1 The impacts of As accumulation under different pH levels:

2 comparing *Ruditapes decussatus* and *Ruditapes*

3 *philippinarum* biochemical performance

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Abstract

Marine bivalves have been used to assess environmental As contamination and the effects of seawater acidification when both factors are acting alone, but limited information is available regarding the impacts of both factors acting in combination. The aim of this study was to compare physiological (glycogen) and biochemical (lipid peroxidation, superoxide dismutase, catalase, glutathione-S-transferase and alkaline phosphatase) responses in both native (*R. decussatus*) and introduced (*R. philippinarum*) clams, when exposed to the combined effects of pH (7.8, control; 7.3) and As concentrations (0 and 4 mg/L). The effect of pH on the health risks associated with clam consumption was also analyzed. Results revealed that both species were able to accumulate As under both pH levels, although higher As concentrations were observed under low pH. Thus, predicted pH decrease will potentiate health risks associated with the consumption of such species, since less amount of clams exposed to As is needed for an adult to exceed the provisional tolerable weekly intake (PTWI). Low pH, As exposure and the combination of both factors did not negatively impact the native species, since clams were able to maintain their physiological and biochemical performance among all conditions. On the other hand, *R. philippinarum* was negatively affected by As exposure at control pH (7.8), inducing biotransformation and antioxidant defense mechanisms against As toxicity. *R. philippinarum* exposed and non-exposed to As presented similar responses under low pH although at this condition the introduced species accumulated twice the amount of As than *R. decussatus*.

Keywords:

Ruditapes species; native and invasive species; Seawater acidification; Metalloid; Oxidative stress

1. Introduction

Global industrialization significantly contributed for the increase of arsenic (As) concentrations in aquatic ecosystems (Chen et al., 2015; Khan et al., 2011), raising public health concerns due its high toxicity for humans (Peshut et al., 2008). For this reason the US environmental Protection Agency list of prioritized pollutants and the US Agency for Toxic substances and Disease Registry defined As as an element of public concern. In the European context this element is not yet among the priority substances list, although in several aquatic ecosystems it is possible to find high concentrations of different organic (dimethylarsinic acid and monomethylarsonic acid) and inorganic (arsenate and arsenite) forms of As in water (0.5 to 8.8 µg/L) and sediment (0.82 to 220 mg/Kg DW) (Smedley and Kinniburgh, 2002; Velez et al., 2015a). The predominant form of As in water and sediment is arsenate, which is the least toxic form of inorganic As (Flora, 2014; Fattorini et al., 2004; Hughes et al., 2011). In marine environments, organisms usually accumulate non-toxic organic As compounds, presenting a low capacity to accumulate arsenate from surrounding seawater (Flora, 2014; Fattorini et al., 2004). Nevertheless, many authors have focused on the As accumulation and its impacts on marine bivalves (Alkarkhi et al., 2008; Alonso-Hernández et al., 2012; Diniz et al., 2008; Liu et al., 2007; Velez et al., 2016a,b), a group of organisms largely studied due to their capacity to accumulate high amounts of metals and metalloids (Gazeau et al., 2013). Both environmental and laboratory studies have revealed the effects of As not only on organisms physiological parameters (e.g. energy metabolism) but also on their biochemical performance (e.g. detoxification mechanisms) (Diniz et al., 2008; Chenglong et al., 2013; Freitas et al., 2016; Rajkumar and Rajkumar, 2013; Velez et al., 2016a,b; Wu et al., 2013a).

Industrialization has also led to the increase of atmospheric CO₂ concentrations, consequently influencing CO₂ and pH levels in the aquatic environment (Billé et al., 2013), and predictions pointing for decreases of up to 0.3–0.5 units of oceanic pH by the end of the 21st century (Caldeira and Wickett, 2005; EPA, 2008; Mohan and Reddy, 2000). Different studies have also used marine bivalves to assess the effects of seawater acidification (Basallote et al., 2011; Berge et al., 2006; Dickinson et al., 2012; Dickinson et al., 2013; Duarte et al., 2014; Freitas et al., 2015; Gazeau et al., 2013). Although marine and especially

estuarine bivalves are exposed to periodic pH fluctuations during seasonal or diurnal cycles of CO₂, studies have already demonstrated that the predicted ocean acidification will have a strong impact on survival, abundance, shell and somatic growth, respiration, ingestion and absorption rate, metal uptake, calcification, immune response and oxidative stress of these organisms (Gazeau et al., 2013; Moreira et al., 2016a; Parker et al., 2013).

Among abiotic factors, low pH can influence As speciation, resulting in the formation of more toxic As forms, namely arsenite (inorganic arsenic) (Sharma and Sohn, 2009; Smedley and Kinniburgh, 2002). Furthermore, both pH and As can change organisms biochemical and physiological performance, increasing their sensitivity to these or other environmental factors. However, to our knowledge, few studies assessed the combined effects of seawater acidification and As on marine invertebrates (Basallote et al., 2015; Moreira et al., 2016a; Ricevuto et al., 2016)

Clam species *Ruditapes decussatus* (Linnaeus, 1758), native from Europe, and *Ruditapes philippinarum* (Adams and Reeve, 1850), native from the southeastern Asia (Indo-Pacific region) and introduced worldwide in different coastal systems (including coastal lagoons in Portugal), are among the most ecologically and economically important marine bivalve species (FAO, 2016a, b). Both species are good bioindicators, presenting high capacity to accumulate metals and As concentrations from water and sediment and to reflect the impacts induced by these elements (Moschino et al., 2012; Velez et al., 2015b). However, although both species co-exist in the same areas (Bidegain et al., 2015; Velez et al., 2015a,b), they may present different responses when exposed to the same contaminants, including As, Hg (Velez et al., 2016b), and Cd (Moraga et al., 2002).

Studies have already demonstrated that metals and metalloids may disturb energy metabolism and osmotic regulation as well as the inducement of oxidative stress in both *Ruditapes* species (Ji et al., 2015; Velez et al., 2016a,b; Wu et al., 2013a; Wu et al., 2013b). *R. decussatus* and *R. philippinarum* also have the capacity to reveal the impacts of predicted ocean acidification, with a reduction in clearance, ingestion and respiration rates and increases on ammonia excretion and metal uptake increase under low pH (pH 6.5, 7.5, López et al., 2010; pH 7.5, Range et al., 2011; pH 7.4, Range et al., 2013; pH 6.1, 6.6 and 7.1, Rodríguez-Romero et al., 2014; pH 7.4, Xu et al., 2016). However, recently Velez et al.

119 (2016c) reported that pH 7.4 did not induce oxidative stress in these two *Ruditapes* species.
120 Nevertheless, to our knowledge no studies have assessed the impacts generated by the
121 combined effect of low pH and As contamination on *R. decussatus* and *R. philippinarum*,
122 which is the focus of the present study. For this, both species were exposed to two pH levels
123 (7.8, control; 7.3) combined with As exposure (0 and 4 mg/L) for 28 days, and clams
124 bioaccumulation capacity, human health risks associated to clams consumption, and
125 alterations on oxidative stress and energy reserves were evaluated.

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2. Materials and methods

2.1 Experimental conditions

R. decussatus and *R. philippinarum* clams were collected in the same area in the Ria de Aveiro (a lagoon at the Northwest coast of Portugal). In order to minimize the effect of body weight on clams responses and accumulation, organisms with similar weight (17-20 g) were selected.

Organisms were maintained during 1-week at 19.0 ± 1.0 °C, pH 7.80 ± 0.10 , 12 light: 12 dark photoperiod and continuous aeration, in artificial seawater (salinity 28) made with artificial sea salt (Tropic Marin® SEA SALT from Tropic Marine Center) and deionized water. During depuration, the water was renewed every other day and organisms were not fed. After this period organisms were divided into different aquaria for pH acclimation. For this, in each aquarium, seawater was bubbled with CO₂. To reach low pH levels, in the corresponding aquaria, pH was gradually decreased (0.2-0.1 units per day), from 7.8 (control) to pH 7.3. Both pH levels (7.3 and 7.8) were continuously monitored and controlled using a pH Stat system (Aquamedic AT Controller). During this acclimation period, organisms were kept at 19.0 ± 1.0 °C, 12 light : 12 dark photoperiod and under continuous aeration. Daily dissolved oxygen concentration was monitored in all aquaria. All organisms were fed with Algamac protein plus (150.000 cells/animal), every 2-3 days. After this period, for each species, four different conditions (3 replicates per condition; 5 individuals per replicate) were tested for 28 days: pH 7.8 and 0 mg/L of As, pH 7.3 and 0 mg/L of As, pH 7.8 and 4 mg/L of As, and pH 7.3 and 4 mg/L of As. Arsenic concentration was prepared with Na₂HAs₄⁻, the less toxic form of inorganic As (Hughes et al., 2011). The As concentration tested was selected taking into account the As tolerance of both species (Velez et al., 2016), the As concentration present in tissues of both species collected from different ecosystems (0.6 to 64 dry weight, DW) (Sfriso et al., 2008; Velez et al., 2015a), as well as the As concentration found in seawater (1.8 to 8.8 mg/L) (Smedley and Kinniburgh, 2002) and sediments (0.82 to 220 mg/Kg DW) (Hédouin et al., 2009; Mamindt-Pajary et al., 2013; Sfriso et 2008; Velez et al., 2015a) from European estuaries. Low pH (7.3) was selected according to the worst predicted scenario of climate change for 2100 (Δ pH = -0.5 units, Caldeira and Wickett, 2005; IPCC, 2013) and the pH range found in the Ria de Aveiro lagoon (6.82 to 7.8) (Velez et al., 2015a).

During the exposure period, organisms were maintained in recirculating water aquaria with artificial seawater (salinity 28 ± 1.5), at 19.0 ± 1.0 °C, 12L:12D photoperiod and dissolved oxygen concentrations were monitored. Animals were fed with Algamac protein plus (150.000 cells/animal), twice a week. Every week, seawater was renewed, and low pH and As concentrations re-established. The pHstat system (Aquamedic AT Controller) was used to control automatically pH in each aquarium, through the addition of CO₂ gas. Water acidification was controlled twice a week using an independent probe (WTW-Wissenschaftlich-Technische Werkstätten GmbH), during the acclimation and exposure period. During experimental period, before water renewal, water samples were collected from each aquarium to quantify total alkalinity by potentiometric titration (Gran, 1952). Total alkalinity (TA), pH, temperature, and salinity were used to determine CO₂ partial pressure (pCO₂), bicarbonate (HCO₃⁻) and carbonate (CO₃²⁻) ions concentrations, and the saturation states of calcite (ΩCal) and aragonite (ΩAg) for each tank. These concentrations were calculated using CO2SYS software (Robbins et al., 2010), with K1 and K2 carbonate dissociation constants (Mehrbach et al., 1973) refit by Dickinson and Millero (1987), and KSO₄ from Dickson (1990). Physico-chemical water parameters and the associated variation, for each condition, are presented in Table I.

Aquaria were daily checked for mortality, and dead organisms removed whenever identified. At the end of the exposure (28 days) organisms were frozen until analysis. Before analysis, organisms were individually pulverized and homogenized with liquid nitrogen and distributed into aliquots, each one containing 0.5 g of tissue.

2.2 Quantification of As and BCF

For As quantification, 0.5 g of soft tissue of each species was digested overnight with 2 mL of concentrated HNO₃:HCl (1:3), at 115 °C in Teflon bombs. After cooling of all samples, the final volume was made up to 15 mL with high purity deionized water and As was quantified by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry), in a certified laboratory at the University of Aveiro. The standard certificated reference material TORT-2 from NRCC (National Research Council of Canada) was analyzed, with a range recovery from 91% to 105%. The results were expressed in µg per g of fresh weight (FW).

Total As concentrations determined were used to calculate: i) the mass of bivalves necessary to be consumed by a 70 Kg adult to exceed the provisional tolerable weekly intake (PTWI); ii) the Bioconcentration Factor (BCF). BCF was obtained dividing the As concentrations determined in organisms from each condition and each species by the nominal exposure concentration (4 mg/L) (McGeer et al., 2003).

2.3 Biochemical markers

For each physiological biochemical parameter determination, 0.5 g of whole soft tissue was used. The extraction for each biomarker was performed with specific buffers according to Moreira et al. (2016a). For this, samples were centrifuged for 10 min at 10 000 g and 4 °C and supernatants were stored at -80 °C or immediately used. The biomarkers analyzed were glycogen (GLY), lipid peroxidation (LPO), glutathione-S- transferases (GSTs), superoxide dismutase (SOD), catalase (CAT), the ratio between reduced (GSSG) and oxidized (GSSG) glutathione, alkaline phosphatase (ALP) and protein (PROT).

GLY content was quantified by the phenol-sulphuric acid method, as described by (Yoshikawa, 1959). The glucose standards were used in concentrations ranging from 0 mg to 5 mg/mL. Samples were incubated at room temperature for 30 min and absorbance was measured at 492 nm. Results were expressed in mg per g of FW.

LPO levels were measured based on Buege and Aust (1978). This method measures the amount of TBARS (thiobarbituric acid reactive substances), based on the reaction of LPO by-products, such as malondialdehyde (MDA), with 2-thiobarbituric acid (TBA), forming TBARS. LPO was determined at 532 nm ($1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ extinction coefficient). The results were expressed in nmol of MDA equivalents per mg of protein.

GSTs activity was measured according to Habig et al. (1974), at 340 nm ($\epsilon = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$) during 5 min. GSTs activity was expressed in U per mg protein. One unit (U) of enzyme was defined as the amount of enzyme that caused the formation of 1 μmol thioether, per min.

SOD activity was measured according to Beauchamp and Fridovich (1971), at 560 nm, after 20 min of incubation at room temperature. The standards of SOD were used with concentrations ranging from 0.25 to 60 U/ mL. Results were expressed in U per mg of protein.

One unit (U) of enzyme activity corresponds to a reduction of 50% of nitroblue tetrazolium (NBT), per min.

CAT activity was determined following Johansson and Borg (1988) method. Formaldehyde standards (0–150 μ M) were used as the standard curve. The absorbance was read at 540 nm. CAT activity was expressed in U per mg of protein. One unit (U) of enzyme activity represents the formation of 1.0 nmol formaldehyde, per min.

GSH and GSSG contents were measured at 412 nm as described by Rahman et al. (2006). GSH and GSSG were used as standards (0–60 μ mol/L). The GSH/GSSG ratio was calculated dividing the GSH content by 2x the concentration of GSSG.

ALP was measured using González et al. (1994) method, at 420 nm. Results were expressed in U per mg of protein. One unit of enzyme (U) is defined as the amount of enzyme that causes the formation of 1 μ mol of nitrophenol, per min.

Protein concentration was assayed using the Biuret method Robinson and Hogden (1940), at 540 nm. Bovine serum albumin (BSA) was used as the standard. Protein concentration was expressed in mg per mL.

2.5 Data analysis

Data on As concentration, BCF values and biochemical markers, from each species and condition (pH 7.8 and 0 mg/L of As, pH 7.3 and 0 mg/L of As, pH 7.8 and 4 mg/L of As, and pH 7.3 and 4 mg/L of As), were submitted to hypothesis testing using permutational multivariate analysis of variance, employing the PERMANOVA+add-on in PRIMER v6 (Anderson et al., 2008).

For each parameter, the null hypotheses tested were: a) for each species and pH level: no significant differences exist between As exposed and non-exposed clams; b) for each species and As exposure concentration: no significant differences exist between clams under different pH levels; c) for each As exposure concentration and pH level: no significant differences exist between species. Significance levels were considered for $p \leq 0.05$. In figures, the minuscule letters represent differences for *R. decussatus* between As concentrations at each pH level (7.8 and 7.3), while majuscule letters represent differences for *R. philippinarum* between As concentrations at each pH (7.8 and 7.3). In figures significant

248 differences between pH conditions, for each species at each As concentration, were
249 presented with asterisks. At each pH and As condition significance levels (Monte Carlo p-
250 values) related to the each biomarker between species are presented in a Table. The same
251 table also presents significance levels between As concentrations (for each species and each
252 pH level) and the significance levels between pH levels (for each species and each As
253 concentration).

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4. Results and discussion

Changes in environmental conditions, namely pH alterations, can modify the bioavailability of As (Sharma and Sohn, 2009; Smedley and Kinniburgh, 2002). The effects of ocean acidification and As contamination on bivalve species has already been shown by different studies when both factors were acting alone (e.g. Alkarkhi et al., 2008; Basallote et al., 2011; Berge et al., 2006; Gazeau et al., 2013; Velez et al., 2016a,b,c,d) but few studies are known on this group of organisms exposed to the combined effect of pH and As (Moreira et al., 2016a). For this, the performance of the native *R. decussatus* and the introduced *R. philippinarum* species was compared when under the combination of As and different pH levels.

4.1 Arsenic accumulation and BCF values

The results obtained showed that the combined exposure of As and pH did not affect the survival of both species, except for *R. philippinarum* exposed to pH 7.8 and 4.0 mg/L of As (7% of mortality).

The present findings revealed that *R. decussatus* and *R. philippinarum* under both pH levels increased As concentrations when exposed to this metalloid, with significant differences only observed between exposed and non-exposed clams at pH 7.3 (Table II). Similar As accumulation capacity was previously reported for marine bivalves (*R. decussatus*, *R. philippinarum*, *Crassostrea gigas*, *C. angulata*, *Saccostrea cucullata* and *Mytilus edulis*) exposed to As (Velez et al., 2016b; Gailer et al., 1995; Moreira et al., 2016a,b; Zhang et al., 2015) (Table IV).

In addition, the present study also demonstrated that, both *R. decussatus* and *R. philippinarum* species exposed to As presented higher concentrations (2.91 ± 0.45 for *R. decussatus* and 6.40 ± 1.87 for *R. philippinarum*) and higher BCF values ($BCF \geq 0.73 \pm 0.02$) under low pH (7.3) than under control pH (7.8) (2.59 ± 0.65 $\mu\text{g/g}$ for *R. decussatus* and 2.18 ± 1.03 $\mu\text{g/g}$ for *R. philippinarum*; $BCF \leq 0.65 \pm 0.20$) (Table II and Table III). These results support the idea that low pH may favor As accumulation by clams, which was more pronounced in the introduced species. It is known that arsenate uptake by organisms is done through phosphate transport systems (such as Na^+ and K^+ -ATPase) which are also used for

pH osmoregulation of estuarine animals (Fan et al., 2015; Monserrat et al., 2007). Due to the absorption of As by the transport systems above mentioned the concentration of As in the organisms may increase. Thus, these mechanisms may explain why, in the present study, organisms accumulated more As at low pH. Similar effects were also observed in marine bivalves *C. virginica*, *Mercenaria mercenaria*, *M. edulis*, *Tegillarca granosa*, and *Meretrix meretrix* exposed to Cu and Cd, and low pH levels (7.40- 7.6) (Götze et al., 2014; Shi et al., 2016) (Table IV).

Our findings also demonstrated that both species accumulated similar As concentrations when chronically exposed to 4 mg/L of As at pH 7.8 (*R. decussatus* 2.59 ± 0.65 mg/L of As and *R. philippinarum* 2.18 ± 1.03 mg/L of As) (Table II). In accordance with present results, similar As accumulation patterns at pH 7.8 between two bivalve species were also demonstrated by others authors (Moreira et al., 2016a; Velez et al., 2016b). When exposed to low pH and As (4 mg/L), the introduced species presented significantly higher As concentration (6.40 ± 1.87 mg/L As) and BCF values (1.60 ± 0.47) than the native species (As concentration 2.91 ± 0.45 mg/L and BCF 0.73 ± 0.02) (Table II and III). These results suggested two possibilities: close related species present different uptake, storage and elimination rates for this metalloid, or the native species was able to keep valves closed as a strategy of protection against As contamination and low pH. The last hypothesis is supported by the lowest BCF observed in this species at low pH and As contamination. It is known that clams under stressful conditions are able to close their valves during long periods to avoid exposure to contaminants and abiotic factors (Carregosa et al., 2014; Freitas et al., 2015; Vernberg, 2012) (Table IV).

4.2 Dietary risk assessment

Regarding the amount of clams soft tissue that a 70 kg adult needs to consume in one week to exceed the PTWI for As, results showed that: for non-exposed clams (0 mg/L As), it is necessary to consume only between 0.58 (pH 7.8) and 0.66 Kg (pH 7.3) of *R. decussatus* and between 0.59 (pH 7.8) and 0.85 Kg (pH 7.3) of *R. philippinarum*; and for exposed clams (4 mg/L As) it is necessary to consume only between 0.41 (pH 7.8) and 0.36 Kg (pH 7.3) of *R. decussatus* and between 0.48 (pH 7.8) and 0.16 Kg (pH 7.3) of *R.*

philippinarum. Similar results were found for *R. decussatus* (0.15 to 0.95 kg) and *R. philippinarum* (0.08 to 0.81 kg), collected from the Ria de Aveiro, Óbidos lagoon (Velez et al., 2015a, b) and from the coast of China (Yang et al., 2013), to exceed the PTWI for As.

Regarding the effects of pH our results also showed that, for contaminated clams, at low pH (7.3) it is necessary to consume lower amounts of either species by an adult of 70 kg to exceed the PTWI compared to clams under pH 7.8. Comparing both species, results showed that under pH 7.8 it is necessary to consume a similar amount of clams for an adult of 70 kg to exceed the PTWI. However, at pH 7.3 it is necessary to consume a lower amount of *R. philippinarum* than *R. decussatus* to exceed the PTWI. Thus, from the present findings, we may conclude that predicted seawater acidification, especially in areas contaminated with As, should be topic of concern particularly in countries with high consumption of seafood, such as Portugal (with an average of 58 kg/cap of seafood consumed per year) (Willemsen, 2003). Furthermore, since less amounts of *R. philippinarum* are needed to exceed the PTWI under an acidified scenario, special attention should be taken in the future when selecting the clam species as food source.

4.3 Physiological parameters

Energy reserves

Glycogen reserves are used by several aquatic organisms as a source of energy in cellular protection systems when exposed to stressful conditions (Duquesne et al., 2004; Hamza-Chaffai, 2014). However, the present results showed that both species maintained the GLY reserves among all tested conditions (pH 7.8 and 7.3, 0 and 4 mg/L of As) (Fig. 1, Table III). The bivalves *C. gigas* and were also able to maintain GLY (carbohydrate) content under ocean acidification and Cd contamination, respectively (Duquesne et al., 2004; Timmins-Schiffman et al., 2014). Nevertheless, according to Velez et al. (2016a) and Almeida et al. (2015), the exposure to As and pharmaceutical drugs, resulted in a reduction of metabolic activity and an increase of GLY content in *R. philippinarum*.

The present study also revealed significantly lower GLY content in *R. philippinarum* than in *R. decussatus*, independently on the As concentration and pH level of exposure (Fig.1, Table III), suggesting different storage and/or use of energy reserves by these two

close related species. Previous studies conducted by Anacleto et al. (2014) reported higher GLY content in the native clam (*R. decussatus*) than in the introduced clam (*R. philippinarum*) under temperature stressful conditions (22 to 38 °C).

4.4 Biochemical parameters

It is known that marine bivalves exposed to stressful conditions increase reactive oxygen species (ROS) production and may also increase the detoxification mechanisms (proteins with high affinity to metals, namely glutathione or metallothioneins, and antioxidant enzymes, namely SOD and CAT) to neutralize ROS and prevent cells from lipid, DNA and protein damage (Amiard-Triquet et al., 2012; Batley and Simpson, 2016). If antioxidant defense systems are not able to eliminate the excess of ROS, the peroxidation of lipids is induced, resulting in physico-chemical changes in cellular membranes (Amiard-Triquet et al., 2012; Catalá, 2009). Regarding glutathione, it is also known that GSH acts as a redox buffer to maintain the cellular redox state and the ratio of GSH/GSSG, and has been used as a biomarker of oxidative stress in many organisms (Flora, 2009; Jozefczak et al., 2012; Marí et al., 2009).

Oxidative stress responses

For each species under the same pH level, results showed no significant differences in LPO levels between exposed and non-exposed clams to As (Fig. 2A, Table III). The native species, *R. decussatus*, under the same pH level did not show significant differences in GSTs and SOD activities between clams exposed and non-exposed to As (Fig. 2B and C, Table III). Similar results were observed for GSTs and SOD activities in *R. philippinarum* exposed and non-exposed to As at low pH (Fig. 2B and C), but at pH 7.8 this species significantly increased both enzymes activities when exposed to As (4 mg/L). CAT activity for *R. decussatus* significantly decreased in clams exposed to As and pH 7.8 while for *R. philippinarum* clams exposed to As increased significantly the CAT activity under pH 7.8. Both species at pH 7.3 showed no significant differences between exposed and non-exposed clams (Fig. 2D and Table III). Regarding, the GSH/GSSG ratio, for each species under the

same pH level, results showed no significant differences between exposed and non-exposed clams (Fig. 3).

The results presented here suggest that the native species was able to regulate the uptake, storage, and As elimination, presenting no damages in the cells membranes and no induction of oxidative stress under chronical As exposure, independently on the pH level tested. Other detoxification mechanisms, namely Metallothioneins (MTs), can also contribute for As sequestration, protecting cells against As toxicity and maintaining the essential ion homeostasis. Recently, some authors demonstrated that marine organisms presented the majority of As bound to the MTs fraction, providing more tolerance and possibly more resistance to As exposure (Casado-Martinez et al., 2012; Diniz et al., 2008; Velez et al., 2016d; Zhang et al., 2015) (Table IV). This may explain why in the present study, As did not induce oxidative stress in the native species. Similar results were observed for *R. philippinarum* exposed to As at low pH, although As accumulation was 2 times higher in this species when compared with the remaining conditions for both species. In accordance with our findings, different authors also demonstrated that As exposure with concentrations up to 4 mg/L did not affect LPO levels and SOD activity in marine species (Velez et al., 2016a; Ventura-Lima et al., 2009; Moreira et al., 2016a; Moreira et al., 2016b) (Table IV). On the contrary, in the present study As affected *R. philippinarum* response at pH 7.8, inducing biotransformation (GSTs) and antioxidant enzymes (SOD, CAT), while LPO levels and GSH/GSSG ratio were maintained. These observations suggest that the induction of biotransformation and antioxidant defense mechanisms were efficient to eliminate the excess of ROS produced by As exposure at pH 7.8. The induction of GSTs activity was also reported in the oysters *S. cucullata* and *C. gigas* and *C. angulata* under As exposure, supporting the important role of these enzymes in the detoxification process of As (Zhang et al., 2015; Moreira et al., 2016a) (Table IV). Velez et al. (2016a) demonstrated that *R. decussatus* acutely exposed to As maintained the activity of SOD and GSTs while increased the CAT activity comparatively to the control conditions (pH 8.0).

Analyzing species responses when under the same As concentration but different pH levels, our results demonstrated significantly higher LPO values at low pH and 4 mg/L of As than at control pH for *R. decussatus* (Fig. 2A) while *R. philippinarum* showed significantly

higher LPO levels at low pH and 0 mg/L of As than at pH 7.8.-(Fig. 2A, Table III). For each species, higher GSTs and SOD activities were found at low pH, but significant differences were only found for GSTs in both species exposed to 0 mg/L of As (Fig 2B, Table III) and for SOD in *R. philippinarum* exposed to 0 mg/L (Fig. 2C and Table III). The activity of CAT showed significantly higher values in *R. decussatus* under pH 7.8 at 0 mg/L and in *R. philippinarum* at the same pH but exposed to As (4 mg/L) (Fig. 2D and Table III). Analyzing GSH/GSSG results showed no significant differences between pH conditions (Fig. 3).

Thus, in general, our results showed higher LPO levels, GSTs and SOD, activity at low pH, indicating that clams were able to activate biotransformation and antioxidant enzymes to prevent cellular damage induced by low pH. The mussel *M. galloprovincialis* maintained LPO levels and the activity of antioxidant and biotransformation enzymes in gills when exposed to low pH (7.4) (Matozzo et al., 2013). Moreira et al. (2016a) reported a similar pattern in LPO levels, SOD and CAT activity in *C. angulata* chronically exposed to As, low pH and the combined effect of both. However these authors also observed an induction of GSTs activity in oysters exposed to As and combined effect of As and low pH when compared with control conditions. In contrast, Freitas et al. (2016) reported for the clam *Scrobicularia plana* exposed to low pH (7.1) higher LPO levels, induction of GSTs and inhibition of CAT, comparing to clams under control conditions (pH 7.8).

Comparing both species, for the same pH level and As concentration, results demonstrated, in general, that the introduced species presented significantly higher LPO levels, GSTs and SOD activity than the native one, independently of the pH level and As concentration (Table III), while for GSH/GSSG ratio, no significant differences were observed between species at the same pH level and As concentration (Table III). Differences among close related species were also found in *C. angulata* and *G. gigas* exposed to the same As concentration (0 and 4 mg/L of As) and pH level (7.8 and 7.3), indicating species-specific oysters cellular damages and defense mechanisms (Moreira et al., 2016a).

Osmoregulation capacity

The activity of ALP in the native species at each pH did not show significant differences among exposed and non-exposed clams (Fig. 4). Similar results were observed at low pH between *R. philippinarum* exposed and non-exposed clams to As (Fig. 4). However, at control pH (7.8), As contamination significantly inhibited ALP activity in *R. philippinarum*. For each species under the same As concentration, no significant differences in ALP activity was observed between pH levels. When comparing both species, at the same pH level and As concentration, the introduced species presented higher ALP activity than the native species in all tested conditions (Table II).

Alkaline phosphatase is a plasma membrane enzyme, described by several authors in marine bivalves, and its activity is influenced by metals (Mazorra et al., 2002; Seitzkalieva et al., 2015). Our results demonstrated that activity of this enzyme remained unchanged in the native species exposed to As at each pH level. Similar results were observed for the introduced species exposed to low pH and As, but at pH 7.8, *R. philippinarum* exposed to As inhibited ALP activity. ALP inhibition was reported in *S. plana* and in *Lamellidens marginalis* under metal and As exposure, respectively (Chakraborty et al., 2010; Mazorra et al., 2002) (Table IV). Nevertheless, Moreira et al. (2016a) demonstrated no changes in ALP activity in *C. gigas* exposed to the combined effect of pH (7.8 and 7.3) and As concentrations (0 and 4 mg/L of As) while the oyster *C. angulata* presented induced ALP activity under As exposure at control pH (7.8) (Table IV). Moreover, the present results indicated that the introduced clam *R. philippinarum* presented higher ALP activity than native species among all tested conditions. The oyster *C. gigas* also presented higher ALP activity than the close related species *C. angulata* under pH 7.8, 7.3 and the combined effect of low pH and As (Moreira et al., 2016a).

5. Conclusion

The present findings demonstrated that both clams accumulated As independently of the pH tested. However, low pH facilitated As accumulation in both species, since the As concentration was higher in clams exposed to low pH than control pH. The results also

indicated that, when exposed to pH 7.3 the amount of clams necessary to be consumed to exceed the PTWI is lower than when organisms are exposed to pH 7.8.

In general, *R. decussatus* exposed to As concentration at the same pH (7.3 and 7.8) maintained the physiological and biochemical responses. Similar results were observed in the introduced species exposed to low pH and As, suggesting that the toxicity of As and low pH was not sufficient to induce oxidative stress under these conditions. On the other hand, at control pH, *R. philippinarum* exposed to As induced the activity of biotransformation and antioxidant enzymes, avoiding oxidative stress. Both species exposed to the same As concentration showed, in general, higher LPO, GSTs, SOD activity under low pH. In addition, results also demonstrated higher levels of LPO and biotransformation, antioxidant and ALP enzymes in the introduced species than in the native congener. These findings suggested that although these species were exposed to the same conditions, physiological and biochemical responses are species-specific.

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Table I -Carbonate system physic-chemical parameters each tested condition pH. Mean values ($\pm$ standard deviation) of measured pH and determined total alkalinity (At) in water each samples collected from each aquarium (temperature 19 ± 1 °C and salinity 28 ± 1 g/L). Partial CO₂ pressure ($p\text{CO}_2$), bicarbonate (HCO_3^-), carbonate ion concentrations (CO_3^{2-}), saturation states of calcite (Ω_{Cal}) and aragonite (Ω_{Ara}) were calculated with CO₂SYS software (Robbins et al., 2010).

	Condition	pH	AT ($\mu\text{mol/Kg}$)	$p\text{CO}_2$ (μatm)	HCO_3^- ($\mu\text{mol/Kg}$)	CO_3^{2-} ($\mu\text{mol/Kg}$)	Ω_{Ara}	Ω_{Cal}
<i>R. decussatus</i>	7.8	7.8 $\pm$ 0.08	2391.7 $\pm$ 131.80	1192.0 $\pm$ 162.35	2233.8 $\pm$ 105.74	78.2 $\pm$ 13.68	1.2 $\pm$ 0.22	1.9 $\pm$ 0.35
	7.3	7.3 $\pm$ 0.05	2897.3 $\pm$ 82.85	4062.6 $\pm$ 742.39	2861.0 $\pm$ 101.11	43.0 $\pm$ 17.85	0.7 $\pm$ 0.28	1.1 $\pm$ 0.43
	7.8 4 mg/L As	7.8 $\pm$ 0.14	2526.6 $\pm$ 211.97	1275.3 $\pm$ 431.61	2348.8 $\pm$ 198.86	88.2 $\pm$ 30.74	1.4 $\pm$ 0.48	2.2 $\pm$ 0.74
	7.3 4 mg/L As	7.3 $\pm$ 0.10	2829 $\pm$ 95.53	3912.6 $\pm$ 96.55	2758.3 $\pm$ 90.92	35.1 $\pm$ 1.54	0.6 $\pm$ 0.03	0.9 $\pm$ 0.04
<i>R. philippinarum</i>	7.8	7.8 $\pm$ 0.08	2350.7 $\pm$ 47.34	1361.2 $\pm$ 242.06	2217.0 $\pm$ 35.01	66.2 $\pm$ 12.44	1.1 $\pm$ 0.20	1.7 $\pm$ 0.32
	7.3	7.3 $\pm$ 0.10	2801.7 $\pm$ 139.54	4074.4 $\pm$ 573.92	2733.6 $\pm$ 131.24	33.9 $\pm$ 6.82	0.7 $\pm$ 0.32	0.7 $\pm$ 0.09
	7.8 4 mg/L As	7.8 $\pm$ 0.11	2419 $\pm$ 183.93	1321.4 $\pm$ 373.60	2265.4 $\pm$ 177.03	76.1 $\pm$ 22.72	1.2 $\pm$ 0.36	1.9 $\pm$ 0.56
	7.3 4 mg/L As	7.3 $\pm$ 0.15	2637.7 $\pm$ 71.99	3984.6 $\pm$ 700.96	2574.9 $\pm$ 73.48	31.1 $\pm$ 3.29	0.5 $\pm$ 0.05	0.8 $\pm$ 0.08

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Table II- Total arsenic concentration and Bioconcentration factor (BCF) in *Ruditapes decussatus* and *Ruditapes philippinarum* ($\mu\text{g/g}$ FW). Significant differences ($p \leq 0.05$) among tested conditions are represented with different letters (a-b) for each species and significant differences between species for each condition are represented with an asterisk.

		Total As in clams		BCF	
		PH 7.8	pH 7.3	PH 7.8	pH 7.3
0 mg/L As	<i>R. decussatus</i>	1.81±0.03 ^a	1.78±0.56 ^a	-	-
	<i>R. philippinarum</i>	1.59±0.94 ^a	1.23±0.22 ^a	-	-
4 mg/L As	<i>R. decussatus</i>	2.59±0.65 ^a	2.91±0.45 ^b	0.65±0.20 ^a	0.73±0.02 ^a
	<i>R. philippinarum</i>	2.18±1.03 ^a	6.40±1.87 ^b	0.54±0.26 ^a	1.60±0.47 ^b

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Table III –Monte Carlo p -values between As concentrations (0 and 4 mg/L), for each species at each pH level (7.8 and 7.3); between pH levels (7.8 and 7.3), for each species at each As concentration (0 and 4 mg/L); and between species (*R. decussatus*, Rd and *R. philippinarum*, Rp) at each pH level and As concentration. Values were obtained for each parameter analyzed: As concentration, Bioconcentration factor, BCF; glycogen content, GLY; lipid peroxidation (LPO) levels; superoxide dismutase (SOD) activity; catalase (CAT) activity; glutathione S-transferases (GSTs) activity; GSH/GSSG; and alkaline phosphatase (ALK) activity. The bold numbers represent significant increases for each clams at each condition ($p \leq 0.05$).

	Species	0 vs 4 mg/L of As		pH 7.8 vs pH 7.3		Rd vs Rp			
		pH 7.8	pH 7.3	0 mg/L of As	4 mg/L of As	7.8 and 0 mg/L of As	7.3 and 0 mg/L of As	7.8 and 4 mg/L of As	7.3 and 4 mg/L of As
As accumulation	Rd	0.096 (=)	0.021 (↑)	0.914 (=)	0.449 (=)	0.706 (=)	0.120 (=)	0.517 (=)	0.009 (↑)
	Rp	0.426 (=)	0.001 (↑)	0.482 (=)	0.008 (↑)				
BCF	Rd				0.454 (=)			0.525 (=)	0.012 (↑)
	Rp				0.007 (↑)				
GLY	Rd	0.497 (=)	0.517 (=)	0.497 (=)	0.622 (=)	0.034 (↓)	0.008 (↓)	0.000 (↓)	0.003 (↓)
	Rp	0.354 (=)	0.057 (=)	0.230 (=)	0.333 (=)				
LPO	Rd	0.182 (=)	0.198 (=)	0.456 (=)	0.014 (↑)	0.023 (↑)	0.003 (↑)	0.004 (↑)	0.028 (↑)
	Rp	0.095 (=)	0.180 (=)	0.031 (↑)	0.480 (=)				
GSTs	Rd	0.328 (=)	0.387 (=)	0.011 (=)	0.161 (=)	0.007 (↑)	0.003 (↑)	0.000 (↑)	0.176 (=)
	Rp	0.0188 (↑)	0.858 (=)	0.006 (↑)	0.247 (=)				
SOD	Rd	0.927 (=)	0.737 (=)	0.051 (=)	0.052 (=)	0.003 (↑)	0.004 (↑)	0.020 (↑)	0.049 (↑)
	Rp	0.046 (↑)	0.967 (=)	0.007 (↑)	0.849 (=)				
CAT	Rd	0.022 (↓)	0.6255 (=)	0.030 (↓)	0.366 (=)	0.024 (↑)	0.004 (↑)	0.000 (↑)	0.004 (↑)
	Rp	0.044 (↑)	0.0705 (=)	0.111 (=)	0.016 (↓)				
GSH/GSSG ratio	Rd	0.580 (=)	0.659 (=)	0.437 (=)	0.662 (=)	0.377 (=)	0.090 (=)	0.002 (↑)	0.708 (=)
	Rp	0.175 (=)	0.051 (=)	0.645 (=)	0.004 (↓)				
ALP	Rd	0.248 (=)	0.344 (=)	0.813 (=)	0.081 (=)	0.001 (↑)	0.001 (↑)	0.026 (↑)	0.008 (↑)
	Rp	0.001 (↓)	0.257 (=)	0.233 (=)	0.152 (=)				

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Table IV – Examples of studies that assessed the effects of chemicals and abiotic factors on marine invertebrates.

Species	Chemicals	Abiotic factors	REF.
<i>Mytilus edulis</i> (bivalve)	As (arsenobetaine and arsenocholine) (100 µg/dm ³)	-	Gailer et. 1995
<i>Chamelea gallina</i> and <i>Mytilus galloprovincialis</i> (bivalve)	-	Salinity (28; 34; 40) & pH (7.4; 7.8; 8.1)	Matozzo et al. (2013)
<i>Saccostrea cucullata</i> (bivalve)	As(III) (1; 5; 20 mg/L) and As(V) (1; 5; 20 mg/L)	-	Zhang et al. (2015)
<i>Mytilus edulis</i> , <i>Tegillarca granosa</i> , and <i>Meretrix meretrix</i> (bivalve)	Cd (0.05 mg/L)	pH (8.1, 7.8; 7.4)	Shi et al. (2016)
<i>Crassostrea virginica</i> and <i>Mercenaria mercenaria</i> (bivalve)	Cu (50 µg/L) and Cd (50 µg/L)	pH (7.60-8.18)	Götze et al., 2014
<i>Crassostrea gigas</i> (bivalve)	-	pH (8.0)	Timmins-Schiffman et al. (2014)
<i>Crassostrea angulata</i> and <i>Crassostrea gigas</i> (bivalve)	As (0; 4 mg/L)	pH (7.8;7.3)	Moreira et al. (2016a)
<i>Crassostrea angulata</i> (bivalve)	As (0; 4 mg/L)	Salinity (10, 20, 30; 40)	Moreira et al. (2016b)
<i>Macoma balthica</i> (bivalve)	Cd (0,01; 0,03; 0,10; 0,30 mg/L)	-	Duquesne et al. (2004)
<i>Venerupis corrugata</i> , <i>Ruditapes decussatus</i> , <i>Ruditapes philippinarum</i> (bivalves)	-	Salinity (0 to 42)	Carregosa et al. (2014)
<i>Ruditapes decussatus</i> and <i>R. philippinarum</i> (bivalve)	-	Temperature (22 to 42) & pH (8.2)	Anacleto et al. (2014)
<i>Ruditapes decussatus</i> and <i>R. philippinarum</i> (bivalves)	As (0 to 70 mg/L) and Hg (0 to 8 mg/L)	-	Velez et al. 2016b
<i>Ruditapes decussatus</i> and <i>R. philippinarum</i> (bivalves)	As (0; 4 mg/L)	pH (7.3;7.8)	present study
<i>Ruditapes philippinarum</i> (bivalve)	As (0; 4; 17 mg/L)	Salinity (10; 21; 28; 35; 42)	Freitas et al. (2016)
<i>Ruditapes philippinarum</i> (bivalve)	As (0; 2 mg/L)	-	Velez et al. (2016a)
<i>Ruditapes philippinarum</i> (bivalve)	-	Salinity (14; 28; 35) & pH (7.8)	Velez et al. (2016c)
<i>Ruditapes philippinarum</i> (bivalve)	Carbamazepine (0.00; 0.03, 0.30; 3.00; 9.00 µg/L)	pH (7.8)	Almeida et al. (2015)
<i>Corbicula fluminea</i> (bivalve)	As (0.1; 0.3; 0.5; 1 mg/L)	pH (7.0)	Diniz et al. (2008)
<i>Venerupis corrugata</i> (bivalve)	Hg (0.00; 0.025; 0.05; 0.10; 0.20 mg/L); Cd (0.0; 0.33; 1.00; 3.00; 9.00 mg/L); As (0.0; 1.60; 4.00;10.00; 25.00 mg/L); Pb (0.00; 0.10; 0.20; 0.40; 0.80 mg/L)	pH (7.8)	Velez et al. (2016d)
<i>Scrobicularia plana</i> (bivalve)	Hg; Cd; Zn; Cu (0.05 to 1 mM)	-	Mazorra et al. (2002)
<i>Scrobicularia plana</i> (bivalve)	Carbamazepine (3.00 mg/L)	pH (7.1; 7.8)	Freitas et al. (2015)
<i>Lamellidens marginalis</i> (bivalve)	As (0 to 5 mg/L)	-	Chakrabortya et al. (2010)
<i>Corbicula fluminea</i> (bivalve)	As (0.1; 0.3; 0.5;1.0 mg/L)	pH (7.0)	Diniz et al. (2008)
<i>Arenicola marina</i> (polychaeta)	As (2, 5, 10; 20 µg/L)	-	Casado-Martinez et al. (2012)
<i>Cyprinus carpio</i> (fish)	As (0; 0.1; 1.0 mg/L)	pH (8.0)	Ventura-Lima et al. (2009)

Figure 1

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Figure 2

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Figure 1 - Glycogen (GLY) content in the native species *Ruditapes decussatus* and in the introduced species *R. philippinarum* exposed to different As conditions (0 and 4 mg/L of As) and pH levels (7.8, 7.3). The minuscule letters represent differences for *R. decussatus* between As conditions at pH 7.8 and 7.3 and majuscule letters represent differences for *R. philippinarum* between As conditions at pH 7.8 and 7.3. Significant differences ($p \leq 0.05$) between pH conditions, for each species at each As condition were presented with asterisks.

Figure 2 - Lipid peroxidation (LPO) (A), glutathione S- transferases (GSTs) (B), superoxide dismutase (SOD) (C) and catalase (CAT) (D) in the native species *Ruditapes decussatus* and in the introduced species *R. philippinarum* exposed to different As conditions (0 and 4 mg/L of As) and pH levels (7.8, 7.3). The minuscule letters represent differences for *R. decussatus* between As conditions at pH 7.8 and 7.3 and majuscule letters represent differences for *R. philippinarum* between As conditions at pH 7.8 and 7.3. Significant differences ($p \leq 0.05$) between pH conditions, for each species at each As condition were presented with asterisks.

Figure 3 - Reduced and oxidized glutathione (GSH and GSSG) ratio in the native species *Ruditapes decussatus* and in the introduced species *R. philippinarum* exposed to different As conditions (0 and 4 mg/L of As) and pH levels (7.8, 7.3). The minuscule letters represent differences for *R. decussatus* between As conditions at pH 7.8 and 7.3 and majuscule letters represent differences for *R. philippinarum* between As conditions at pH 7.8 and 7.3. Significant differences ($p \leq 0.05$) between pH conditions, for each species at each As condition were presented with asterisks.

Figure 4- Alkaline phosphatase (ALP) in the native species *Ruditapes decussatus* and in the introduced species *R. philippinarum* exposed to different As conditions (0 and 4 mg/L of As) and pH levels (7.8, 7.3). The minuscule letters represent differences for *R. decussatus* between As conditions at pH 7.8 and 7.3 and majuscule letters represent differences for *R. philippinarum* between As conditions at pH 7.8 and 7.3. Significant differences ($p \leq 0.05$) between pH conditions, for each species at each As condition were presented with asterisks.

Figure 3

Figure 3

Figure 4

Figure 4