from coastal areas along the Gulf of Gabès (Tunisia) (Machreki-Ajmi and Hamza-Chaffai, 2008) were also higher than the concentrations found in the present study (0.07 to 0.03 mg/kg FW).

The present findings further revealed that Cu and As concentrations, in the most contaminated areas (E and F), reached the sediment quality guidelines for ERL and TEL.

In the present study, cockles from the lowest (area A) and the highest (areas E and F) contaminated areas showed the highest contamination levels (34.68–66.61 mg/kg DW). The cockle *Austrovenus stutchburyi* from Creek Estuary and Pegasus bay (New Zealand) showed similar or even lower contamination levels (18.2–59 mg/kg DW, Marsden et al., 2014) than cockles from the present study. Higher concentrations were reported for other bivalves such as *R. philippinarum* from Venice lagoon (Italy) (19–81 mg/kg DW, Sfriso et al., 2008; Moschino et al., 2012) and *Mercenaria mercenaria* from Indian River Lagoon (Florida, United States) (103–237 mg/kg DW; Trefry and Trocine, 2011).

Regarding each element in *C. glaucum*, the present study showed that Ni was the most abundant element while Pb, Hg and Cd were the least abundant ones. Higher accumulation of Ni was also found in *C. edule* cockles from the Ria de Aveiro (Freitas et al., 2014). El Nemr et al. (2012) also reported Ni as one of the most accumulated elements in bivalves collected along the Egyptian Mediterranean coast and Cd one of the less accumulated elements. Also Cheggour et al. (2005) reported Ni as one of the most abundant metals found in *Scrobocularia plana* from Moroccan estuaries while Cd and Cu were the less abundant elements. In *R. philippinarum* from polluted areas in the Venice lagoon (Italy) Cd, Co and Pb were the less accumulated elements (Sfriso et al., 2008).

The concentration of Ni, As, Cu, Cr, Pb, Hg and Cd (lower than 3.47, 1.78, 1.77, 1.81, 0.50, 0.02 and 0.02 mg/kg DW, respectively) found in *C. glaucum* from the Óbidos lagoon were lower than values found for *Tapes philippinarum* from polluted areas of the Venice lagoon (Italy) (Sfriso et al., 2008). Also Machreki-Ajmi and Hamza-Chaffai (2006) found higher Cd and Pb concentrations (0.08–2.5 mg/kg and 0.13–1.07 mg/kg DW, respectively) in *C. glaucum* from Gulf of Gabès (Tunisia) than in *C. glaucum* from present study. Similar higher concentrations of Cu (16.2–20 mg/kg DW), Cd (0.19–1.64 mg/kg DW) and Ni (23.6–37.5 mg/kg DW) were found in *S. plana* from Moroccan Atlantic estuaries (Africa) (Cheggour et al., 2005), when compared with the present results.

In the present study a moderate correlation (0.54) was found between total element concentration present in *C. glaucum* and total elements present in sediment. Furthermore, although the present study reported that As, Cu, Cr, Cd, Hg and Pb were the least bioaccumulated elements in this species, a different pattern was observed in the sediments where Cu, Cr and Pb were the most abundant. These results may indicate that elements in sediment presented low bioavailability and their accumulation in cockles did not accurately indicate the true contamination levels in the environment. Therefore, according to our results, we can assume that in the present study *C. glaucum* could not be considered a good sentinel species. Freitas et al. (2012) also reported that the metal concentrations in the soft tissues of *C. edule* did not reflect the metal concentrations observed in sediment from the Ria de Aveiro (Portugal). Studies reported by Giarratano et al. (2010) and Duarte et al. (2011) also showed that the total metal concentration found in *Mytilus edulis chilensis* and sediment from the coastal waters of the Beagle Channel (Argentina) presented no significant correlations, suggesting that differences in bioavailability may be markedly influenced by local conditions. Sfriso et al. (2008) showed that metal concentrations in clams from Venice lagoon (Italy) were more correlated to concentrations in the suspended particles than to the concentration in surface sediments.

Our findings also showed that although As was one of the least accumulated elements by cockles, its concentration in organisms from the majority of the sampling areas was higher than the regulatory maximum levels (1 mg/kg FW) for shellfish defined by FSANZ. Taking As concentrations into consideration, the consumption of cockles from Óbidos lagoon represents a moderate risk to human health, since it is necessary for an adult (70 kg) to consume only 0.60 kg of cockles in one week to exceed PTWI. This fact must be taken into consideration since in general coastal populations regularly consume large amounts of bivalves. In Portugal around 58 kg/cap/year of seafood is consumed, being cockles among the most consumed species (Willemsen, 2003; INE, 2013; DGRM, 2014). Similar findings were observed by Figueira et al. (2011) showing that the ingestion of less than 1 kg of cockles from the Ria de Aveiro (Portugal) would result in exceeding the PTWI threshold for As. Li and Gao (2014) showed that the daily consumption of *M. edulis* (0.63–0.96 g) and *Sinonovacula constricta* (0.44–0.83 g) in China reached the PTWI limit for As (0.87 mg/person/week BFMAC, Bureau of Fisheries of the Ministry of Agriculture of China). Velez et al. (2015a) and Yang et al. (2013) reported that it is necessary for an adult with 70 kg to consume a lower amount of *R. philippinarum* (0.08 kg and 0.65 kg) from the Ria de Aveiro (Portugal) and the coast of China, respectively, to exceed the PTWI for As, representing a higher risk for human consumers.

The present study demonstrated that elements in cockles were equitably distributed between the soluble and insoluble fractions, with a similar percentage of total elements concentration in both fractions. However, *C. glaucum* tends to allocate more Ni and As in the soluble fraction. These elements may be in their free form or bound to molecules in the cytoplasm (Wallace et al., 2003). The allocation of As to the soluble fraction of *C. edule*, *R. philippinarum*, *R. decussatus* and *Venerupis corrugata* from the Ria de Aveiro was also reported in previous studies (Freitas et al., 2012; Velez et al., 2014, 2015a). Nevertheless, the present results demonstrated that the majority of Cu, Cr and Pb was found in the insoluble fraction, indicating that these elements were in their precipitated form or bound to membranes (Wallace et al., 2003). Rainbow and Smith (2010) assessed the subcellular compartmentalization of elements in the mussel *M. edulis*, the clam *R. philippinarum*, the scallop *Aequipecten opercularis* and the oyster *Crassostrea gigas* from Poole Harbor (England) and reported that the majority of Zn, Cd and Ag found in these species was in the insoluble fraction. Similar metal partitioning was reported by Geffard et al. (2004) for *M. edulis* and *C. gigas* with higher concentration of Ag, Cd, Cu, Zn in the insoluble fraction. Pellerin and Amiard (2009) showed that in the gills of *Mya arenaria* and *M. edulis* from contaminated St. Lawrence maritime estuary (Quebec, Canada) most of the Cd and Cu was allocated in the insoluble fraction. Metal partitioning of Pb in *R. decussatus* exposed to Ag, Cd and Zn revealed predominant percentage of these metals in the insoluble fraction (Ng and Wang, 2005). Also, Blackmore and Wang (2004) found lower percentages of Cd (20%) and Zn (14%) in the soluble fraction of *Saccostrea cucullata* exposed to these metals.

The present findings further revealed that the cockle *C. glaucum* presented higher elements bioaccumulation (BSAF > 1) in the areas located in the middle of the lagoon comparing with cockles from the most contaminated areas in the Bom Sucesso arm (BSAF < 1). The areas in the middle of the lagoon presented lower TOM and fine particles than areas in the Bom Sucesso arm. High TOM and high percentage of fine particles could explain this pattern since these parameters could influence the bioavailability of different elements, limiting their accumulation in cockles tissues. According to previous studies (Duarte et al., 2011; Diop et al., 2015), elements accumulation in organisms may be related to the capability of elements to be associated with sediment, since the later plays a special role in the fate of elements. Also, the bioavailability of these metals and metalloids in sediments may be influenced by different factors, such as organic matter, environmental parameters (pH, salinity and temperature), and organisms behavior (Chapman and Wang, 2001). In fact, according to Eggleton and Thomas (2004) and Hyun et al. (2006), the organic matter and fine particles have a strong capacity to adsorb metals and metalloids, affecting the bioavailability of these elements in sediments. Also Velez et al. (2015a) suggested that the higher concentration of elements found in sediments from the Ria de Aveiro and low accumulation levels in clams (*R. decussatus*, *R. philippinarum* and *V. corrugata*) may be related to the organic matter content and the high percentage of fine particles found in the sampling areas.

4.2. Morphological characterization of Cerastoderma *spp.*

Along the Óbidos lagoon both *C. glaucum* and *C. edule* are found. To ensure the correct use of *C. glaucum* specimens in the present study, cockles collected were submitted to morphological characterization. The common European cockles *Cerastoderma edule* and *C. glaucum* are very similar to each other, the former inhabits coastal areas and estuaries and the latter is often found in non-tidal areas such as lagoons and salt marshes, although their distribution may overlap and confusion in their distinction is possible. These species present few morphological distinctive features, often problematic for an unequivocal separation between them. Machado and Costa (1994) proposed a set of criteria to distinguish the Portuguese cockles populations. The most useful characters for a reasonable separation of those species are: valve profile, ventral junction shape, type of calcareous scales and shell color (cf. Machado and Costa, 1994). Using one or two features (particularly the least useful characteristics appointed by Machado and Costa, 1994) may not be enough for an unequivocal validation due to species variability and/or shared morphological traits and, for this reason, the presence of *C. edule* in lagoons is often overlooked. However, with this approach it is possible to fairly separate the two species even when there is a similar shape and signs of worn scales in the shells that cast some doubts. In the present study the two species were separated by close observation, using a large set of criteria (except scales in most cases).

4.3. Genetic analyses

Besides the morphological characterization of cockles, to unequivocally guarantee that *C. glaucum* was the species used for bioaccumulation and biochemical analyses, specimens were subjected to genetic analyses, using mitochondrial DNA *COI* gene. The results obtained revealed two point mutations for *C. glaucum*, however some haplotype and nucleotide diversity for mitochondrial *COI* gene within populations of *C. glaucum* were also previously observed (Tarnowska et al., 2010). The mitochondrial *COI* gene is considered a conserved gene, but the relative nucleotide divergence obtained between the two studied species – 19% – is comparable to nucleotide sequence divergence of *C. edule* and *C. glaucum* from a previous work, which presented 26.50% of divergence (Tarnowska et al., 2012). Moreover, the previous genetic study of Tarnowska et al. (2010) also showed a clear separation of both species by nuclear markers.

4.4. Oxidative stress biomarkers

The uptake of metals and As by marine organisms occurs from sediments, suspended particles, water column and food sources, depending on the dietary and ecological lifestyle of the organisms (Livingstone, 2001). High accumulation of metals and As, could lead to numerous adverse biochemical effects, namely related with oxidative stress such as enhanced lipid peroxidation (Valko et al., 2005; Dunlop et al., 2009; Paul-Pont et al., 2010). However, organisms have the capability to increase their antioxidant defenses to prevent these damages, such as enzymatic antioxidants (SOD, CAT), biotransformation enzymes (GSTs) and also non-enzymatic antioxidants (GSH), which directly neutralize several reactive species through its oxidation to GSSG (Regoli and Giuliani, 2014). Cells also have essential metal binding proteins involved in metal homeostasis and detoxification processes in living organisms including MTs (Paul-Pont et al., 2010).

The present study showed that *C. glaucum* from areas in the middle of lagoon accumulated higher metal and As concentrations than the sediment from the same location (BSAF > 1), being the majority of these elements present in the insoluble fraction (i.e. precipitated elements or bound to the membranes) inducing low oxidative stress. For this reason, oxidative stress biomarkers and total concentration of elements accumulated by *C. glaucum* presented a weak correlation, reflecting the tolerance of this species to metals and As contamination by allocating most of the elements to the insoluble fraction. Also studies conducted by Freitas et al. (2012) reported no correlation between oxidative stress biomarkers and accumulation of metals and As in *C. edule* from the Ria de Aveiro (Portugal), reflecting the higher percentage of metals allocated to the insoluble fraction. Nevertheless, in the present study, a slight increase in lipid peroxidation and in GSTs activity was recorded in areas where the organisms accumulated higher element concentrations (areas A, E and F). Chandurvelan et al. (2015) revealed correlation between metal accumulation, metallothionein-like protein content, antioxidant enzyme (CAT) and lipid peroxidation in the green-lipped mussel *Perna canaliculus* from South Island (New Zealand). The mussel *M. guyanensis* from polluted mangroves on Santa Catarina Island (Brazil) also increased LPO levels and GSTs activity in the most polluted areas (Torres et al., 2002). The increase in lipid peroxidation and GSTs activity in the present study may be explained by the capability of cockles to activate their oxidative stress responses, such as GSTs (involved in cellular detoxification processes), against damages in the cellular membrane caused by ROS (González-Fernández et al., 2015). In addition, cells also have cytosolic scavengers (namely GSH), which neutralizes reactive oxygen species through its oxidation to GSSG. Due to these mechanisms cells have the capacity to maintain membrane integrity and homeostasis by repairing oxidatively damaged lipid components (Valavanidis et al., 2006; Alves de Almeida et al., 2007). In the present study, the GSH content was not significantly different in cockles from different areas, suggesting a normal oxidation process through conversion of GSH to GSSG, and reconversion of GSSG to GSH. Dafre et al. (2004) showed an increase of total GSH and GSSG content in mussels *Perna perna* exposed to Pb.

Furthermore, a similar pattern was found in the protein content and antioxidant enzymes (SOD and CAT) along the study areas, with no significant differences when comparing cockles from all sampling areas. According to Box et al. (2007), SOD activity in the digestive gland of the mussel *M. galloprovincialis* showed no significant differences between polluted and non-polluted areas, while CAT activity was induced in polluted areas. Similar results were obtained by Torres et al. (2002) in the mussel *M. guyanensis* from polluted mangroves on Santa Catarina Island (Brazil). Also Geret et al. (2003) reported no alteration in cytosolic SOD activity present in the digestive gland of *R. decussatus* from areas with different levels of contamination, while CAT activity was higher in clams from a low contaminated area. Results reported by Freitas et al. (2012) showed low variation in SOD and CAT activities in *C. edule* from different areas along the Ria de Aveiro (Portugal).

In the present study, MTs levels were higher in cockles with low elements concentration, suggesting that their induction may not be related to metals and As concentration in cockles. According to other authors (Baudrimont et al., 1997; Kägi, 1991; George and Langston, 1994), the induction of MTs could reflect not only the presence of metals (like Cd, Hg, Ag) but a wide range of factors, such as hormones, cytotoxic, oxidizing agents (e.g. H_2O_2), endogenous and exogenous agents, and physical stress. Machreki-Ajmi et al. (2011) showed that in *C. glaucum*, from the Gulf of Gabès area (Tunisia), MTs levels were significantly variable due to the physiological changes caused by gonad development and food abundance. Also, Baudrimont et al. (1997) reported that metal accumulation in the clam *Corbicula fluminea* seemed not to be involved in the variation of MTs levels, but influenced by hormonal secretions implicated during reproductive phenomena. Figueira et al. (2012) further demonstrated that MTs in *C. edule* were induced not only by Cd but also by the presence of H_2O_2.

Taking into consideration that cockles from different areas showed similar biochemical behavior we suggest caution when assuming *C. glaucum* as a good bioindicator species, at least when studying low to moderately contaminated systems such as the Óbidos lagoon. Also Machreki-Ajmi et al. (2008), studying the biochemical responses of this species in contaminated systems, suggested the need for deeper investigation before this species could be suggested as a bioindicator species.

5. Conclusions

The present study ensured the correct identification of *C. glaucum* from the Óbidos lagoon through the identification of cockles morphological characteristics and genetic analyses. Results also revealed that this species was present in areas with different levels of contamination and with different sediment characteristics (TOM content and percentage of fine particles).

Overall, our data showed that *C. glaucum* from areas A, E and F presented higher total elements concentrations than cockles from the remaining areas. The total element concentrations found in *C. glaucum* were moderately correlated with the sediment contamination levels, which suggests precaution when considering this species as a sentinel.

Except for As, the concentrations of all elements found in *C. glaucum* were below the maximum regulatory levels defined by international organizations, reporting low health risk from the consumption of this species in what regards to the contaminants analyzed.

The present study also showed that, in general, *C. glaucum* from areas in the middle of the lagoon accumulated higher metals and As concentrations than the sediment (BSAF > 1). However, the majority of these elements were present in the insoluble fraction inducing low damages to the cells. Due to that we suggest a precautious use of *C. glaucum* as a bioindicator species, at least in sediment from areas with low to moderate contamination levels.

Overall, although *C. glaucum* has been identified as a good sentinel and bioindicator species, caution should be taken when studying areas characterized by low or even moderate pollution levels.

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