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CÁTIA SOFIA VELEZ SILVA

PODERÃO AS ESPÉCIES *RUDITAPES DECUSSATUS* (NATIVA) E *RUDITAPES PHILIPPINARUM* (INTRODUZIDA) VIVER EM SIMPATRIA?

IS IT POSSIBLE FOR NATIVE AND INTRODUCED *RUDITAPES* SPECIES TO LIVE IN SYMPATRY?

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o júri

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palavras-chave

Ecotoxicologia, contaminação por metais/metaloides, alterações climáticas, bivalves, consumo de marisco, stress oxidativo, biomarcadores

resumo

Nos ecossistemas marinhos os organismos estão expostos a uma enorme variedade de stresses tais como a contaminação por metais/metaloides, as alterações climáticas e a introdução de espécies invasoras. Uma das espécies invasoras mais bem-sucedidas é a amêijoa *Ruditapes philippinarum*, no entanto esta espécie pode também coabitar em ecossistemas costeiros com espécies nativas de amêijoas tais como *Ruditapes decussatus*. Embora alguns estudos utilizem estas espécies para avaliar a contaminação ambiental, pouca informação está disponível relativamente à abundância e distribuição de *R. decussatus* e *R. philippinarum* em diferentes ecossistemas. Além disso, os impactos induzidos por contaminantes e por fatores relacionados com as alterações climáticas em ambas as espécies continuam pouco conhecidos. Posto isto, a presente tese teve como objetivos perceber se a espécie nativa, *R. decussatus*, e a espécie introduzida, *R. philippinarum*, coabitam na Ria de Aveiro e na Óbidos lagoon, estudar a acumulação de metais e arsénio, a sua compartimentação e as alterações bioquímicas induzidas nestas espécies quando no ambiente. Para além destes objetivos, pretendeu-se também avaliar, através de ensaios laboratoriais, a resposta de ambas as espécies quando expostas a um stress isolado (contaminação por arsénio e variações de salinidade, temperatura ou pH), ou à combinação de diferentes stresses (contaminação por arsénio e variações de pH). Os estudos de campo efetuados na Ria de Aveiro e na Óbidos lagoon mostraram que as duas espécies coabitam e que a espécie introduzida em 2013 e 2014 ainda não tinha substituído a espécie nativa. Quando presentes na mesma área, ambas as espécies apresentam concentrações e compartimentação semelhante de metais e de arsénio, independentemente da contaminação de cada área e ecossistema. Esta semelhança reflete-se também ao nível da resposta bioquímica. Este estudo evidenciou também que, no ambiente ambas as espécies bioacumularam maior concentração de metais e arsénio nas áreas menos contaminadas do que nas áreas mais contaminadas. Para além dos trabalhos de campo, os estudos de laboratório mostraram que ambas as espécies quando expostas a arsénio apresentaram tolerância semelhante, sendo capazes de tolerar a toxicidade do arsénio até 8.2 mg/L. Contudo, quando expostas a um cenário de contaminação acima desta concentração, ambas as espécies apresentaram elevada mortalidade e reduzida capacidade em manter os seus mecanismos de desintoxicação. Relativamente à exposição a temperaturas elevadas, a espécie nativa provou ser a mais tolerante, uma vez que se verificaram menores alterações celulares e moleculares nesta espécie comparativamente à espécie introduzida. A espécie nativa também evidenciou uma elevada tolerância face à acidificação da água do mar comparativamente à espécie introduzida. No entanto, salinidades altas (35) e baixas (14) afetaram negativamente a espécie nativa. O mesmo se verificou para a espécie introduzida a salinidade 35. Em relação à exposição combinada a pH e arsénio os resultados mostraram que a resposta bioquímica de ambas as espécies não foi afetada negativamente. Contudo a espécie introduzida acumulou mais arsénio que a espécie nativa, pelo que a acidificação da água do mar poderá potenciar o risco associado ao consumo desta espécie. Em conclusão, os resultados reportados nesta tese contribuem para uma melhor compreensão sobre a coexistência de ambas as espécies em diferentes ecossistemas, da acumulação e toxicidade de metais/metaloides e da resposta de ambas as espécies quando expostas a diferentes condições ambientais tais como contaminação por arsénio, aumento da temperatura, flutuação da salinidade, e acidificação da água do mar.

keywords

Ecotoxicology, metal(loid)s contamination, climate change, bivalves, shellfish consumption, oxidative stress, biomarkers

abstract

In marine ecosystems, organisms are exposed to a variety of stressors such as metal/metalloid contamination, climate change, and introduction or spread of invasive species. One of the most successful invasive species is the clam *Ruditapes philippinarum* but this species may also co-habit in coastal ecosystems with the native species (*Ruditapes decussatus)*. Although some researchers have studied these species to assess environmental contamination, little information is available about both species from different ecosystems. In addition, the effects induced by contaminants and climate change related factors on both species remain poorly understood. Therefore, the present thesis aimed to understand if the native species, *R. decussatus*, and the introduced species, *R. philippinarum*, co-exist in the same areas of the Ria de Aveiro and the Óbidos lagoon, and to study their metal(loi)s accumulation, partitioning, and biochemical performances when co-habiting. In addition, in the laboratory, it was also assessed the response of sympatric clam species when exposed to single (As concentrations and salinity, temperature, pH shifts) or combined stressors (pH and As concentrations). Field studies in the Ria de Aveiro and Óbidos lagoon revealed that native and introduced species co-exist in both marine ecosystems and in 2013/2014 the introduced species has not yet supplanted the native one. When present in the same area, both species presented similar metal(loid)s concentrations, cellular metal partition, and biochemical performance, independently of the contamination level of each area and ecosystem. This study also revealed that both species from both ecosystems accumulated higher metal(loid)s concentrations in the least contaminated areas than in the most contaminated areas. In addition to the fieldwork, laboratory studies showed that both species exposed to As contamination presented similar tolerance, being able to regulate As toxicity up to 8.2 mg/L. However, both species under As contamination scenario above 8.2 mg/L were negatively affected, not being able to maintain detoxification mechanisms against As exposure leading to its death. Relatively to temperature exposure, the native species was the most tolerant species to future chronic warming environmental conditions since the temperature rises induced less subcellular and molecular changes in native, than in introduced species. In addition, native species were also tolerant to seawater acidification (7.3) when compared to introduced one. However, low (14) and high (35) salinities affected negatively native species. Similar results were observed for introduced species at high salinity. Regarding the combined effects exposure, results showed that both species were not negatively affected by the predicted seawater acidification. However, the introduced species accumulated higher amount of As than the native clams, suggesting that the predicted seawater acidification may potentiate health risks associated with the consumption of this species. In conclusion, findings reported in this thesis improved the knowledge and provide an important data base about the distribution, contamination and the performance of both species from different ecosystems and the response of both species exposed to different environmental conditions, such as arsenic contamination, temperature increase, salinity shifts, and seawater acidification.

Thesis publications

The present work is based on the following manuscripts:

Velez, C., Figueira, E., Soares, A.M.V.M., Freitas, R., 2015. Spatial distribution and bioaccumulation patterns in three clam populations from a low contaminated ecosystem. Estuarine Coastal And Shelf Science. 155, 114-125.

Velez, C., Galvão, P., Longo, R., Malm, O., Soares, A.M.V.M., Figueira, E., Freitas, R., 2015. *Ruditapes philippinarum* and *Ruditapes decussatus* under Hg environmental contamination. Environmental Science And Pollution Research. 22, 15, 11890-11904 .

Velez, C., Leandro, S., Figueira, E., Soares, A.M.V.M., Freitas, R., 2015. Biochemical performance of native and introduced clam species living in sympatry: The role of elements accumulation and partitioning. Marine Environmental Research. 109, 81-94.

Velez, C., Freitas R., Antunes S.C., Soares A.M.V.M., Figueira E., 2016. Clams sensitivity towards As and Hg: A comprehensive assessment of native and exotic species. Ecotoxicology And Environmental Safety. 125, 43-54.

Velez, C., Figueira E., Soares, A. M.V.M, Freitas R., 2017. Effects of seawater temperature increase on economically relevant native and introduced clam species. Marine Environmental Research.123, 62-70.

Velez, C., Figueira E., Soares, A. M.V.M, Freitas R., 2016. Native and introduced clams biochemical responses to salinity and pH changes. Science of the Total Environment. 566-567, 260-268.

Velez C., Figueira E., Soares, A. M.V.M, Freitas, R., 2016. The impacts of As accumulation under different pH levels: Comparing *Ruditapes decussatus* and *Ruditapes philippinarum* biochemical performance. Environmental Research. 151, 653-662.

Other publications

Velez, C., Figueira, E., Soares, A. M.V.M, Freitas, R., 2016. Bioaccumulation patterns, element partitioning and biochemical performance of *Venerupis corrugata* from a low contaminated system. Environmental Toxicology. 31, 5, 569-583.

Velez, C., Figueira, E., Soares, A. M.V.M, Freitas, R., 2016. Combined effects of seawater acidification and salinity changes in *Ruditapes philippinarum*. Aquatic Toxicology*. 176, 141-150.*

Velez, C., Pires A., Sampaio L., Cardoso P., Moreira A., Leandro S., Figueira E., Soares A.M.V.M., Freitas, R., 2016. The use of *Cerastoderma glaucum* as a sentinel and bioindicator species: Take-home message. Ecological Indicators. 62, 228-241.

Velez, C., Figueira, E., Soares A. M.V.M, Freitas R., 2016. Accumulation and cellular partitioning of metals and As in the clam *Venerupis corrugata*: different strategies towards different elements. Chemosphere. 156, 128-134.

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List of figures and tables

Abbreviations

ALP - Alkaline phosphatase

BAX- bcl-2-like protein 4

BCF- Bioconcentration Factor

BSAF - Biota-Sediment Accumulation factor

CA - Carbonic Anhydrase

CAT - Catalase

CI - Condition Index

Cox-1 - cytochrome C oxidase subunit I gene

DW - Dry Weight

EFSA - European Food Safe Authorities

ERL - Effects Range Low

ERM - Effects Range Median

ETS – Electron Transport System

FSANZ - Food Standards Australia and New Zealand

FW -Fresh Weight

gadd45 - growth arrest and DNA damages

GLY - Glycogen

GR - Glutathione reductase

GSH - Glutathione reduced

GSHt - Total glutathione

GSSG - Glutathione oxidized

GSTΩ - Glutathione S-transferase omega

GSTs - Glutathione S-transferase

Hsp - Heat shock protein

IPCC - Intergovernmental Panel on Climate change

JECFA - Joint FAO/WHO Expert Committee on Food Additives

LPO - Lipid Peroxidation

MDA - Malondialdehyde

MLs - Maximum permissible limits

MRG - Metal-rich granules

MTs - Metallothioneins

PCO - Principal Coordinates Analysis

PEL - Probable Effects Levels

PROT - Protein

PTWI - Provisional Tolerable Week Intake

ROS – Reactive Oxygen Species

SOD – Superoxide dismutase

TA - Total alkalinity

TBA - Thiobarbituric acid

TEL- Threshold Effect Level

THg – Total mercury

TOM - Total Organic Matter

USFDA - United States Food and Drug Administration

Chapter 1

General introduction

1.1. Metals and As in marine ecosystems

Coastal ecosystems are among the most relevant and dynamic systems in terms of productivity and biodiversity (Beaumont et al., 2007; Costanza et al., 1997). In addition to their ecological importance, these systems also provide notable economic resources, serving as food provision for economically relevant species and humans (Beaumont et al., 2007; Miththapala, 2013). However, the natural equilibrium of marine ecosystems can be rapidly disturbed by pollution, posed either by natural or anthropogenic sources (Green-Ruiz and Páez-Osuna, 2001; Poulos et al., 2000). Among pollutants, metals and metalloids are one of the most dangerous groups, not only because of their high toxicity even at low concentrations, but also due to their accumulation in the environment, including inhabiting organisms (Acton, 2011; ATSDR, 2016). This fact encouraged in the last decades' researchers to identify different sources and levels of metal(loid)s in the aquatic environment (Abreu et al., 2000; Dixit et al., 2015; Donnici et al., 2012; Pereira et al., 2008; Randall and Chattopadhyay, 2013; UNEP, 2010), as well as their impacts on inhabiting biota (Al-Subiai et al., 2011; Chandurvelan et al., 2015; Cravo et al., 2012).

Arsenic (As) is among the most toxic metalloids found in the environment (ATSDR, 2016). A wide range of As concentrations is reported in a vast amount of published literature (e.g. Mamindy-Pajany et al., 2013; Martins et al., 2013; Mora et al., 2004; Smedley and Kinniburgh, 2002). Pesticides, wood preservatives, biosolids, ore mining and smelting are the most common sources of As in the environment (Abreu et al. 2000; Donnici et al., 2012; Pereira et al., 2008; Randall and Chattopadhyay, 2013). Arsenic can occur in the environment in several oxidation states and its toxicity varies widely with its concentration and speciation, being inorganic As (arsenate, AsV, and arsenite, AsIII) the most toxic form, while organic As compounds (methylarsonic acid, MMA, and dimethylarsinic acid, DMA) the least toxic (Neff, 1997). Inorganic As compounds are the most common forms found in seawater, particle matter and sediments while organic As is generally predominant in marine organisms (Fattorini et al., 2004; Neff, 1997). Often, the inorganic As can be converted to less toxic forms such as MMA and DMA, by aerobic and anaerobic sediment bacteria (Rahman et al., 2014; Wang et al., 2014). These reactions are influenced by oxidation-reduction potential, pH, temperature, salinity, metal sulfide and sulfide ion concentrations, iron concentration, and distribution and composition of the biota (ATSDR, 2016). Owing to its harmful effects in marine ecosystems, many investigators determined the levels of As in sediments and marine food (Alonso-Hernández et al., 2012; Rahman et al., 2012).

Besides As, mercury (Hg), lead (Pb), cadmium (Cd), nickel (Ni) and chromium (Cr) are also listed as priority hazardous substances in the US Agency for Toxic Substances and Disease Registry (ATSDR, 2016), due to their high persistence, potential for bioaccumulation, and toxicity (Ahmad et al., 2012; Rahman et al., 2014). These elements have their origin in marine ecosystems from anthropogenic and natural sources (Dixit et al., 2015; UNEP, 2010). Metals are present in sediments or in suspended particulate matter, water column, and food sources (Solomon, 2008;

Spada et al., 2012). In sediments and water, metals can be converted into toxic organic forms, by bacteria and/or fungi (Cempel and Nikel 2006; Chiarelli and Roccheri, 2014; Correia et al., 2013; Pak and Bartha, 1998; Solomon, 2008; Ullrich et al., 2001; UNEP, 2010). This conversion and the toxicity of these metals are strongly dependent on abiotic factors such as pH, salinity, and temperature (Celo et al. 2006; Pedro et al., 2013; Solomon, 2008; UNEP, 2010; Velma et al., 2009). It is known that once introduced into the aquatic environment, metals can be accumulated in the sediments in concentrations above the threshold defined levels, resulting in ecosystems deterioration (Long et al., 1995; Long and MacDonald1998; Macdonald et al., 1996). Natural (tidal movement and storms) and anthropogenic (human activities such as dredging, dredge) disturbance of surface sediments may affect the bioavailability of metals in the marine environment and, consequently, their toxicity (e.g. Eggleton and Thomas, 2004; Pan and Wang, 2012; Zhang et al., 2014). Atkinson et al. (2007) revealed that the physical disturbance of sediment release metals more rapidly than biological disturbance (e.g. bioturbation by benthic bivalves) being this process influenced by sediment pH and dissolved oxygen concentration. Zhang et al. (2014) revealed that sulfites, organic fraction, sediment texture, redox potential, salinity, and temperature are among the factors that highly influence metal bioavailability in sediments. These authors also observed that the increase of salinity was related to the increase of metals bioavailability. Also, low pH can weaken the strength of metal association and impede the retention of metals by sediments, leading to their release (Belize et al., 2004; Guven and Akinci, 2013). On the other hand, Atkinson et al. (2007) demonstrated that high pH and dissolved oxygen generate low dissolved metal concentrations in the waters upper layer. Chakraborty et al. (2012) revealed that total organic carbon is also one of the key factors which play a crucial role in controlling speciation of Pb and Cd in sediments.

Once bioavailable, metal(loid)s can be accumulated by organisms (e.g. Alonso-Hernández et al., 2012; Baudrimont et al., 2005; Chandurvelan et al., 2015; Govind and Madhuri, 2014). Newman (2009) defined bioaccumulation as the accumulation of a contaminant in and on an organism from all sources. The bioconcentration concept may be viewed as a special case of bioaccumulation, which is related to the concentration of a contaminant only from water into organisms (McGeer et al., 2003; Newman, 2009). The bioaccumulation term is derived from measurements in the natural environment and includes accumulation from water, food, sediment and fine particles suspended in water, while the bioconcentration is associated with metal exposure under laboratory conditions with accumulation resulting only from water (McGeer et al., 2003). High metal(loid) concentration may have toxic effects on biota and ultimately death in most living organisms (Fig 1.1) (Govind and Madhuri, 2014). Up to date, several authors have investigated the metal(loid)s accumulation and the effects of their accumulation in different organisms (e.g. Ahmad et al., 2012; Alonso-Hernández et al., 2012; Faganeli et al., 2012; Rahman et al., 2014).

Once a contaminant enters in the organism it becomes available for biotransformation, detoxification, sequestration, redistribution, or activation and elimination (Fig.1.1) (Newman, 2009).

Metal(loid)s are subjected to biotransformation, resulting in sequestration (metallothionein or similar functioning molecules) or elimination, which means that excretion or metabolization of a metal(loid)s results in a decrease in the amount of contaminants within the organism (Newman, 2009). However, high metal(loid)s concentration may elicit toxic effects on biota and ultimately death in most living organisms (Fig 1.1) (Govind and Madhuri, 2014). Due to that up to date several authors have investigated the levels of metal(loid)s in sediment and water, their speciation and bioavailability patterns in marine ecosystems, as well as the accumulation and the induced effects in biota (e.g. Ahmad et al., 2012; Alonso-Hernández et al., 2012; Faganeli et al., 2012; Rahman et al., 2014). More important, metal(loid)s bioavailable can be taken up by marine organisms and potentially be transferred to the upper trophic levels (biomagnification), which eventually lead to adverse effects on organisms and humans due to the consumption of contaminated seafood (Fig. 1.1) (Wang, 2002).

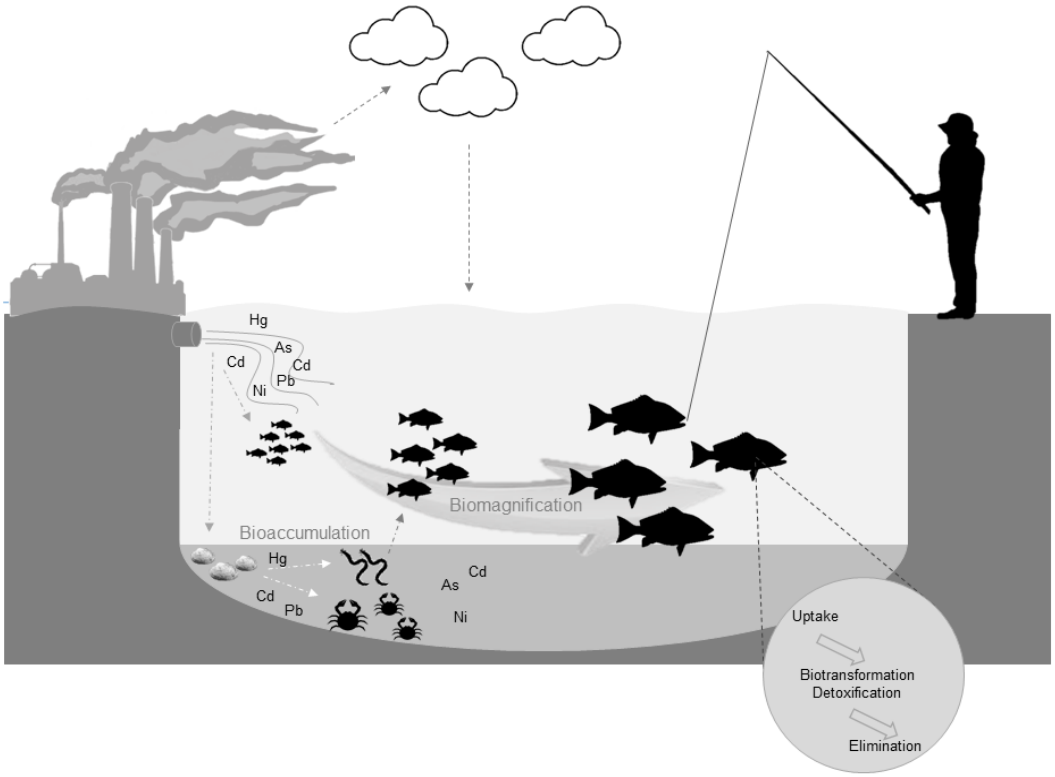

Figure 1.1 – Bioaccumulation of metal(loid)s in different marine organisms and biomagnification through the food chain.

The consumption of different marine species that present great ability to accumulate metal(loid)s, and the level of metals in seafood, lead the international organizations (European Food Safety Authority, EFSA, United States Food and Drug Administration, USFDA, or Food Standards Australia and New Zealand, FSANZ) to establish maximum permissible limits (MLs),

above which edible seafood cannot be marketed. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) defined the provisional tolerable weekly intake (PTWI) for contaminants that a human can accumulate in its body. Tolerable intakes are expressed on a weekly basis because the contaminants given this designation may accumulate within the body over a period of time (Altug, 2002).

Knowing the capacity of metal(loid)s to be accumulated both in the environment and inhabiting organisms, monitoring programs have become widely established. One of the most famous marine monitoring program is the World Mussel Watch (WMW) which began in the 1970s in the United States, being the longest running contaminant monitoring program in the world (Kimbrough et al., 2008). This program used the concentration of contaminants in bivalves and sediments, to assess their health. Different monitoring programs based on 'Mussel Watch' (Goldberg, 1986) have been used to monitor the environmental status of different coastal regions, such as Taiwan (Jeng et al., 2000), Morocco, Africa (Sparks et al., 2014), Spain (Spanish Maritime Monitoring Program, Besada et al., 2011), Turkey (Kucuksezgin et al., 2010) and Italy (Spada et al., 2013). In addition to field studies, many authors also performed ecotoxicological tests to assess the effects of metal(loid)s, revealing that metal(loid)s exposure may result in reduced survival, respiratory disruption, impaired reproduction, reduced growth and changes in enzyme and genetic levels of aquatic biota (e.g. Baudrimont et al., 2005; Eisler 1988; Figueira et al., 2012; Freitas et al., 2014; Velma et al., 2009).

1.2. Climate change

Besides pollution pressures, marine ecosystems may be also affected by climate change (e.g. Botana et al., 2015; Coll et al., 2008; Gazeau et al., 2013). The greenhouse gases (carbon dioxide, CO_2, methane, CH_4, nitrous oxide, N_2O, and fluorinated gases) from human activities contributed to significant global climate change during the last decades (IPCC, 2007). According to the Intergovernmental Panel on Climate change (IPCC, 2014), "human influence on the climate system is clear and growing, with impacts observed across all continents and oceans".

It is expected that climatic change related factors, including changes in temperature, pH, and salinity, will influence coastal systems physical, biological and biogeochemical characteristics (IPCC 2007; 2014; Kennedy et al., 2002; VijayaVenkataRaman et al., 2012). It is known that marine organisms are exposed to natural salinity, pH, and temperature changes during the tide shifts, being tolerant to a wide range of values. However, when such tolerance ranges are exceeded, negative consequences may take place. Furthermore, changes in such abiotic factors may affect the sensitivity of organisms to pollutants (Moe et al., 2013) and may also alter their toxicity (Botana et al., 2015; Lee et al., 2013; Schiedek et al., 2007). In addition, the interaction of

global warming, salinity changes, and ocean acidification may result in a complex, and amplified impact on individuals, populations, and communities (IPCC, 2014).

1.2.1. Global warming

Global warming corresponds to the increase of the average temperatures on the earth due to the rise of greenhouse gases (namely CO_2) influenced by agriculture, land clearing and burning of fossil fuels (Li and Lin, 2015; Morales et al., 2014). According to the most recent report of the IPCC (2014), "the climate system warming is irrefutable". Over the next century, the IPCC (2014) predicts that the global average warming will increase by 1.0 to 4.0, and consequently the seawater surface temperatures would increase (EPA, 2008; IPCC, 2013, 2014; Kennedy et al., 2002). In fact, according to the United States Environmental Protection Agency (EPA, 2016) increases in the surface ocean temperatures have already been observed with an estimated average increase of 1°C from 1901 to 2015 (IPCC, 2014). However, these temperature shifts are ubiquitous for all ocean waters. There is a significant variation at the regional and national scales with more evident temperature increase at high latitudes (Burgiel and Muir, 2010).

Ocean warming is directly related to the sea-level rise and oceanic circulation patterns (Kennedy et al., 2002). The sea-level rise promoted by melting glaciers, coupled with different water temperatures results in changes of ocean circulation patterns (Herr and Galland, 2009), which will also affect coastal areas as recently demonstrated by Mackenzie and Schiedek (2007). The temperature changes in coastal ecosystems may influence dissolved oxygen concentrations in water, resulting in a possible risk of hypoxia due to the eutrophication (Eissa and Zaki, 2011). Additionally, the marine organism's biology (mortality, reproduction, growth, chemical performance and behaviour) and ecological processes (e.g. productivity and species interactions) may be affected by temperature changes (Anacleto et al., 2014; Anestis et al., 2007; Han et al., 2007; Monari et al., 2007), since many organisms already live close to their thermal tolerance limits (Nguyen et al., 2011; Somero, 2005; Stillman and Somero, 2000). It is known that many marine species are sensitive to temperature shifts and just a few degrees can affect their geographical distribution (Brierley and Kingsford, 2009; Sorte et al., 2011). Those species that are unable to migrate can compete for resources, which contribute to local extinctions and provides exceptional opportunities for the dispersal and growth of invasive species (Kennedy et al., 2002; Masters et al., 2010). Due to that, different authors have been focused on the effects of temperature changes on marine organisms (e.g. Deschaseaux et al., 2011; Duarte et al., 2014; Monari et al., 2007).

Despite the effects of temperature in organisms, temperature changes may also change the chemistry of contaminants present in marine ecosystems resulting in a significant alteration in their toxicities and the sensitivity of organisms to these pollutants (Moe et al., 2013; Schiedek et al., 2007).

1.2.2. Ocean acidification

Natural phenomena and the continuous combustion of fossil fuels, their transportation, cement production, agriculture, and deforestation, are predicted to increase the atmospheric levels of CO_2 over time (Branch et al., 2013; Canadell et al., 2007; Turley et al., 2010). Since the industrial revolution, oceans absorbed approximately 30% of the CO_2 emitted by these anthropogenic activities (IPCC, 2014), providing a valuable service to human societies by lowering the rate and severity of climate change. Although this buffer capacity poses effective results, continues absorption of CO_2, ocean warming and changes in wind patterns reduces the capacity of ocean to take up additional CO_2 from the atmosphere (Herr and Galland, 2009). At the end of the century, the predicted partial pressure of CO_2 (pCO_2) is expected to increase current values from 390 to 700-1,000 µatm in the state of the earth ocean models (Caldeira and Wickett, 2005; Melzner et al., 2012; Turley et al., 2010). However, in coastal lagoons and estuaries, the magnitude of expected changes in pCO_2 may be higher than in oceans. According to Melzner et al. (2012), coastal hypoxic regions may expect high pCO_2 values (3,400 and 4,500 µatm) which depend on salinity. Global surface ocean pH is expected to decline from 0.3 to 0.5 units by the end of the century (Caldeira and Wickett, 2005; IPCC, 2007, 2014). The decrease in pH of the earth´s oceans due to CO_2 uptake from the atmosphere is known as ocean acidification (IPCC, 2007).

It is known that the massive increase of CO_2 in seawater will generate changes in the seawater carbonate chemistry of coastal and estuarine ecosystems (Billé et al. 2013; Hilmi et al., 2015). Once dissolved in seawater, CO_2 reacts with water, forming carbonic acid (H_2CO_3). Then, this acid dissolves rapidly to form hydrogen (H^+, an acid) and bicarbonate ions (HCO_3^-, a base) (Fig. 1.2). This process lowers pH (due to the increase of H^+), increases the concentration of HCO_3^- and decreases the availability of carbonate minerals (CO_3^{2-}) (Fig. 1.2). The decrease of CO_3^{2-} available also decreases the affinity of CO_3^{2-} with calcium (Ca^{2+}), which may induce negative effects on calcifying organisms, since some marine organisms extract Ca^{2+} and CO_3^{2-} from seawater. Calcium and CO_3^{2-} have shell-forming capacity to originate calcium carbonate ($CaCO_3$) such as calcite and aragonite (Hardege et al., 2011; Turley et al., 2010).

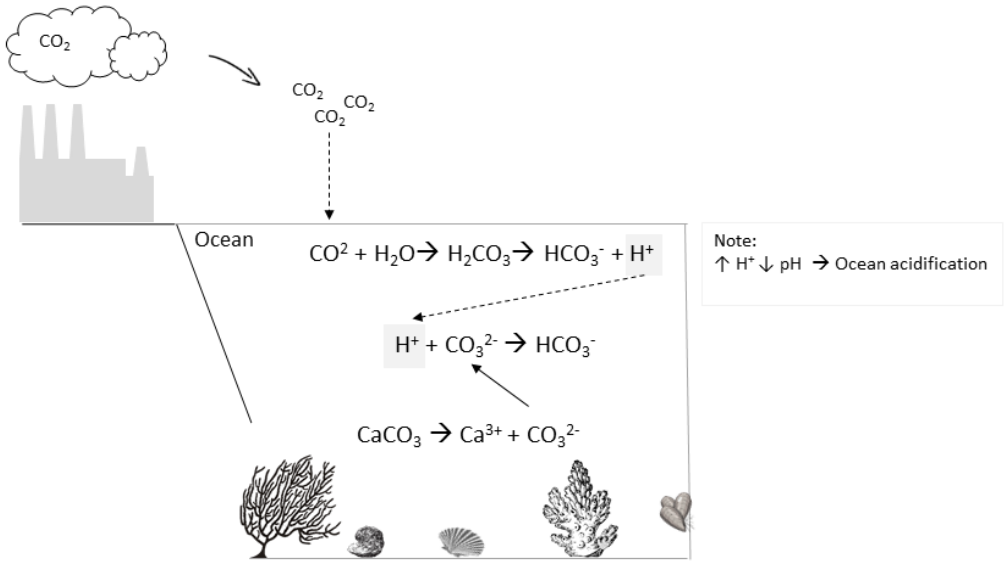

Figure 1.2 – The atmospheric CO_2 is absorbed by the ocean. The continuous increase of dissolved CO_2, increase the hydrogen ions (H^+), leading to ocean pH decrease and consequently ocean acidification. The extra H^+ ions react with carbonate ions (CO_3^{2-}) to form more bicarbonate, which decreases its availability to organisms to build their calcium carbonate ($CaCO_3$) shells and exoskeletons.

Calcifying organisms such as coral reefs, shellfish and zooplankton are among the first potential victims of ocean acidification (Dupont et al. 2010; Kleypas et al., 2006). Recent works recognized pH decrease as a key factor that affects many biological processes, especially, calcification, respiration, regulation of internal pH, energy metabolism, gene expression, feed, growth and reproduction (Berge et al., 2006; Dickinson et al., 2012; Duarte et al., 2014; Gibson et al., 2011; Kleypas et al., 2006; Orr et al., 2009). In a macroscale, it influences biodiversity, trophic interactions, and other ecosystem processes (Kleypas et al., 2006; Royal Society, 2005). In addition to the effects of seawater acidification on organisms, it is also known that seawater acidification may favour the desorption of metals from sediments, their toxicity and the susceptibility of organisms to these pollutants (Hong et al., 2011; López et al., 2010; Riba et al., 2003).

1.2.3. Salinity changes

Ocean acidification, the rise of seawater temperatures and the seawater levels are the most commonly discussed consequences of anthropogenic climate change for the global oceans (IPCC 2007, 2014). However, according to IPCC (2007), ocean salinity changes may be used as a sensitive indicator of climate change, providing important information on changes in precipitation, evaporation, river runoff and ice melt, as well as changes in the Earth's hydrological cycle over the oceans. It is known that global warming may also contribute to the occurrence of extreme weather events, such as acceleration of the global rainfall and evaporation cycle, as well as enhanced

freshwater input by rivers and melting glaciers into the ocean, estuarine and coastal areas, promoting salinity shifts (Coughlan et al., 2009). Since 1950, there is a similar tendency of salinity spatial patterns and the mean distribution of evaporation minus precipitation, i.e., high-salinity regions where evaporation dominates have become more saline, while low-salinity regions where rainfall dominates have become fresher (IPCC, 2013).

It is well known that estuaries and coastal lagoons are subjected to tidal and seasonal salinity changes (Kusler, 1990; Menéndez et al., 2012; Robins et al., 2016). However, due to the increase in frequency and severity of such events, salinity is expected to drastically change, affecting seawater chemistry and changing the buffering capacity of water (Hamer et al., 2008). When the salinity tolerance of marine organisms is exceeded, marine biota is affected, which ultimately may cause changes in biodiversity and productivity of ecosystems. The effects of salinity changes have been investigated by several authors on marine biota, revealing the impacts of salinity on the respiration rate (Resgalla et al., 2007), growth (Javanshir, 2013), host–pathogen interactions (Bussell et al., 2008; Matozzo et al., 2007; Perrigault et al., 2012), immune responses (Matozzo et al., 2007; Reid et al., 2003), metabolic and physiological alterations on marine organisms (Carregosa et al., 2014; Coughlan et al., 2009; Sarà et al., 2008). Moreover, salinity changes may also influence the toxicity of contaminants and physiological regulation processes in organisms involved in the detoxification of hazardous substances, making organisms vulnerable to additional chemical stress (Lee et al., 2013; Schiedek et al., 2007).

1.3. Biological invasions and introduced species

Besides metal(loid) contamination and climate change, invasive species may pose negative effects on marine ecosystems (Burgiel and Muir, 2010; Moe et al., 2013; Robins et al., 2016). In recent decades, hundreds of thousands of species are released intentionally or unintentionally worldwide into new environments (Nentwig, 2007). This phenomenon was accelerated by rapid globalization and increasing trends of trade, travel, and transport (Hulme, 2009). The species that are introduced outside of their natural range either intentionally or unintentionally by human activities are defined as introduced species (also described as alien, exotic, non-native or nonindigenous species) (EPA, 2008). The introduced species may play a beneficial role in ecosystem functioning, resulting in a net gain of species and in an increase of biomass production (FAO, 2009; Minchin and Rosenthal, 2002). However, in marine ecosystems, introduced marine species may rapidly become invasive and displace the native ones (Katsanevakis et al., 2016) (EPA, 2008). Nowadays, the introduction and spread of invasive species have been identified as a major ecological threat to coastal marine communities (Pimentel et al., 2005). The International Union for Conservation of Nature and Convention on Biological Diversity (2016) defines invasive as “a species introduced by humans – either intentionally or accidentally - outside of its natural past or present distribution whose introduction and/or spread, threaten biological diversity”. In fact, it is

known that biological invasions may have different impacts on biodiversity, natural resources and ecosystem services (Stylianos et al., 2014; Occhipinti-Ambrogi and Savini, 2003). Invasive species have been responsible for biodiversity loss and the homogenization of the invaded habitat (Occhipinti-Ambrogi and Savini, 2003). The replacement of native species by the invasive ones lead to the decline of populations, local extinctions, changes in community composition, and effects on entire ecosystem processes (Blackburn et al., 2014; Katsanevakis et al., 2016; McNeely et al., 2001). Invasive species also have the potential to alter ecosystem processes such as water or nutrient cycling, reducing the quantity and quality of natural resources and creating substantial economic losses (McNeely et al., 2001). Due to these reasons, several authors have studied the interactions between the introduced and native marine species in order to correctly identify potential outcomes/ threats of species introduction (Bidegain et al., 2015a; Bielen et al., 2016; Stylianos et al., 2014).

Many species become invasive due to their dispersal characteristics, life-history strategies, growth rates, distributional ranges, and tolerance towards environmental stressors (McNeely et al., 2001; Walther et al., 2009). According to McKenzie et al. (2012) and Piola and Johnston (2006), the tolerance of invasive species to metal-contaminated habitats may facilitate their establishment and spread in marine ecosystems. However metal bioavailability may be affected by climate change scenarios and consequently affect invasive species (Lee et al., 2013; Moe et al., 2013).

Changes in climate (e.g. pH, temperature and salinity) can alter the sensitivity of organisms to pollutants, namely metals and metalloids, which may alter the conditions for the establishment and spread of invasive species. Also, environmental factors related to climate change may alter the suitability of local climates for native species and the nature of interactions among native communities (EPA, 2008). In addition, climate change may also influence the toxicity of contaminants (Schiedek et al., 2007). Several authors recognized the interaction of climate change associated with contaminants (Moe et al., 2013), and invasive species as responsible for the decline of biodiversity (Harris and Tyrrell, 2001; IPCC, 2007; 2014; Masters and Norgrove 2010; Moe et al., 2013).

Thus, the study of interactions between the introduced and native marine species has become a focus of interest, especially when dealing with economically relevant species. Among marine animals, bivalves are one of the most successful invasive organisms (Assis et al., 2015). It is known that successful invasive bivalves' species may compete with native ones. Examples of these bivalves are the mussel *Arcuatula* (*Musculista*) *senhousia* that compete with the mussel *Mytilus galloprovincialis* (young individuals) (Otero et al., 2013). Also the Pacific oyster *Crassostrea gigas* has been introduced in different ecosystems, contributing to the decline of the Mediterranean native oyster *Ostrea edulis* and the native mussel *Mytilus edulis*, essentially by outcompeting them for food and space (Diederich, 2006; Wrange et al., 2010). The invasive clam *Ruditapes philippinarum* competes for food and space with other native filter-feeding invertebrates, such as *Ruditapes decussatus* (Otero et al., 2013).

1.4. Marine bivalves as test organisms

Besides biotic factors, the abiotic factors such as pH, salinity, temperature and metal(loid)s may also influence marine bivalves species (Anacleto et al., 2014; Carregosa et al., 2014; Parker et al., 2013). Thus, in the last decades, marine bivalves have been used in several fields, namely ecotoxicology, since they represent one of the most important biological groups on estuaries. Playing an essential role in the community, bivalves contribute to the energy flow, water column purification besides being an important food resource for human populations (e.g. Figueira et al., 2012; Santos et al., 2011; Sheehan and McDonagh, 2008; Verdelhos et al., 2014). The global aquaculture production of molluscs (mostly bivalves) was ~75% (13.9 million tons) of total aquaculture, representing an economically relevant activity for worldwide population (Costanza et al., 1997).

Bivalves, which are mostly filter-feeder organisms, present a sessile nature in the adult stage (e.g. reflecting site-specific conditions), high abundance and widespread distribution (Hamza-Chaffai, 2014). Due to these characteristics, bivalves are excellent candidates as sentinel and/or bioindicator organisms (Hamza-Chaffai et al, 2014). Beeby (2001) defined sentinel species as "species insensitive to a pollutant, able to accumulate bioavailable contaminants in their tissues, showing a simple correspondence between tissue and ambient levels and with high abundance, sedentary or with a limited home-range". On the other hand, bioindicator species were 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).

Acting as sentinels, bivalve species can help detect marine pollution because bivalves present a great capacity to bioaccumulate chemicals (Al-Subiai et al., 2011; Jebali et al., 2014; Moschino et al., 2012; Pellerin and Amiard, 2009). However, bivalves present a broad tolerance range to metal(loid)s toxicity, reflecting or not the effects of their bioaccumulation (bioindicator) (Bergayou et al., 2009; Box et al., 2007; Cravo et al., 2012; Vlahogianni et al., 2007). The bivalve characteristics led to the use of these as sentinel and/or bioindicator species in marine pollution studies around the world, signaling the presence of toxic substances and changes in the environment pollutant status (e.g. Bergayou et al., 2009; Cravo et al., 2012; Mora et al., 2004; Moschino et al., 2012; Sheehan and McDonagh, 2008).

The majority of bivalves are also tolerant to a wide range of abiotic factors (e.g. Carregosa et al., 2014; Compton et al., 2007; Hahn et al., 2012). However, increasing frequency of extreme weather events, the limit of abiotic tolerance can be reached, with authors considering bivalves as sensitive indicators for climate change (Bielen et al., 2016; Rodrigues et al., 2015; Sorte et al., 2011). Due to that several studies on the effects of acidification, temperature and salinity on marine invertebrates use bivalves as model organisms (Anestis et al., 2007; Carregosa et al., 2014; Dickinson et al., 2012; Parker et al., 2013), namely *Mytilus edulis* and *Serripes groenlandicus,*

which have been recognized as bioindicators to assess the effect of climate change (Carroll et al., 2009; Caza et al., 2016).

1.4.1. *Ruditapes decussatus* and *R. philippinarum*

The marine bivalves' species *Ruditapes decussatus* (Linnaeus, 1758) and *R. philippinarum* (Adams & Reeve, 1850) have been proposed as sentinel or bioindicator species, due to their wide distribution, ecological and economic interest and high capacity to accumulate pollutants (Bebianno et al., 2004; Ji et al., 2006; Matozzo et al., 2012; Smaoui-Damak et al., 2004). Several authors have been using both species to assess the health status of ecosystems (Baudrimont et al., 2005; Bebianno et al., 2004; Cravo et al., 2012; Matozzo et al., 2012; Usero et al., 1997). More recently, these organisms have also been employed to assess the effects of climate change (e.g Freitas et al., 2014; Han et al., 2007; Parker et al., 2013; Wang et al., 2012).

Nowadays, the clam *R. philippinarum* is among the most commercialized species, having a considerable economic importance, in terms of aquaculture, and/or as a harvested 'wild' resource (FAO, 2016a). This species, native from the Indo-Pacific waters, have been introduced in several parts of the world, such as the Atlantic and Mediterranean coast, for culture purposes, in the early 70's: first in France, and later in England, Spain, Italy and more recently, in Portugal (Chiesa et al., 2017; FAO, 2016a; Flassch and Leborgne, 1992; Moschino et al., 2012). *R. philippinarum* was introduced into European waters due to overfishing and irregular yields of the native (European) grooved carpet shell, *R. decussatus* (Chiesa et al., 2017; FAO, 2016b; Gosling, 2003). According to FAO (2016a), since 80th, the global aquaculture production of *R. philippinarum* increased, being reported to be approximately 4 million tons in 2014. Due to its high distribution, fast growth, great ability to adapt to new environments and to compete for food and space, *R. philippinarum* is considered one of the most successful invasive species in the Mediterranean (Otero et al., 2013; Pranovi et al., 2006; Streftaris and Zenetos, 2006). Up to now, in several European systems, such as the Santander Bay (Spain), Arcachon bay (France) and Poole Harbour (south coast of England) *R. philippinarum* is considered an introduced species, living in sympatry with the native ones (Bidegain and Juanes, 2013; Blanchet et al., 2004; Dang et al., 2010; Humphreys et al., 2007; 2015). In Portugal, according to Chainho et al. (2015), *R. philippinarum* is currently the dominant bivalve species in the Tagus estuary and abundant in both Sado estuary and in Ria de Aveiro, comparing with the native species. Recently Moura et al. (2017) reported that the introduction of *R. philippinarum* in Tagus Estuary was responsible for the decline of *R. decussatus* population since this ecosystem has near-ideal environmental conditions for the *R. philippinarum* growth.

Despite the negative impacts that *R. philippinarum* can bring to the population of native clams, this invasive species brought new ecosystem services including new fisheries activity and regulation of benthic processes (e.g. oxygen consumption, nitrification, and denitrification) (Tsujimoto et al., 2012; Welsh et al., 2015). The clams, *R. philippinarum* and *R. decussatus* co-

exist in different ecosystems (Bidegain and Juanes, 2013; Usero et al., 2007). The grooved carpet shell clam *R. decussatus* is native from Europe and its distribution goes along the Atlantic coast, from Norway to Congo, English Channel, Mediterranean Sea and in the Red Sea, being the most cultivated species through the Atlantic coast (France, Spain, Portugal) and in the Mediterranean basin (FAO, 2016b; Gosling, 2002). The European clam has a great economic value and a consequent high commercial importance, representing an important source of income (Matias et al., 2013). According to FAO (2016b), from 2010 to 2014 the global aquaculture production of this species was around 4 tons. In Portugal, this species is commonly produced and harvested, representing a large portion of the aquaculture production (27% in 2009; DGPA, 2011).

The clam *R. philippinarum* is characterized by a solid, equivalent and inequilateral shell, with many variations in colour and pattern, generally brownish, while *R. decussatus* is characterized by its yellowish colour with brown stains, radial and concentric ridges (FAO, 2016a, b). *R. philippinarum* is distinguished from *R. decussatus* by its much more pronounced striation pattern than *R. decussatus* (Fig 1.3 A, B) (Hurtado et al., 2011). The habitat requirements of the native and introduced species are similar, living buried in sand, gravel or mud bottoms sediments in bays, estuaries and coastal lagoons (FAO, 2016a,b). *R. decussatus* and *R. philippinarum* are usually found buried 15-25 cm and 2-4 cm, respectively, below the surface in the intertidal zone (FAO, 2016a,b; Gosling, 2003). *R. philippinarum* has lower tendency to bury in comparison to *R. decussatus*, suggesting that this species is highly vulnerable to predation which may play an important role in its expansion (Bidegain et al., 2015b*;* Gosling, 2003). Since both clams species share similar habitats and requirements, they compete both in the natural medium and aquaculture farms (Usero et al., 1997). Also, it is known that *R. philippinarum* is more resistant to physical stress, pathogens and present a faster growth than *R. decussatus* (FAO et al., 2016a,b; Gosling, 2003). Although both species present similar habitat requirements, the growth rate of native species is associated to the primary production of the water column (Page and Lastra, 2003), while *R. philippinarum* seems to mainly feed on particulate organic matter resuspended from the sediment (FAO, 2016a; Watanabe et al., 2009). In both clams species, the feeding and breathing are accomplished by two separated siphons but while in *R. decussatus* the siphons are separated along its whole length (Fig 1.3 A), in *R. philippinarum* they are merged along almost their entire length (Fig 1.3 B) (Hurtado et al., 2011).

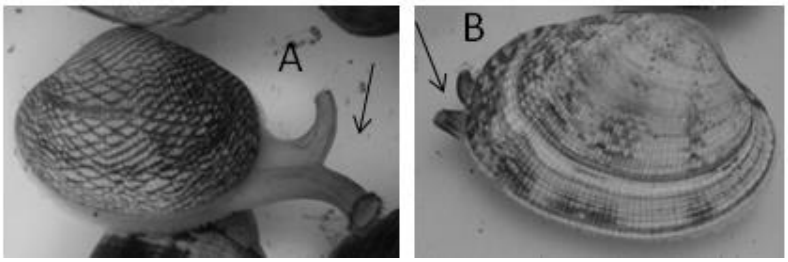

Figure 1.3 - Shell and siphon morphology in *Ruditapes decussatus* (A) and *R. philippinarum* (B).

Both species are euryhaline, which means that *R. decussatus* and *R. philippinarum* are tolerant to a wide range of salinities (e.g. Carregosa et al., 2014; Gazeau et al., 2013).

The sexual maturation of *R. decussatus* and *R. philippinarum* begins at a small size, between 10 –21 mm shell length for *R. decussatus*, while *R. philippinarum* starts at a shell length of 5 mm (Gosling, 2003). The reproduction in both species is external, occurring in water, being dependent on exogenous factors such as temperature and food supply (Gosling, 2003). For both species, reproduction occurs mainly during summer in the wild and/or hatcheries (FAO, 2016a,b). However, both species may adopt distinctive reproductive dynamics depending on their geographical origin (Çolakoğlu and Palaz, 2014; Matias et al., 2013; Moura et al., 2017; Smaoui-Damak et al., 2012). For example, in the Cork Harbour (Ireland) the spawning season occurs between Jully and August for *R. decussatus*, while for *R. philippinarum* it goes from August to November (Xie and Burnell, 1994). On the contrary, in the Çardark lagoon (Turkey), the spawning season starts in May until October for *R. decussatus* and from April to October for *R. philippinarum* (Genez et al., 2015).

1.5. Tools used to assess the effects of contaminants and abiotic factors

Different studies used bivalves to assess the environmental contamination, providing information only about the metal(loid)s concentrations in bivalves tissues and sediments (e.g. Kucuksezgin et al., 2010; Mora et al., 2004; Usero et al., 2007). However, recent monitoring studies and research studies are also interested in the effects of contaminants, as well as climate change effects on organisms (e.g. Anacleto et al., 2014; Dickinson et al., 2012; Hamza-Chaffai, 2014; Verdelhos et al., 2014; Wang et al., 2012). Thus, a wide range of biomarkers has been studied (e.g. Hamza-Chaffai, 2014; Regoli and Giuliani, 2014; Pal et al., 2007) and recommended as active monitoring tools for impact evaluation of contaminants in marine organisms and for risk assessment in coastal systems (Fernández et al., 2010; Vlahogianni et al., 2007; Wang et al., 2012). Biomarkers are defined as a biochemical, physiological or behavioural responses, as well as changes in the expression of genes, that can be measured in tissue, body fluid samples, or in whole organism resulting from external stressors (Hamza-Chaffai, 2014; Monserrat et al., 2007; Pal et al., 2007; Solé et al., 2009). They represent early and sensitive signs of injuries imposed to organisms (Monserrat et al., 2007). Different biomarkers have been used in marine species to predict potential detrimental effects induced by different contaminants and climate change (e.g. Bergayou et al., 2009; Hamza-Chaffai, 2014; Monari et al., 2007; Valavanidis et al., 2006).

Measuring the same biomarkers in the same species but in different sites provides information about the pollution status, allowing a better comprehension of organisms' responses

(Chandurvelan et al., 2015). Moreover, recent studies have shown that traditional biochemical biomarkers may provide useful information about the performance of organisms exposed to extreme temperatures, ocean acidification and salinity changes (e.g. Beaulieu and Costantini, 2014; Deschaseaux et al., 2011; Pfeifer et al., 2005). Several authors reported the induction/inhibition of traditional biochemical biomarkers in marine bivalves when exposed to pollutants (Figueira et al., 2012; Geret et al., 2003; Wang et al., 2012) or climate change scenarios (Botana et al., 2015; Parker et al., 2013). Among biochemical biomarkers, there are some that play an important role in physiological processes (carbonic anhydrase (CA) activity) (Lionetto et al., 2006), cell differentiation (alkaline phosphatase (ALP) activity), energy reserves (glycogen (GLY) content), metabolism and/or energy consumption (mitochondrial electron transport (ETS) activity) (Taylor and Mayer, 2010), oxidative stress (lipid peroxidation levels (LPO), antioxidant enzymes, glutathione reduced/oxidized ratio (GSH/GSSG)) (Box et al., 2007), as well as detoxification mechanisms (glutathione S-transferases) (Taylor et al., 2007) of marine organisms. In addition, metallothioneins (MTs) have been considered as a biochemical biomarker for metal pollution in marine ecosystems (Hamza-Chaffai et al., 2000).

It is known that CA activity has been suggested by different authors as a potential biomarker of exposure to metal pollution, as well as a suitable tool to salinity shifts and ocean acidification (Fabry et al., 2008; Le Roy et al., 2014; Lionetto et al., 2006; Zhang et al., 2012). It is well known that this enzyme is a widely-distributed metalloenzyme, which catalyses the reversible hydration/dehydration reactions of CO_2, through its conversion to carbonic acid (H_2CO_3) (Lionetto et al., 2000), a reduction in CA activity limits bicarbonate formation, negatively affecting physiological processes of organisms (Dickinson et al., 2012). Carbonic anhydrase plays an important role in osmoregulation and acid-base balance, as well as, in calcification processes of estuarine organisms (Fabry et al., 2008; Le Roy et al., 2014; Lionetto et al., 2006; Zhang et al., 2012). Recent studies demonstrated that CA activity may be affected by ocean acidification, salinity shift and metal contamination, being inhibited or induced by these stressors to compensate osmoregulation and/or acid-base balance (Beni ash et al., 2010; Lionetto et al., 2006; Moreira et al., 2016). Also, the ALP play an important role in oxygen carrying systems, osmoregulation, and biomineralization (Lovett et al., 1994; Mazorra et al., 2002; Morthorst et al., 2014; Seitkalieva et al., 2015; Viarengo and Nott, 1993). This enzyme belongs to a group of phosphomonoesterases enzymes involved in the hydrolyses of phosphate (Vroon and Israili, 1990). The suitable use of ALP as a biomarker have been studied, since this enzyme revealed to be sensitive to metal contamination and ocean acidification in different marine bivalve species (Chakraborty et al., 2010; Mazorra et al., 2002; Moreira et al., 2016; Morthorst et al., 2014; Seitkalieva et al., 2015).

Another important biomarker is GLY. The determination of GLY has been used in organisms from different field and laboratory studies (e.g. Duquesne et al., 2004; Freitas et al., 2016; Hamza-Chaffai, 2014) since the amount of GLY stored in bivalves is considered a good indicator of body

content (Ansaldo et al., 2006; Patterson et al., 1999). Several marine organisms use GLY reserves when exposed to stressful conditions (Cruz et al., 2016; Duquesne et al., 2004; Hamza-Chaffai, 2014). Glycogen reserves are the main energy source in cellular protection systems being broken down to yield glucose molecules when energy is needed (Berg et al., 2002).

Several authors also have used biomarkers of oxidative stress to assess the effects of different stressors (Bergayou et al., 2009; Carregosa et al., 2014; Fernández et al., 2010; Vlahogianni et al., 2007). Organisms under normal physiological conditions produce and eliminate naturally reactive oxygen species (ROS) (Lushchak, 2011). However, metal(loid)s and/or abiotic factors may promote an imbalance between ROS production and elimination, leading to oxidative stress (e.g. Abele et al., 2011; Lushchak, 2011; Sheehan and McDonagh, 2008; Tomanek, 2012). The main ROS generated by cellular metabolism are the single oxygen ($^1O_2$), the superoxide anion (O_2^-), the hydrogen peroxide (H_2O_2) and the hydroxyl radical (·HO) among others (Fig. 1.4) (Valavanidis et al., 2006; Regoli and Giuliani, 2014). These molecules, produced as by-products during the ETS of aerobic respiration, oxidative phosphorylation, or by oxidoreductase enzymes and metal catalysed oxidation, have the potential to cause a number of deleterious events, namely damages in lipids, proteins and nucleic acids (Shinde et al., 2012). In addition, damages in mitochondria caused by superoxide ion can lead to apoptosis (cellular suicide) (Abele et al., 2011). One of the most common biomarkers used to measure the oxidative damage is the LPO. To prevent the damages caused by ROS imbalance, cells are able to increase detoxification mechanisms, such as antioxidant (superoxide dismutase, SOD; and catalase, CAT) and biotransformation enzymes (e.g. GSTs), as well as employ sequestration mechanisms (glutathione or MTs) that can neutralize the toxic compounds into a less harmful form (Ramos-Gómez et al., 2011). According to previously published studies, this detoxification strategy protects molecular targets against oxidative injuries and could be the reason why organisms increase their tolerance when exposed to polluted environments (e.g. Livingstone, 2001; Marques et al., 2016).

The antioxidant enzymes SOD and CAT convert superoxide anion (O_2^-) into H_2O_2, and H_2O_2 into molecular oxygen and water, respectively (Fig. 1.4) (Alves de Almeida et al., 2007; Duracková, 2010). Several authors reported the induction of SOD and CAT activities in marine bivalves when exposed to non-essential metals (Cd, Hg, Pb; Figueira et al., 2012; Geret et al., 2003; Wang et al., 2012). Hydrogen peroxide is also the substrate for glutathione peroxidase (GPx), using GSH as the electron donor to catalyze the reduction of H_2O_2 into H_2O (Fig. 1.4). The GSH is also an antioxidant useful tool to regulate intracellular homeostasis cycle and plays a significant role in protection of organisms against oxidative damage (Box et al., 2007). The GSH/GSSG ratio is essential for the ordinary functioning of cell metabolism, being a suitable biomarker of oxidative stress and indicates the redox status of the cell (Box et al., 2007). Glutathione *S*-transferases reduce hydroperoxides to alcohols with the simultaneous oxidation of GSH (Regoli and Giuliani, 2014). Glutathione peroxidase and GSTs are enzymes also involved in the detoxification of electrophilic compounds through biotransformation processes (Regoli and Giuliani, 2014).

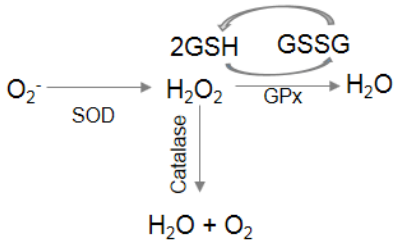

Figure 1.4 – Oxidative stress mechanisms involved in detoxification mechanisms (Adapted from Regoli and Giuliani, 2014; Huggett, 1992).

Metallothioneins were identified as a defense mechanism through chelation of metals to their ionic form (Bebianno and Serafim 1998) and later to the formation of granules (Marigómez et al., 2002). Additionally, MTs have been used as a monitoring tool, namely as a contaminant-specific, marker of metal exposure (Hamza-Chaffai et al., 2000). These proteins have been involved in the protection of oxidant damage, metabolic regulation, and homeostasis of essential metals (Paul-Pont et al., 2010; Serafim and Bebianno, 2010). Other authors also revealed that MTs may be affected by abiotic and biotic factors such as temperature and reproduction cycles (Geffard et al., 2007).

The induction/inhibition of mechanisms involved in oxidative stress is associated to gene expression adjustment performed by organisms in response to environmental changes (Buckley et al., 2001). The gene expression has been used as a molecular approach, elucidating how the intertidal field environment affects the physiology of its inhabitants (Chaney and Gracey, 2011). The expression of genes may be up or down-regulated to maintain homeostasis in organisms under environmental stress (Liu et al., 2012; Todgham and Hofmann, 2009). Recently, different authors studied the regulation of gene expression in marine bivalves under different stressors (e.g. Arini et al., 2015; Hamdoun et al., 2003; Liu et al., 2012; Park et al., 2009). The gene expression adjustment provides to the organisms a source of physiological plasticity (Buckley et al., 2001). In fact, it is known that the gene expression regulation can be one of the most rapid and sensitive responses to environmental stressful conditions (Gracey et al., 2008; Zippay and Hofmann, 2010). Examples of genes affected by environmental stressors include bcl-2-like protein 4 (*Bax*), cytochrome C oxidase subunit I gene (*Cox-1* or *coi*), *16S*, superoxide dismutase (*SOD*), heat shock protein (*Hsp*) and growth arrest and DNA damages (*gadd45)* (e.g. Binias et al., 2014; Hamdoun et al., 2003; Meistertzheim et al., 2007; Park et al., 2009; Pil et al., 2008).

The expression of *Bax* has been assessed in different organisms, highlighting the regulation of apoptosis in the mitochondria (Floros et al., 2006). Among the mitochondrial markers are the genes *Cox-1* and *16S*, involved in the mitochondrial metabolism (Lakra et al., 2009). Another gene implicated in the stress signaling of physiological or environmental stress is *gadd45*. This gene provides information about cell cycle arrest, DNA repair, cell survival, and senescence, or

apoptosis (Liebermann and Hoffman, 2008). The upregulation of genes involved in mitochondrial metabolism (*Cox-1* and 1*6S*) and DNA repair (*gadd45*) was reported in marine bivalves exposed to stress conditions (Binias et al., 2014). Other important genes include the *MnSOD* (mitochondrial) and *CuSOD* (cytosol) involved in the oxidative stress response. Park et al. (2009) and Pil et al. (2008) showed an induction of genes responsible for antioxidant defense in bivalves under increasing temperatures. Among a variety of genes currently employed to assess the physiological effects of thermal variations in organisms, the *Hsp70* has been showing an important role in thermal stress response (Anestis et al., 2008; Osovitz and Hofmann, 2005). The *Hsp* gene expression is related to thermotolerance at the organism level (Buckley et al., 2001; Hamdoun et al., 2003). Temperature and pH may be responsible for the up-regulation of *Hsp70* expression in bivalves (Liu et al., 2012), while metal exposure may be related with the down-regulation of *Hsp70* genes (Taylor et al., 2013; Thompson et al., 2012).

1.6. Aim and outline of the thesis

Metal and metalloid contamination, climate change and invasive species represent three of the greatest threats to biodiversity and services of ecosystems. Although it has been already described that the introduced *R. philippinarum* may live in sympatry with the native species *R. decussatus* in different ecosystems, such as Santander Bay and San Simon bay (Spain) (Bidegain and Juanes, 2013; Hurtado et al., 2011), Ria de Aveiro (Maia and Gaspar, 2014), Tagus estuaries (Garaulet, 2011) or may replace the native ones (Mediterranean sea, Otero et al., 2013; Pranovi et al., 2006), becoming invasive (Otero et al., 2013; Pranovi et al., 2006), little information is available regarding the Portuguese coast (namely the Ria de Aveiro, the Óbidos lagoon). In particular, scarce information is known about the effects/responses of these close related species coexisting in the same area under the same stressors, namely pollution (derived from metals and metalloids) and climate change related factors (temperature increase, ocean acidification and salinity changes), and how these stressors influence the spread of the introduced species.

In order to bring light to the subject "Is it possible for native and introduced *Ruditapes* species to live in sympatry?", the present thesis presents a study addressing the following questions: i) Do the native species, *Ruditapes decussatus*, and the introduced species, *Ruditapes philippinarum*, co-exist in the same areas of two coastal systems (the Ria de Aveiro and the Óbidos lagoon)?; ii) If so, do they present similar metal(loid) accumulation, metal(loid) partitioning and biochemical performance?; iii) Are there any potential health risks to humans due to their consumption?; iv) Do both species respond in a similar way to pollutants and climate change related factors?. To investigate these questions environmental and laboratory studies were conducted, evaluating species spatial distribution and densities, bioaccumulation capacity and elements partitioning, species responses to pollutants (metal(loid)s) and climate change related factors.

This thesis was divided into five chapters, including the current general introduction (Chapter 1), Chapter 2 describing the environmental studies conducted in the Ria de Aveiro and the Óbidos lagoon; Chapters 3 and 4 describing laboratory experiments testing the impacts of single and combined exposures; and a final chapter summarizing the general conclusions of this thesis (Chapter 5).

In detail:

Chapter 2 is entitled “Native and introduced species from contaminated ecosystems” and assess the coexistence of both bivalves species, the risks associated with consumption of both species, the environmental metal(loid) concentration, metal(loid) accumulation and partitioning, as well as the biochemical response of both species from two contaminated ecosystems.

Chapter 3 is entitled “Organisms response to single stress”, assesses and compares the response of the native clam, *R. decussatus*, and the introduced clam, *R. philippinarum*, when exposed to single stressors, namely temperature, salinity and pH changes, as well as As contamination, through different biomarkers approach.

Chapter 4 is entitled “Organisms response to combined stressors”, assesses the combined effects of different pH levels and As contamination (pH 7.8 and 7.3; 0 and 4 mg/L of As) on bioaccumulation capacity of native and introduced bivalve species, human health risks associated with clams consumption, and alterations on oxidative stress and energy reserves of both species.

Chapter 5 is entitled “General discussion and final considerations”, and results and findings of Chapters 2, 3 and 4 are summarized, and future research perspectives proposed.

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Chapter 2

Native and introduced species from contaminated ecosystems

This chapter is based on the following manuscripts:

Velez, C., Figueira, E., Soares, A.M.V.M., Freitas, R., 2015. Spatial distribution and bioaccumulation patterns in three clam populations from a low contaminated ecosystem. Estuarine Coastal And Shelf Science. 155, 114-125

Velez, C., Galvão, P., Longo, R., Malm, O., Soares, A.M.V.M., Figueira, E., Freitas, R., 2015. *Ruditapes philippinarum* and *Ruditapes decussatus* under Hg environmental contamination. Environmental Science And Pollution Research. 22, 15, 11890-11904

Velez, C., Leandro, S., Figueira, E., Soares, A.M.V.M., Freitas, R., 2015. Biochemical performance of native and introduced clam species living in sympatry: The role of elements accumulation and partitioning. Marine Environmental Research. 109, 81-94.

2.1. Introduction

Coastal systems are subjected to a large amount of contaminants from natural and anthropogenic sources, contributing to their deterioration (Green-Ruiz and Páez-Osuna, 2001; Naidu et al., 2016; Poulos et al., 2000; Tornero and Hanke, 2016). Among different contaminants, metal(loid)s are a worldwide problem, since they can be toxic even at low concentrations (Acton, 2011; ATSDR, 2014). Due to their persistence and toxicity, particular attention has been given to identify the key factors which control the spatial distribution, the concentration, the bioavailability and the mobility of metal(loid)s in marine ecosystems (Chakraborty et al., 2012; Fernando, 1995). However, in the environment, various factors can influence the metal(loid)s bioavailability and metal(loid)s sequestration strategies of different marine organisms (Campana et al., 2015). Some factors that possibly influence the bioavailability and, consequently, the toxicity of metal(loid)s are the remobilization events and/or physicochemical water changes (pH, salinity, temperature) affecting the release of metal(loid)s to the water column (e.g Annibaldi et al., 2015; Allen and Horn, 2006; Eggleton and Thomas, 2004; Xu et al., 2015).

In marine bivalves, the metal(loid)s uptake, from the surrounding environment, may occur by facilitated diffusion and active transport, being these contaminants accumulated in their tissues (Fukunaga and Anderson, 2011). Bivalves are among the marine mollusks which present a high environmental bioaccumulation potential, by contacting directly with contaminated water and/or sediments (Hamza-Chaffai, 2014; Moschino et al., 2012; Pellerin and Amiard, 2009; Sfriso et al., 2008). Due to their feeding behavior, sedentary lifestyle, wide distribution, abundance and the bioaccumulation potential, these organisms have been recommended as a possible bioindicator and/or sentinel species (Bergayou et al., 2009; Jena et al., 2009). Thus marine bivalves represent an important tool to assess the ecosystem quality (Jebali et al., 2014; Saavedra et al., 2004; Zuykov et al., 2013).

Bivalves may accumulate a large amount of metal(loid)s experiencing or not toxic effects (Rainbow and Smith, 2010). They developed specific strategies to survive in contaminated ecosystems by enhancing their tolerance during metal(loid)s accumulation (Wallace and Luoma, 2003; Wallace et al., 2003). When accumulated metal(loid)s present different subcellular partitioning, distributed by the cellular debris, metal-rich granules (MRG), organelles, heat-sensitive proteins (“enzymes”) and heat-stable proteins (metallothioneins, MT) (Wallace and Luoma, 2003; Wallace et al., 2003). MT and MRG constitute the compartment defined as biologically detoxified metal (BDM), corresponding to detoxification mechanisms developed by organisms, which may be involved in their tolerance and possible resistance to a given contaminant (Wallace et al., 2003). On the other hand, metals bound to sensitive cellular components (e.g. organelles and enzymes) can provide information about potential toxicity mechanisms, corresponding to the compartment containing metal-sensitive fractions (MSF) (Wallace et al., 2003). Wallace and Luoma (2003) reported that metal associated with metal-rich granules and cellular debris (insoluble fraction) are

less available, while organelles, enzymes and MT (soluble fraction) can be trophically available for transfer through the food chain (biomagnification).

Therefore, the study of metal(loid)s fractioning in response to accumulation provides information regarding the potential toxicity and fate of accumulated elements, as well as, their trophic availability (Wallace and Luoma, 2003; Wallace et al., 2003). Since metal(loid)s can be accumulated in lower trophic levels and biomagnified through the food chains (Hosseini et al., 2013).

Although different studies have assessed the metal(loid)s concentrations in different marine sediments and bivalves, the effects of this accumulation in the performance of different species coexisting in the same area are not commonly evaluated, despite proved to be of prime relevance (Alves de Almeida et al., 2007; Freitas et al., 2012; Fukunaga and Anderson, 2011; Li and Gao, 2014; Moschino et al., 2012; Valavanidis et al., 2006). Nevertheless, it has been shown that metal(loid)s can induce oxidative stress in marine bivalves, through the production of ROS, being the antioxidant and biotransformation enzymes induced to prevent lipid, protein and DNA damages (Alves de Almeida et al., 2007; Regoli and Giuliani, 2014).

Marine bivalves present not only great ecological importance as filter feeding organisms, but also play an important socio-economical role since they represent a commercial source for human consumption (Guillen and Motova, 2013). For these reasons, since the 1990s, the world production of bivalves has been steadily increasing (Rees et al., 2010). Due to high quantities of bivalves consumed by humans, international organizations (EFSA, USFDA or FSANZ) established maximum permissible limits (MLs) of metal(loid)s in seafood, above which edible seafood cannot be marketed. However, different bivalve species may accumulate different pollutants, representing different risks to public health.

The Manila clam, *Ruditapes philippinarum*, native from the Indo-Pacific region, presents a significant commercial value (FAO, 2016). The fast adaptability to a new environment and its fast growth makes this species a very suitable species for aquaculture, in several European estuaries (Flassch and Leborgne, 1994; Gaspar et al., 2012; Mistri, 2004; Usero et al., 1997). However, the introduction of *R. philippinarum* has greatly changed the exploitation living resources in different aquatic systems (Gaspar et al., 2012; Usero et al., 1997). It is known that the habitat requirements of the native European clam, *Ruditapes decussatus*, are similar which in many cases co-habit the same areas, increasing the possibility of the introduced species supplant the native one or live in sympatry, as described in Santander Bay (Spain) (Juanes et al., 2012; Occhipinti-Ambrogi and Savini, 2003; Otero et al., 2013).

Thus, the objectives of the present study were to understand if the native species, *Ruditapes decussatus*, and the introduced species, *Ruditapes philippinarum,* co-exist in the same areas of two different ecosystems at the Portuguese Coast (Ria de Aveiro and Óbidos lagoon) and to assess if both species present different accumulation, metal partitioning, and biochemical

performance. For that, the abundance of *R. decussatus* and *R. philippinarum* in different sampling areas of the Ria de Aveiro (Portugal) and Óbidos lagoon (Portugal) was assessed, as well as, the physicochemical characteristics and contamination levels in the sediments, the bioaccumulation pattern of both clam species, the human health risks associated with its consumption and the biochemical performance of both species in different areas of each ecosystem.

2.2. Materials and Methods

2.2.1. Study area and sampling procedure

2.2.1.1. Ria de Aveiro

The Ria de Aveiro is a shallow lagoon located on the northwest coast of Portugal (40°38´ N, 8°45´ W) (Fig. 2.1). This aquatic system presents 45 km long and 10 km wide, covering an area between 66 and 83 km^2, at low and high tide (spring tide), respectively. According to Dias (2001), the tidal range of this lagoon varies between 3.2 and 0.6 m for the strong spring and neap tides. The tides forces contribute to well-mixed-system (Lopes and Dias, 2007). This system connected to the Atlantic Ocean through an artificial channel with a depth of about 20 m, comprising several channels (S. Jacinto, Ílhavo, Mira, Ovar, and Murtosa) and distinct intertidal zones, such as mud flats and salt marshes (Dias et al., 2000). Mira channel is considered the least impacted channel (Castro et al., 2006), while the most impacted areas are in the Estarreja Channel and in the Laranjo Bay due to substantial contamination in bottom sediments (Pereira et al., 2008a). Among of the Ria de Aveiro channels, S. Jacinto and the Espinheiro channels present the strongest currents (reaching values of about 2 m/s near the mouth, during spring tides and flood period) and they connected to the mouth of the lagoon (Lopes and Dias, 2007). This ecosystem receives fresh water from the Antuâ, Vouga, Cáster, Gonde and Boco rivers, typically with river flows discharges into the Ria de Aveiro ranging between 1.0 to 61.3 m^3/s (Dias et al., 2003; Santos et al., 2014). However, the main input of fresh water is from the Vouga river located in the Espinheiro channel with average flows of 50 m^3/s (Dias, 2001; Dias and Lopes, 2006). This ecosystem is exposed to seasonal and spatial salinity changes, presenting a longitudinal salinity gradient (0-36) (Vaz and Dias, 2008). Beyond natural physicochemical changes, this aquatic system has been submitted to anthropogenic pressure, such as agriculture, cattle rearing, industries (chemical, metallurgic, ceramics, tannery and pulp milling) and mining activity from abandoned mines near to Vouga river (Delgado et al., 2000). Furthermore, this ecosystem is historically contaminated with Hg, since effluents from a Hg cell chloralkali plant located in the Estarreja industrial complex were discharged in this ecosystem, from the 1950s until 1994 (Pereira et al., 2009a). In the last decade, the Hg discharge diminished considerably but the concentrations of this metal in the surface sediments of some areas of this system are still higher than pre-industrial levels (0.05 mg/kg) (Ahmad et al. 2012; Cardoso et al. 2013; Pereira et al., 2009a). These discharges induced an environmental

contamination gradient of Hg inside the lagoon and consequently a high Hg accumulation in organisms (Abreu et al. 2000; Castro et al. 2009). According to Marques et al. (2009), the discharge of pyrite residues directly into the ground and industrial wastewaters discharge into the Ria de Aveiro also contribute to an As contamination scenario in this ecosystem, being possible to find in sediments from "Estreito de Estarreja" As concentrations above the limits established by Directive 86/278/CEE. Regarding the organisms biodiversity, Rodrigues et al. (2011) demonstrated that the Ria de Aveiro has an important biodiversity in terms of macrobenthic communities. Bivalves, such as *Cerastoderma edule*, *Venerupis corrugata*, *R. decussatus* and *R. philippinarum*, are among the most commonly encountered organisms in this ecosystem, presenting not only ecologically important, but also has an economic, social, and cultural importance (Maia and Gaspar, 2014). According to Maia and Gaspar (2014) the presence of *R. philippinarum* was not reported in 2006/2007, but in 2011 this species was found in banks likely to be commercially exploited. Taking into account different anthropogenic sources and the presence of the clams *R. decussatus* and *R. philippinarum* in the Ria de Aveiro, 15 areas (3 sites in each area) (named from A to O) were surveyed in September 2013 (August) (Fig. 2.1). At each site pH, salinity and temperature were measured in water and sediments with specific probes. After that, all clam individuals present in a square of 50 x 50 cm were collected for relative abundance (the percentage of each species relative to the total number of organisms, %), density (number of organisms by m^2) (Elliott, 1977), Condition Index, CI, metal(loid)s and biochemical parameters determination. In each site, sediment samples were also collected, for sediment grain size analysis, total organic matter (TOM) quantification and determination of elements (chromium, Cr; nickel, Ni; copper, Cu; lead, Pb; cadmium, Cd; mercury, Hg; arsenic, As) concentration.

After sampling, specimens and sediments were transported on ice (0ºC) to the laboratory.

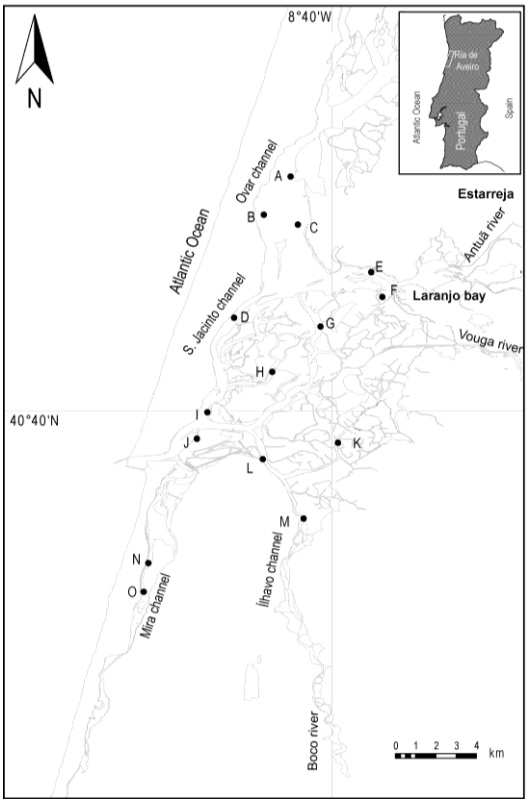

Figure 2.1 – The Ria de Aveiro, showing the positioning of the sampling areas (A-O).

2.2.1.2. Óbidos lagoon

The Óbidos lagoon is a small and shallow coastal system located in the west Portuguese coast with a mean depth of 1 to 2 m, covering a wet area of approximately 7 km^2 (Pereira et al., 2009b) (Fig. 2.2), and it is permanently connected to the sea by a narrow inlet (on the order of 100 m) (Oliveira et al., 2006). The waves directions are almost perpendicular to the beach (faces 315°N, and wave periods range from 5 to 20 s) and due to the very energetic (wave heights exceeding 1m during 88% of the time) waves regime, this system is constantly subject to dredging operations undertaken mainly in the inlet and downstream channels to promote a permanent sea connection (Fortunato and Oliveira, 2007; Oliveira et al., 2006). This behavior can be justified by the length, the depth, the tidal ranges, small surface area of the lagoon, as well as the sediment inputs in the upper lagoon (Oliveira et al., 2006). The Óbidos lagoon is characterized by extensive intertidal sandbanks partially separated by channels and small freshwater contributions (Carvalho et al., 2005). The freshwater inflow enters in the lagoon by the Cal River at the Barrosa arm, Vala do Ameal at the Bom Sucesso arm and by the Arnóia River discharge (between both lagoon arms) (Oliveira et al., 2006). The Arnóia River contributes about 90% of freshwater fluxes into the lagoon, being the major source of sediments, whose deposition has created an extensive sand bank (Oliveira et al., 2006). This lagoon was classified as a system with moderate metal contamination by Pereira et al. (2009c, d) and Carvalho et al. (2011). Previous studies showed that the Barrosa

arm receives agriculture and urban effluents from the Caldas da Rainha City, resulting in an area with high nutrient availability, being previously classified as eutrophic (Pereira et al., 2009c). Concerning metals and As contamination, Oliveira et al. (2006) and Pereira et al. (2008b) showed that the major source of these contaminants was related to wastewater discharges in Cal River (Barrosa arm), with maximum concentrations of Ni, Cu and Cd in periods of high inflow and remobilization from sediments in summer months (Pereira et al., 2009b). The Bom Sucesso arm is also a confined area but receives a smaller freshwater flow (Vala do Ameal) with better water quality than the Cal River, according to the Portuguese categorization of freshwater systems. In this area, metal remobilization from sediments is less likely due to the greater depth (Pereira et al., 2009b,c). According to Carvalho et al. (2005), in this lagoon, three different macrobenthic communities are identified, corresponding to the inlet, central and inner parts of the system, which seem to reflect the adaptations of the communities to the environmental gradients established within the lagoon due to tidal currents and fluvial discharge. The bivalves, *Cerastoderma edule*, *R. decussatus* and *Venerupis corrugata* are among the most characteristic bivalve species of the intermediate area of the lagoon (Rodrigues et al., 2012). Due to that 5 sampling areas (3 sites in each area) (named from A to E) were selected in June 2014 to survey the presence of *R. decussatus* and *R. philippinarum* specimens (Fig. 2.2). At each site pH, salinity and temperature were measured in water and sediments with specific probes. These areas were also selected taking into account the levels of contamination in this ecosystem. In each area, clams were collected in 3 different sites with bullrakes (65 cm x 25 cm, with minimum mesh size 13 mm). After that density, relative abundance, CI, metal(loid)s and biochemical parameters of clams were determined. Also, at each area, three replicates of sediments were collected (one per site) for sediment grain size analysis, total organic matter (TOM) content determination, and quantification of metal(loid)s.

After sampling, specimens and sediments were transported on ice (0ºC) to the laboratory.

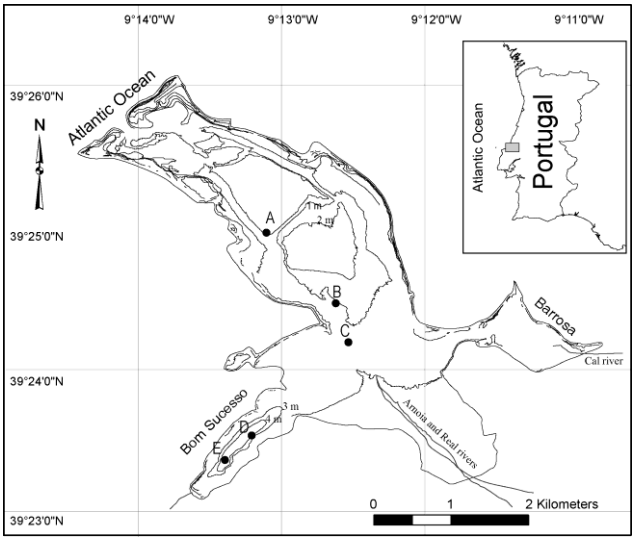

Figure 2.2 - The Óbidos lagoon, showing the positioning of the sampling areas (A-E).

2.2.2. Laboratory analyses

In the laboratory, at least 6 individuals of each species from each area of the Ria de Aveiro and 12 individuals of each species from each area of the Óbidos lagoon were collected. Both species, in each area, were weighed, measured (length and width) and used to determine the Condition Index (CI). The remaining organisms were preserved at -80 ºC until further analyses. Sediment for the TOM content and metal(loid)s quantification were preserved at -20ºC, while the remaining sediments for grain size analysis were preserved at room temperature.

2.2.2.1. Biometric data

Soft tissue of each organism was carefully separated from shells and washed with distilled water to remove dirt. Both soft tissue and shells were put in an oven at 60ºC for 48 h and then weighed for CI determination. CI values were calculated according to the following equation described by Matozzo et al. (2011):

$$\text{the } \frac{\text{dry weight of soft tissues}}{\text{the dry weight of the shells}} \times 100$$

Condition index provides an indication of the general physiological status of the animals.

2.2.2.2. Grain size analysis

Sediment grain-size determination was performed using 3 replicates per area from the Ria de Aveiro and the Óbidos lagoon. This determination was analyzed by wet and dry weight following the procedure described by Quintino et al. (1989). For this analysis, approximately 120 g of each sediment were used. Sediment was washed with distilled water. After washing, hydrogen peroxide was added in successive concentrations (30, 60 and 120 volumes) for chemical organic matter destruction. Then samples were dried at 60ºC until obtaining a constant weight (from 24 to 48 h) and the total weight was determined (*P1*). Tetra-sodium pyrophosphate (30 g/L) was added to dry sediment for 24 h to promote the particles disaggregation. After disaggregation of particles, sediment was sieved through a 63 µm mesh and the material retained at this mesh was dried at 60 °C until to obtain a constant weight (*P2*). The weight of fraction lower than 63 µm was determined by the difference between *P1* and *P2*. The remaining sediments were sieved through a battery of sieves (0.063-4.000 mm) spaced at 1 *phi* (Φ) ($\Phi = -\log_2$ the particle diameter (mm)). The percentage of silt and clay fraction (fine particles, with diameter <0.063 mm), gravel fraction (particles with diameter >2.000 mm) and sand fraction (0.063-2.000 mm) was determined in relation to the total dry weight of sediment. Data was used to calculate the median value, P50, expressed in phi (Φ) units (dry weight, DW). The median and the percentage of fines were used to classify the sediment,

according to the Wentworth scale: very fine sand (median from 3 to 4 Φ); fine sand (2-3 Φ); medium sand (1-2 Φ); coarse sand (0-1 Φ); very coarse sand (-1 to 0 Φ) (Table 2.1).

The final classification adopted the description 'clean', 'silty' or 'very silty' when the silt and clay fraction ranged from 0% to 5%, from 5% to 25% and from 25% to 50%, respectively, of the total sediment (DW) (Doeglas, 1968). Samples with more than 50% fines content were classified as mud (Table 2.1).

Table 2.1 - Classification of sediments, adapted from Wentworth (Doeglas, 1968).

Median (f)	Sediment classification		Fines content (%)		
			<5	5-25	25 – 50
(-1) - 0		Very coarse			
0 - 1		Coarse			
1-2	Sand	Medium	Clean	Silty	Very silty
2-3		Fine			
3-4		Very fine			
> 4	Mud		Above 50%		

2.2.2.3. Total organic matter

For TOM determinations 3 replicates were used per area from the Ria de Aveiro and the Óbidos lagoon. TOM was determined according to Byers et al. (1978), corresponding to the percentage of weight loss in 1 g of dried sediment, after combustion at 450 ºC (with minimal risk of volatilizing inorganic carbon) during 5 h.

2.2.2.4. Metal(loid)s quantification

Metal(loid)s determination was performed in sediments (3 replicates/area) and in both species *R. decussatus* and *R. philippinarum* (3 individuals/species/area) from the Ria de Aveiro and the Óbidos lagoon.

For Metal(loid)s quantification in sediments, 2 g of homogenized air-dried sediment was digested overnight at 115ºC with 10 mL of $1HNO_3$:3HCl (Suprapur, Merck) in digestion Teflon bombs (sealed chambers). To prevent the loss of metal(loid)s by volatilization, chambers were only opened when completely cooled. The cooled digest was made up to 50 mL with deionized water.

For element quantification in *R. decussatus* and *R. philippinarum*, organisms with similar size were selected. Organisms were thawed, wet weighed and mechanically homogenized with liquid nitrogen, and subjected to subcellular fractionation by centrifugation at 1450 *g*, for 15 min at 4ºC. Fractionation resulted in the isolation of two distinct fractions: soluble and insoluble. In the first fraction, elements are soluble in the cytosol while in the second elements are in the organelles, metal-rich granules (MRG) and cellular debris (Wallace and Luomo, 2003; Wallace et al., 2003). The samples for element quantification in all soft tissue and in the soluble and insoluble fractions were digested overnight at 115ºC with 2mL of $1HNO_3$:3HCl. The cooled digest was made up to 5 ml with deionized water.

The concentration of seven elements (Cr, Ni, Cu, Pb, Cd, Hg, As) was measured in sediments and organisms (soluble and insoluble fraction) from the Ria de Aveiro and the Óbidos lagoon by inductively coupled plasma-mass spectrometry (ICP-MS) in a certified laboratory at the University of Aveiro. Regarding the quality controls, the calibration 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 certified referent material (CFR) NIST 1643e. The whole procedure was verified with standard certificated reference materials (CRM): MESS-3 (for sediments) and TORT-2 (for clams soft tissues), both from NRCC (National Research Council of Canada). The values obtained for the whole of the CRM analysis ranged from 80% to 110%. All samples below this accuracy level were rejected and the analyses repeated.

Regarding the historic of Hg contamination, in the Ria de Aveiro, and the distance of the contamination source, organisms from areas E, K and N were selected to determine the MeHg concentration. These concentrations were quantified according to the EPA Method 1630. Samples were weighted, pulverized with liquid nitrogen, and divided into aliquots (FW). These samples were lyophilized (DW), weighted (0.03 g), and digested with KOH/ methanol (25%) (Sigma®, purity levels 85 and 99%, respectively), during 6 h, at 60 °C. After 48 h, the digested samples and sodium acetate at pH 4.9 ethylated by sodium (Aldrich, purity level 97%) was added in 40-mL Teflon line borate glass bottles. The MeHg was measured with an automated analytical system (MERX, Brooks Rand). The standard reference material NIST 2976 was also analyzed (121–123%). MeHg determinations were not performed in sediments from the Ria de Aveiro, since according to Válega et al. (2008) and Ramalhosa et al. (2011), the MeHg percentage found in sediments near to Laranjo bay were very low. MeHg quantifications were not performed in organisms and sediments from the Óbidos lagoon since the Hg concentrations found were below 0.03 mg/L of Hg for sediments and organisms.

In order to obtain the total concentration of elements present in both clams species, the concentration of elements present in soluble and insoluble fraction was added up. The concentration of each element (soluble and insoluble fraction, and total) in both species was expressed in mg per kg fresh weight (FW), to compare with maximum permissible limits (MLs) expressed in mg/kg FW, but also in dry weight (DW) according to Ponsero et al. (2009), to allow

better comparison with literature data and to allow the determination of the Biota-Sediment Accumulation Factor (BSAF).

Taking into consideration the elements concentration of both species, in the most and the least contaminated areas of Ria de Aveiro and Óbidos lagoon, the amount of clams tissue that a 70 kg adult need to consume in one week to exceed Provisional Tolerable Weekly Intake (PTWI) was determined. This value was obtained dividing the PTWI of a 70 kg adult by the concentration of a given element in organisms.

The concentration of elements in sediments was expressed in mg per kg of dry weight (DW), since the sediments were previously dried during 48 h at 25 ºC to allow the comparison between the present data and sediment quality guideline values (threshold effect level, TEL; effects range low, ERL; probable effects level, PEL; effect range median, ERM) (Burton, 2002; Long et al., 1995; Long and Morgan, 1990; Macdonald et al., 1996).

2.2.2.5. Biota-Sediment Accumulation Factor (BSAF)

Biota-Sediment Accumulation Factor (BSAF) was determined dividing the total concentration of a given element in the organism (mg/Kg DW) by the concentration of that element in the sediment (mg/Kg DW), under environmental conditions where organisms are influenced by water and dietary sources (McGeer et al., 2003).

2.2.2.6. Biomarkers

For biomarkers analyses at least 3 organisms of each species per area from the Ria de Aveiro (areas B, C, E, F, K, M, N, and O) were used. The biochemical analyses were not performed in organisms from area G due to the low density of both species in this area. For the Óbidos lagoon 6 individuals were used per species and from each area. For these analyses, organisms (whole soft tissues) were, individually, pulverized with liquid nitrogen and 0.5 g of soft tissue was used. Samples were extracted with specific buffer for each biochemical parameter and centrifuged (10,000*g*) during 15 min at 4ºC. Supernatants were stored at -80ºC or used immediately. A Thermo Scientific Multiskan GO UV/Vis Microplate Spectrophotometer was used for spectrophotometric quantifications.

The biomarkers determined were: total soluble protein (PROT) content, lipid peroxidation (LPO) levels, superoxide dismutase (SOD) activity, catalase (CAT) activity and glutathione *S*-transferase (GSTs) activity.

Protein (PROT) content

The content of PROT was determined according to Robinson and Hogden (1940), following the Biuret method, and using bovine serum albumin (BSA) as standard (0–40 mg/mL) (Calbiochem®, purity level 98%). Sample extraction was performed with phosphate buffer at pH=7.0 (50 mM disodium hydrogen phosphate dihydrate; 1 mM ethylenediamine tetraacetic acid disodium salt dehydrate (EDTA); 1% (v/v) TritonX-100; 1% (v/v) polyvinylpyrrolidone (PVP); 1 mM dithiothreitol (DTT). The reaction mixture in a final volume of 325 µL consisted of 25 µL of sample extract and 300 µL of Biuret reagent. The incubation was for 10 min, at 30ºC. At the end of this period, absorbance was read at 540 nm. The results were expressed in mg per g of FW.

Lipid Peroxidation (LPO) levels

The quantification of LPO levels was based on the reaction of LPO by-products, such as malondialdehyde (MDA), with 2-thiobarbituric acid (TBA) (MERCK®), forming ThioBarbituric Acid Reactive Substances (TBARS), according to the protocol described by Buege and Aust (1978). Samples extraction was performed with 20% of trichloroacetic acid (TCA, (MERCK®) in proportion 1:2 (w/v). After extraction, samples were treated with TBA (0.5% in TCA solution). The reaction occurred at 95 ºC for 25 min. The amount of TBARS, namely MDA, was measured at a wavelength of 532 nm. The calculation of MDA concentration was made using its extinction coefficient (1.56 x 10^5 M^{-1} cm^{-1}). The results were expressed as nmol of MDA equivalents per g of FW.

Superoxide dismutase (SOD) activity

For SOD activity quantification, the method described by Beauchamp and Fridovich, (1971) was followed, with slight modifications (Freitas et al., 2012). The cell extraction was performed with potassium phosphate buffer (50 mM disodium hydrogen phosphate dihydrate; 1 mM EDTA; 1% PVP; 1 mM DTT, pH 8.0. The standard curve was performed with SOD standards (0.25–60 U/mL) (Sigma®). The assay mixture was performed with 250 µL of the reaction buffer (50 mM Tris–HCl, pH8, 0.1mM (diethylenetriaminepentaacetic acid) DTPA , 0.1mM hypoxanthine (Sigma®), and 68.4 mM nitrobluetetrazolium (NBT, AMRESCO), 25 µL of xanthine oxidase (56.1 mU/ml) and 25 µL of extract sample. Xanthine-xanthine oxidase complex produces superoxide radicals that react with NBT to produce a colored formazan. Samples and standards were incubated with agitation for 20 min, at room temperature. The activity of SOD was measured at 560. Results were expressed in U per g of FW. One unit of enzyme (U) corresponds to a reduction of 50% of nitroblue tetrazolium (NBT), per min.

Catalase (CAT) activity

The activity of CAT was measured according to Johansson and Borg (1988), with some modification according to Freitas et al. (2012). The cell extraction was performed with potassium

phosphate buffer (50 mM disodium hydrogen phosphate dihydrate; 1 mM EDTA; 1% PVP; 1 mM DTT, pH 7.0. For CAT activity quantification, 25 µL of extract sample and standards of formaldehyde (0-150 µM) were added 125 µL of reaction buffer (50 mM potassium phosphate, pH 7.0), 37.5 µL of ethanol and 25 µL of H_2O_2 (35.28 mM) to initiate the reaction. Samples and standards were incubated at room temperature for 20 min. After the reaction, 37.5 µL of potassium hydroxide (KOH) (10 M) were added to finish the reaction and 37.5 µL of 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (purpald) as a chromogen, representing the formaldehyde produced. The samples were incubated once again for 10 min in a stirrer at room temperature and was added 12.5 µL of potassium periodate. The absorbance was read at 540 nm. The results were expressed as U of g FW. One unit of enzyme (U) is defined as the amount of enzyme that it is responsible by the formation of 1.0 nmol formaldehyde, per min.

Glutathione S-transferase (GSTs) activity

For the GSTs activity, cell extracts were homogenized in 50 mM phosphate buffer containing Triton X-100 at 0.1% (pH=7.0). The activity of GSTs was determined following the method described by Habig et al. (1974). These enzymes catalyze the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione, forming a thioether. For GSTs activity determination 100 µl of extracted sample and 200 µL of reaction solution were used (60 mM CDNB, reduced glutathione (GSH) and 0.1 M potassium phosphate buffer, pH 6.5). This formation was followed by the absorbance increment at 340 nm, intervals of 10 s during 5 min, at room temperature. For the enzyme activity quantification, it was selected a time interval (5 min) during which the activity was linear. The activity of GSTs was determined using extinction coefficient of 9.6 mM^{-1} cm^{-1} for CDNB. Results were expressed as U per g of FW. One unit of enzyme (U) corresponds to the amount of enzyme that caused the formation of 1 µmol of thioether, per min.

2.2.2.7. Statistical analysis

The biometric data, the environmental parameters, the concentration of metal(loid)s in sediments and both species, the BSAF values, the soluble and insoluble fraction of elements, and the biochemical parameters for the Ria de Aveiro and Óbidos lagoon were submitted to hypothesis testing using the PERMANOVA (permutational multivariate analysis of variance) + add-on in PRIMER v6 (Anderson et al., 2008). The pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance. When the main test revealed statistically significant differences ($p \leq 0.05$), pairwise comparisons were performed. The *t*-statistic in the pair-wise comparisons was evaluated in terms of significance among different areas and species. Values lower than 0.05 were considered as significantly different. The null hypotheses tested were: (i) for environmental parameters: no significant differences exist among areas; (ii) for sediment contamination: no significant differences exist among areas; (iii) for metals and As concentrations in organisms: no

significant differences exist among species and areas; (iv) for BSAF values: no significant differences exist among species and areas; (v) for soluble and insoluble fraction: no significant differences exist between species and areas: (vi) for each biochemical parameter: no significant differences exist between areas and species.

The results obtained were expressed as distinct letters for *R. decussatus* (lowercase letters) and for *R. philippinarum* (uppercase letters) to indicate differences among areas. To indicate differences between species asterisks were used.

The Spearman correlation was used to evaluate: the correlation between metals and As concentration present in sediments and in both species (Ria de Aveiro and Óbidos lagoon); the correlation between elements concentration in sediments and physicochemical characteristics from the Ria de Aveiro and the Óbidos lagoon; the correlation between clams density and elements concentration in sediments and physicochemical characteristics. The Spearman correlation value was classified as weak (0.26 to 0.49), moderate (0.50 to 0.69), strong (0.70 to 0.89) or very strong (0.90 to 1.00) ($p \leq 0.05$) (Munro, 2001). The confidence level of the Spearman correlations was 0.05.

For each species from the Ria de Aveiro and the Óbidos lagoon, the biochemical parameters were used to calculate the Euclidean distance similarity matrix. This similarity matrix was simplified through the calculation of the distance among centroids matrix based on biochemical parameters (r>0.70), which was then submitted to ordination analysis, performed by Principal Coordinates analysis (PCO). Spearman correlation vectors of environmental data, metal(loid)s concentrations in sediments and clams tissues descriptors were provided as supplementary variables being superimposed on the top of PCO graph.

Metal(loid)s concentrations in sediments from the Ria de Aveiro and the Óbidos lagoon were used to calculate the Euclidean distance similarity matrix. This similarity matrix was simplified through the calculation of the distance among centroids matrix based on metal(loid)s concentrations, which was then submitted to ordination analysis, performed by PCO. Spearman correlation vectors (r>0.7) of environmental data, metal(loid)s concentrations in sediments and the concentration of each element descriptors were provided as supplementary variables being superimposed on the top of PCO graph.

The environmental data matrices, metal(loid)s concentrations in sediments and organisms were related to the biological data using the BIOENV procedure (BEST routine) (Clarke and Gorley 2015).

All statistical analyses above mentioned were conducted using PRIMERv6® (Phymouth Marine Laboratory) (Anderson et al., 2008).

2.3. Results

2.3.1. Ria de Aveiro

2.3.1.1. Species biometric data and density

Results revealed that *R. decussatus* was present in seven of fifteen areas, while *R. philippinarum* was present in nine of the fifteen areas (Fig. 2.3). Both species co-existed in seven of the fifteen areas. The native species presented higher relative abundance and densities values in areas O, N, and K than the introduced species, while higher relative abundance and density values for *R. philippinarum* were obtained in areas F, G and M (Fig. 2.3, Table 2.2). In general, the introduced species showed wider spatial distribution in this ecosystem than the native one (Fig. 2.3).

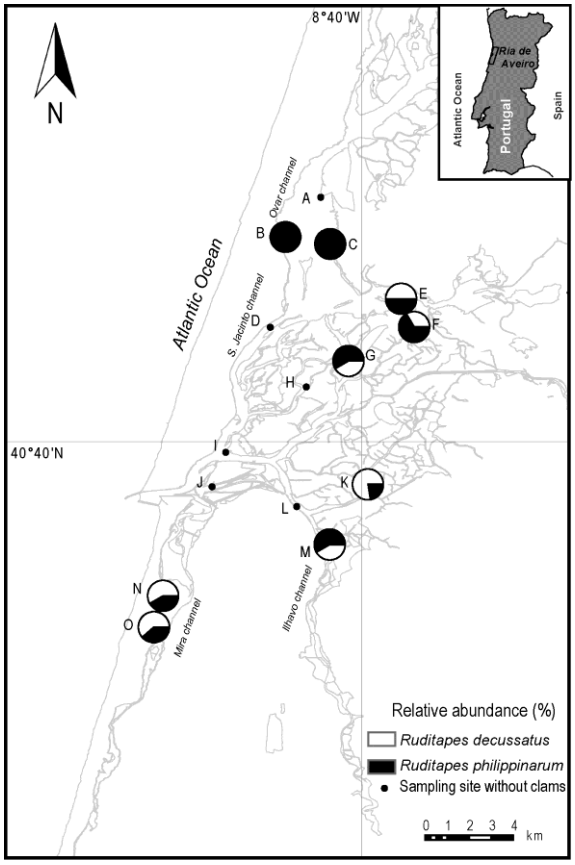

Figure 2.3 - Relative abundance (%) of the *Ruditapes decussatus and R. philippinarum* along the 15 sampling areas from the Ria de Aveiro (A-O).

Both species presented similar weight, length, and width (Table 2.2), with no significant differences between species from the same area. Similar results were also observed in CI values for both species (9.27 ± 1.63 to 11.63 ± 1.59% for *R. decussatus* and 7.69 ± 1.11 to 10.78 ± 3.38% for *R. philippinarum*) (Table 2.2).

Table 2.2 – Density (# ind./m^2), weight (g), length (cm), width (cm) and Condition Index (CI, %) for *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp), in each area (mean±SD). For each parameter and for each species, significant differences ($p \leq 0.05$) among areas are represented with distinct letters (a-c for Rd and A-C for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with asterisks. Areas are presented according to the increase of sediment contamination gradient. Highlighted values represent the species with the highest density. **no organisms were available.

Area	Species	Density	Weight	Length	Width	CI
O	Rd	10.67±4.62^{a}	21.6±7.83^{a}	3.30±0.45^{a}	4.50±0.70^{a}	9.96±2.44^{a}
	Rp	6.67±2.89^{A}	28.8±17.06^{A,B}	4.00±0.50^{A}	4.80±0.66^{A}	**
B	Rp	5.33±2.31^{A}	24.88±5.74^{A,B}	4.33±0.29^{A}	3.38±0.26^{B}	**
N	Rd	9.33±2.31^{a}	29.74±9.41^{a}	4.95±0.72^{b}	3.60±0.50^{a}	9.27±1.63^{a}
	Rp	6.67±4.62^{A}	30±12.43^{A}	4.43±0.56^{A}	3.70±0.47^{A,B}	9.01±0.75^{A}
C	Rp	10.00±4.00^{A}	12.84±6.27^{B}	3.52±0.60^{A,B}	2.70±0.47^{C}	10.72±2.00^{A}
E	Rd	10.67±2.31^{a}	24.32±11.48^{a}	4.96±0.52^{b}	3.92±0.38^{a}	11.05±4.77^{a}
	Rp	10.67±9.24^{A,B}	25.78±9.35^{A,B}	4.52±0.59^{A}	3.69±0.48^{A}	10.78±3.38^{A}
G	Rd	1.33±2.31^{b}	19.2±0.00^{a}	4.90±00^{b}	3.20±00^{b}	**
	Rp	2.67±2.31^{B}	23.35±17.75^{A,B}	3.70±0.14^{B}	2.40±0.42^{C}	**
F	Rd	8.00±5.20^{a}	17.93±4.23^{a}	3.90±0.25^{a}	3.12±0.19^{b}	**
	Rp	16.00±10.58^{A}	22.90±7.14^{A,B}	4.20±0.42^{A}	3.22±0.36^{C}	7.69±1.11^{A}
K	Rd	41.33±22.74^{c}	17.6±9.98^{a}	3.70±1.08^{a,b}	2.60±0.82^{b}	11.16±1.59^{a}
	Rp	12.00±10.58^{A,B}	21.7±8.77^{A,B}	4.30±0.45^{A}	3.20±0.45^{B}	10.40±7.79^{A}
M	Rd	5.33±2.31^{a,b}	17.55±16.75^{a}	3.88±1.07^{a,b}	2.83±0.83^{b}	**
	Rp	9.33±2.31^{A}	25.29±8.73^{A,B}	4.60±0.64^{A}	3.47±0.51^{B}	**

2.3.1.2. Physicochemical data and metal(loid)s contamination

Regarding environmental data, among the sampling areas, results showed that salinity varied between 28 and 40, pH from 6.82 to 8.53 and temperature between 18.0 and 22.6 ºC (Annex I). It was also possible to demonstrate that in areas with high salinity (36-40, J, I, L, H, D, and A) no clams were recorded.

The sediment grain size analysis revealed that, in general, areas located in Mira, S. Jacinto and Ovar channels (I, D, B, N, A and J) were classified as clean medium/corse sand, with the fines fraction below 4% and the median ranging from 1 to 2 Φ (Annex I). The remaining areas were classified as very/silty medium/fine sand (areas O, H, C, E, G, F, K, and L) or mud (area M) (Annex I). Furthermore, the results obtained showed that, in general, areas E, G, F K, M, and L presented

a higher percentage of fines particles (15.42 to 73.35%) and TOM (3.03 to 6.15%) than remaining areas (Annex I).

The data for metal(loid)s concentrations in sediments, from each of the fifteen sampling areas, are shown in Fig. 2.4. Considering the total metal(loid)s concentration in sediments, results showed that areas are located in Mira, S. Jacinto, and Ovar channels were the areas with lowest contamination levels (Fig. 2.4). The results obtained also showed that area L was the most contaminated area (49.73±2.83 mg/kg DW), with significant differences compared to the remaining areas, while area I was the least contaminated area, with no differences compared to the areas D, O and B (4.31±0.09 mg/kg DW) (Fig. 2.4, Annex II). Areas I, D, O, B, N, A and J presented eleven to five times lower total element concentrations than the most contaminated area (L) (Fig. 2.4 and Annex II). Results noticed that, in general, the most contaminated areas (14.16±0.74 to 49.73±2.83 mg/kg DW, E, G, F, K, M, L) presented significantly higher fines and TOM content than the least contaminated areas (4.31±0.09 to 14.13±0.75 mg/kg DW, I, D, N, J) (Annex I and II).

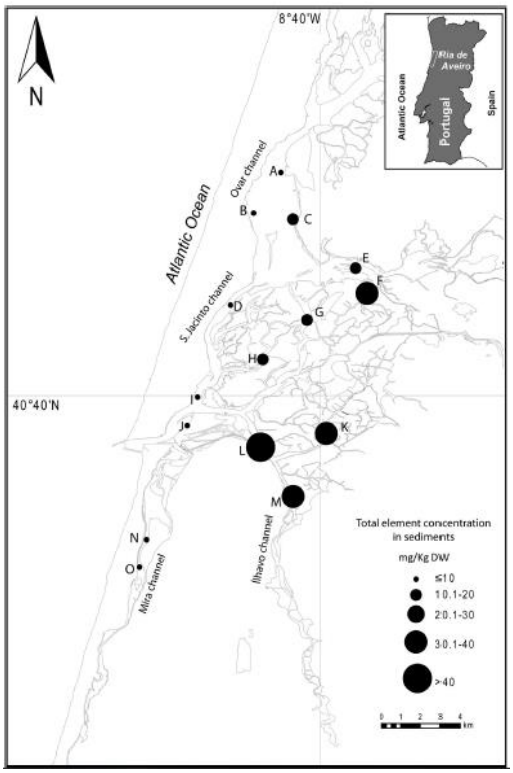

Figure 2.4 - Total element concentrations (mg/kg DW) in sediments from the Ria de Aveiro (Areas A to O).

The data for physicochemical parameters and sediment contamination was correlated, and a strong correlation was observed between sediment contamination and fines content (0.78), while TOM and sediment contamination presented a moderate correlation (0.6). When correlating the physicochemical parameters, and metal(loid)s concentrations found in sediments with the density of *R. decussatus* and *R. philippinarum* results revealed a poor correlation (< 0.5) for *R. decussatus*

in all parameters analyzed, while for *R. philippinarum*, density presented a moderate correlation with total contamination (0.69) and temperature (0.66).

In most of the areas, As (0.82±0.16 to 6.14±2.75 mg/kg DW), Cr (1.02±0.28 to 16.95±3.21 mg/kg DW) and Pb (0.93±0.02 to 15.25±4.25 mg/kg DW) were the elements with the highest concentrations, while Cd (0.02±0.00 to 0.20±0.05 mg/kg DW) and Hg (0.01±0.01 to 0.15±0.11 mg/kg DW) were the elements with the lowest concentrations in sediments (Annex II). Nevertheless, except for Hg (0.15±0.11 mg/kg DW) in area F and Cu (26.22±8.99 mg/kg DW) in area L, along the Ria de Aveiro, in all areas the elements presented concentrations lower than the corresponding TEL (Threshold Effect Level, 0.13 mg/kg DW for Hg and 18.70 mg/kg DW for Cu) values (Annex II). Metal(lois)s did not exceed the ERL, PEL, and ERM values. For most of the elements, significant differences were found between areas with the lowest (areas I-B) and the highest (areas F, K, M, L) contamination levels (Annex II).

2.3.1.3. Metal(loid)s concentrations in organisms and BSAF values

Total metal(loid)s concentration found in organisms is shown in Fig. 2.5. Results revealed that both species from areas with the lowest (O) and the highest levels of sediment contamination (K and M) presented higher metal(loid)s concentrations than organisms from the remaining areas (Fig. 2.5). Furthermore, results obtained showed that, except for areas O, G, and M, no significant differences exist between species from the same area. When correlating sediment contamination with metal(loid)s concentrations present in *R. decussatus* and *R. philippinarum*, a moderate correlation was observed for both species (<0.55).

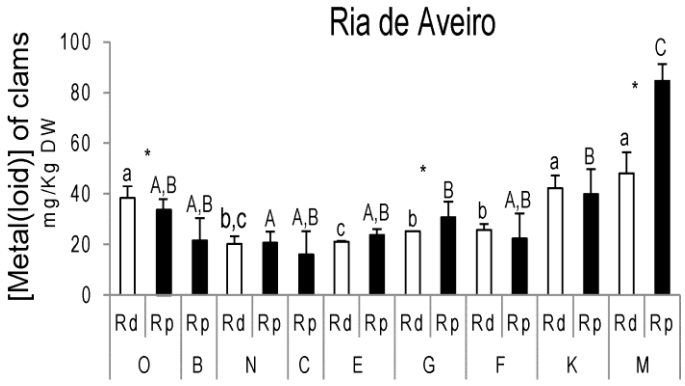

Figure 2.5 - Total metal(loid)s concentrations (mg/kg DW) in *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp) among areas (mean±SD). Areas are presented according to the increase of sediment contamination gradient. For each species, significant differences ($p \leq 0.05$) among areas are represented with different letters (a-c for Rd and A-C for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with an asterisk.

The BSAF values in both species of clams are presented in Fig. 2.6. Results showed that both species from the least contaminated areas (O, B and N) tend to have higher BSAF values than organisms from the most contaminated areas (F, K, and M) (Fig. 2.6). Results also showed that, except in area M, no significant differences between species were recorded (Fig. 2.6).

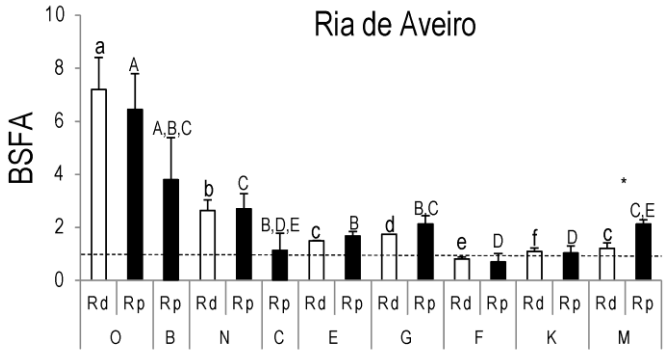

Figure 2.6 - Biota-Sediment Accumulation Factor (BSAF) in *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp) among areas (mean±SD). Areas are presented according to the increase of sediment contamination gradient. For each species, significant differences ($p \leq 0.05$) among areas are represented with different letters (a-f for Rd and A-E for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with an asterisk.

Regarding the element concentration of each element, it was possible to observe that both species presented higher As (7.38±3.41 - 69.73 mg/kg DW), Ni (2.00±0.45 - 8.12±5.01 mg/kg DW) and Cu (2.44±1.10 - 8.90±1.33 mg/kg DW) concentrations compared to the remaining elements (0.78±0.10 - 2.05±0.00 mg/kg DW for Pb; 0.23±0.05 - 0.43±0.12 mg/kg DW for Cd; 0.05±0.01 - 0.24±0.06 mg/kg DW for Hg; 0.04±0.01 - 0.09±0.01 mg/kg DW for MeHg), in the majority of areas (Annex III and IV). For MeHg concentrations, results showed higher MeHg values in both species from area E than organisms from areas N and K (Annex IV). Regarding the concentration of each element in both species from each area, in general, results showed no significant differences between species, except in area M for As and in area N for Cu (Annex III and IV).

The BSAF values of each element demonstrated that, in general, both species from all areas tend to bioaccumulate preferentially As, Cu, and Cd (BSAF >1) (Annex III and IV). Both organisms also tend to bioaccumulate Cr, Pb and Ni in the least contaminated areas (BSAF>1) (Annex, Table III and IV). Both species from areas E and F tend to bioaccumulate higher Hg concentration than both species from the remaining areas (Annex IV). When comparing both species, in the same area, results demonstrated that both species tended to bioaccumulate similar concentrations in the same area, except in area M for As and Cr and in area N for Cu and Hg (Annex III and IV).

2.3.1.4. Subcellular partitioning

Analyzing the concentration of elements in the soluble and insoluble fractions (Annex V), results showed that native and introduced species presented the majority of metals concentrations in the insoluble fraction, independently on the area. However, in the most of the areas, the majority of As concentration was found in the soluble fraction (Annex V).

2.3.1.5. Human health risk from clams consumption

The concentration of all metals quantified in native and introduced species from the Ria de Aveiro was below the USFDA, FSANZ and EFSA MLs (Table 2.3). When compared the total As concentrations present in both species with total As MLs for USFDA, results showed that in both species As concentration were below the USFDA MLs. According to USFDA, inorganic As is approximately 10% of total As. When applied this ratio to the present data for total As concentrations, it is possible to estimate that inorganic As concentration in clams from the Ria de Aveiro (0.2-1.4 mg/Kg) were above the MLs for inorganic As established by FSANZ (1 mg/Kg) (Table 2.3).

The element content in the whole soft parts of the clams allowed the calculation of the mass of bivalves necessary to be consumed by a 70 Kg adult to reach the PTWI (Table 2.3). Despite, the PTWI values established by JECFA and FSANZ for As, only refer its inorganic form in seafood, the results in the present thesis are mentioned as total As concentrations for both species. But assuming the inorganic arsenic estimate in clams from the Ria de Aveiro (0.2-1.4 mg/Kg), overall, As appeared to be the element of concern regarding *R. philippinarum* consumption. The ingestion of more than 0.80 of *R. philippinarum* per week results in exceeding the PTWI threshold for As in the most of the areas considered in the present study (Table 2.3). For the remaining elements, larger amounts (>1.48 Kg) of both clams fresh tissues is needed to be consumed in one week to exceed the PTWI standard values (Table 2.3).

Table 2.3 - Concentrations of different elements (mg/kg fresh weight, FW) in *Ruditapes decussatus* and *R. philippinarum* from the Ria de Aveiro and the amount of clams that a 70 kg adult needs to consume to exceed PTWI (Provisional Tolerable Week Intake). max corresponds to the highest concentration found for each element among the sampling areas. min corresponds to the lowest concentration found for each element among the sampling areas. Highlighted values are the lowest values needed to exceed the PTWI. *** inorganic arsenic. # inorganic arsenic estimative.

			As*	Cr*	Ni*	Pb*	Cu*	Cd*	Hg*	MeHg**
Maximum permissible limits (MLs., *mg/Kg or **µg/Kg)										
	EFSA					1.50		1.00	0.50	
	USFDA		86.00	13.00	80.00	1.70		4.00	1.00	
	FSANZ***		1.00***			2.00		2.00	0.50	
	Ipolyi et al. (2004)									44
PTWI (mg/Kg/week)	JECFA		0.015***		0.035	0.025	3.5	0.007	0.005	3.3
	FSANZ		0.015***			0.025		0.007	0.005	
Metal concentration in clams (mg/Kg FW)										
R. philippinarum		max	1.40	4.33	1.16	0.34	1.78	0.08	0.04	12.60
		min	0.16	1.31	0.40	0.16	0.55	0.05	0.01	4.80
R. decussatus		max	0.72	3.68	1.66	0.41	1.67	0.08	0.05	12.20
		min	0.20	1.58	0.51	0.16	0.59	0.05	0.01	5.90
Amount of clams consumed per week to exceed PTWI (Kg), based on JECFA values										
R. philippinarum		max	0.80#		2.11	5.16	137.64	5.83	8.97	17.80
		min	6.50#		6.13	10.94	444.11	10.89	27.63	46.20
R. decussatus		max	1.50#		1.48	4.27	147.00	5.98	7.61	19.30
		min	5.30#		4.76	10.94	414.09	10.12	55.26	38.50

Current consumption guidelines for elements set by different organizations are also presented: EFSA - European Food Safe Authorities; USFDA - United States Food and Drug Administration; FSANZ - Food Standards Australia and New Zealand; JECFA - Joint FAO/WHO Expert Committee on Food Additives.

2.3.1.6. Biomarkers

Protein (PROT) content

The content of PROT in *R. decussatus* and *R. philippinarum* along of the sediment contamination gradient is present in Fig. 2.7. The results showed that *R. decussatus* presented the highest PROT content in area E, while the lowest protein content was observed in organisms from area F. On the other hand, the introduced species presented the highest PROT content in area N, while the lowest protein content was found in organisms from areas C, F, and M (Fig. 2.7). In terms of PROT content, significant differences between species were only observed in area N.

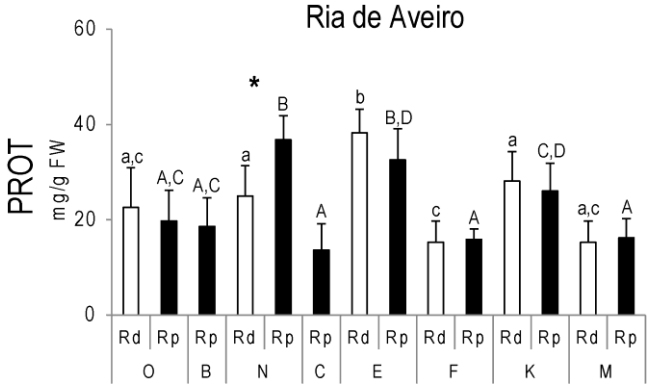

Figure 2.7 – Total protein content (PROT, mg/g FW) in *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp) among areas (mean±SD). Areas are presented according to the increase of sediment contamination gradient. For each species, significant differences ($p \leq 0.05$) among areas are represented with different letters (a-c for Rd and A-D for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with an asterisk.

Lipid peroxidation (LPO) levels

The results of LPO showed that the native species presented significantly higher values in organisms from areas E and K than in the remaining areas (Fig. 2.8). For *R. philippinarum* significantly higher LPO values were found in species from area E when comparing with organisms from the remaining areas (Fig. 2.8). When comparing *R. decussatus* and *R. philippinarum*, in the same area, results showed that significant differences between both species were only observed in areas N and E, with the native species showing lower LPO values than the introduced species and in area K, with the native species presenting the highest LPO values.

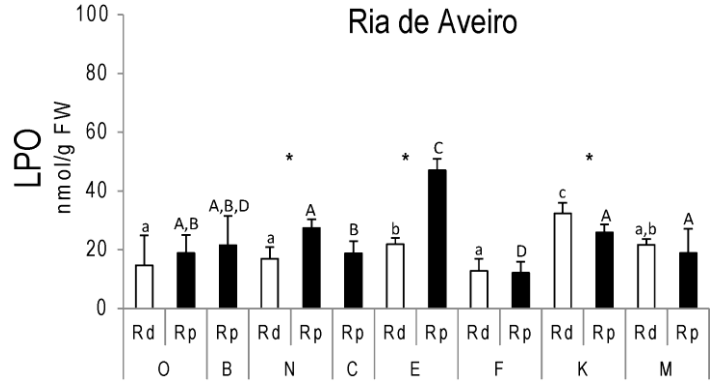

Figure 2.8 – Lipid peroxidation levels (LPO, nmol/g FW) in *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp) among areas (mean±SD). Areas are presented according to the increase of sediment contamination gradient. For each species, significant differences ($p \leq 0.05$) among areas are represented with different letters (a-c for Rd and A-D for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with an asterisk.

Superoxide dismutase (SOD) activity

Regarding SOD activity, results demonstrated higher values in *R. decussatus* from areas N, E, and M when compared to organisms from the remaining areas (Fig. 2.9). The introduced species presented the highest SOD activity in areas E and K. When comparing both species, in the same area, results showed significant differences between species only at area E and M, with *R. decussatus* presenting higher SOD activity than *R. philippinarum* (Fig. 2.9).

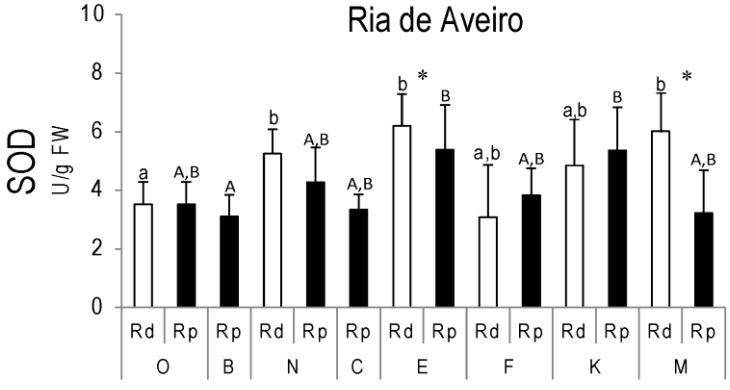

Figure 2.9 – Superoxide dismutase activity (SOD, U/g FW) in *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp) among areas (mean±SD). Areas are presented according to the increase of sediment contamination gradient. For each species, significant differences ($p \leq 0.05$) among areas are represented with different letters (a-b for Rd and A-B for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with an asterisk.

Catalase (CAT) activity

The activity of CAT showed higher values in *R. decussatus* from areas N, E, and K than in organisms from the remaining areas (Fig. 2.10). Similar results were found for *R. philippinarum*. When comparing both species, in each area, significant differences were only observed at area N, with *R. decussatus* presenting significantly lower CAT activity than *R. philippinarum* (Fig 2.10).

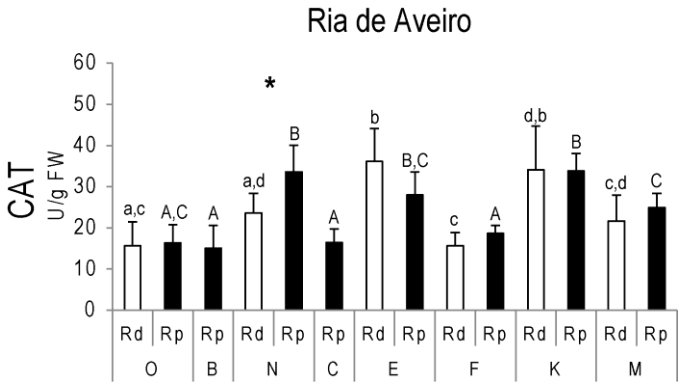

Figure 2.10 – Catalase activity (CAT, U/g FW) in *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp) among areas (mean±SD). Areas are presented according to the increase of sediment contamination gradient. For each species, significant differences ($p \leq 0.05$) among areas are represented with different letters (a-d for Rd and A-C for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with an asterisk.

Glutathione S-transferase (GSTs) activity

The results of GSTs showed higher values in *R. decussatus* from areas N, E, and M when compared with organisms from the remaining areas (Fig. 2.11). On the other hand, the introduced species presented no significant differences in GSTs activity among areas, except in area E where it was the highest value. When comparing both species, in the same area, present results showed significant differences between *R. decussatus* and *R. philippinarum* in area E, with *R. decussatus* presenting lower GSTs activity than *R. philippinarum*, and in area M, where *R. decussatus* presented higher GSTs activity than *R. philippinarum* (Fig. 2.11).

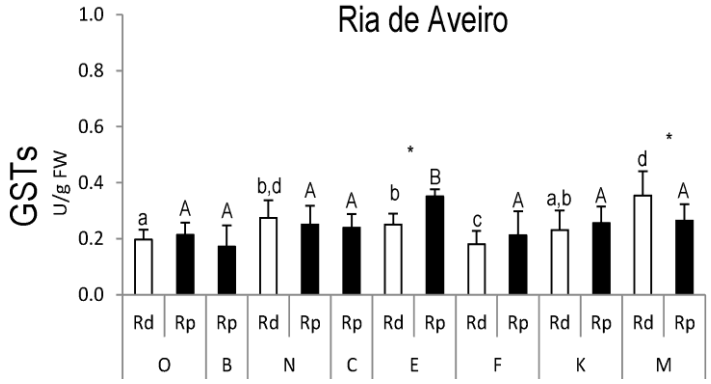

Figure 2.11 **-** Glutathione *S*-transferases activity (GSTs, U/g FW) in *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp) among areas (mean±SD). Areas are presented according to the increase of sediment contamination gradient. For each species, significant differences ($p \leq 0.05$) among areas are represented with different letters (a-d for Rd and A-B for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with an asterisk.

2.3.1.7. Relation between physicochemical, metal(loid)s concentrations and biochemical performance - multivariate analysis

According to the BIOENV procedure, TOM content, Pb contamination in sediments and As, Pb and Cd concentrations in *R. decussatus* were the variables best correlated with the biochemical parameters for *R. decussatus* (p=0.743). On the other hand, for *R. philippinarum* pH was the variable best correlated with biochemical parameters (p=0.423).

For *R. decussatus*, PCO analysis was based on the biochemical parameters and the superimposed vectors were the physicochemical data and sediment and species metal(loid)s concentration data (Fig. 2.12 A). The PCO axis 1 explained 60.8% of the total variability, separating the areas K and E on the negative side of the axis; areas M and N near to the origin axis and areas O and F on the positive side of the axis. The PCO axis 2 explained 31.0% of the total data variation, where areas K and M (on the positive side of the axis) were separated from the areas E, N O, and F (on the negative side of the axis). In areas E and K, *R. decussatus* were characterized by higher SOD, CAT and PROT content than organisms from the remaining areas. TOM percentage, Cr concentration in sediments, As concentration and the total metal(loid)s concentrations in *R. decussatus* were the main factors related to the biochemical responses of organisms presented in area M. On the other hand, Pb concentration present in *R. decussatus* tissues was the main factor related to the biochemical responses of organisms from area O.

Regarding the PCO analysis for *R. philippinarum* from the Ria de Aveiro, PCO analysis was based on the biochemical parameters and the superimposed vectors were the physicochemical data and sediment and species metal(loid)s concentration data (Fig. 2.12 B). Results showed that the PCO axis 1 gathered 65.7% of the total variation and showed a clear distinction among areas E, N K (on the negative side of the axis) and the remaining areas (areas M, G, C, B, and F, on the positive side of the axis) (Fig. 2.12B). The PCO axis 2 explained 20.2% of total variance, with areas E, M, O and B on the positive side of the axis, while area C is near to the origin axis and areas N, K and F are present on the negative side of the axis. The species *R. philippinarum* was characterized by higher PROT and CAT values in area E and higher CAT and SOD activity in organisms from areas N and K when compared with the remaining areas. The Pb and As contamination in sediments were the main factors related to biochemical responses of *R. philippinarum* from area F.

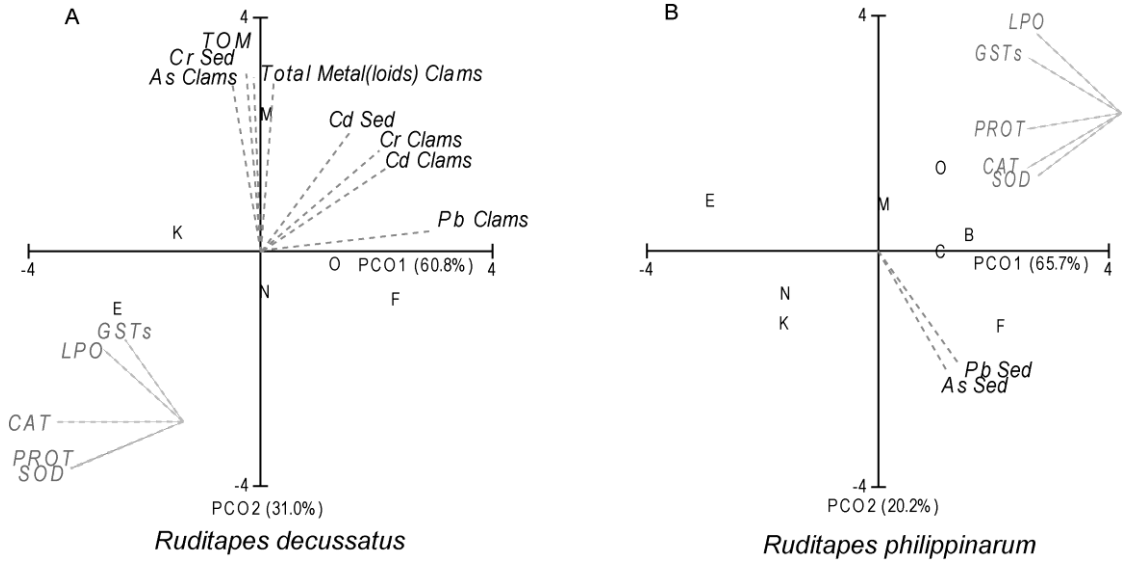

Figure 2.12 - Centroid ordination diagram (PCO). The matrix was based on biochemical parameters for *R. decussatus* (A, $r > 0.70$) and for *R. philippinarum* (B, $r > 0.70$) and spearman correlation vectors are superimposed as supplementary variables, namely physicochemical parameters, contamination in sediments and organisms.

2.3.2. Óbidos lagoon

2.3.2.1. Species biometric data and density

In the Óbidos lagoon, the presence of *R. decussatus* was recorded in all the sampling areas, while the introduced species was reported in four (areas B to E) of the five areas, coexisting with *R. decussatus* in all these areas (Fig. 2.13).

When comparing both species, in the same area, results revealed that native species presented higher abundance and density than the introduced one (Fig. 2.13, Table 2.4). The clam *R. philippinarum* was the species with the highest weight (Table 2.4). The native species presented lower length and higher width than *R. philippinarum* in all areas (Table 2.4).

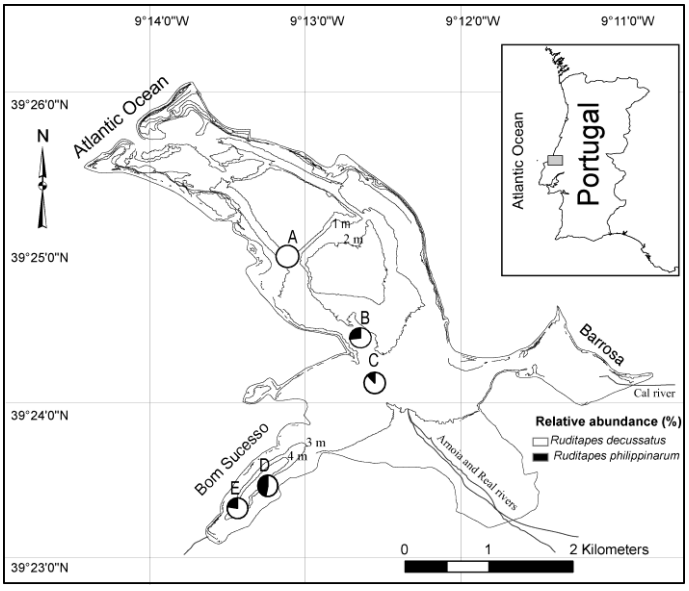

Figure 2.13 - Relative abundance (%) of *Ruditapes decussatus* and *R. philippinarum* along the 5 sampling areas from Óbidos lagoon.

The results obtained revealed that *R. decussatus* presented higher CI values when compared with *R. philippinarum*, in all the areas (Table 2.4)

Table 2.4 - Density (# ind./m^2), weight (g), length (cm), width (cm) and Condition Index (CI, %) for *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp), in each area (mean±SD). For each parameter and for each species, significant differences ($p \leq 0.05$) among areas are represented with distinct letters (a-c for Rd and A-C for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with asterisks. Areas are presented according to the increase of sediment contamination gradient. Highlighted values represent the species with the highest density.

Area	Species	Density	Weight	Length	Width	CI
A	Rd	72.22±11.1^{a,b}	12.4±3.3^{a,b}	3.8±0.3^{a}	2.6±0.3^{a}	14.6±2.1^{a}
B	Rd	55.56±14.70^{a,c*}	10.6±1.6^{a,b}	2.4±0.1^{b,c}	3.5±0.2^{b,c}	19.0±4.7^{a,b*}
	Rp	20.37±6.42^{A,B}	11.5±2.6^{A}	2.5±0.1^{B}	3.2±0.2^{A}	10.1±1.6^{A}
C	Rd	94.44±11.11^{b*}	12.4±2.5^{a}	2.6±0.2^{b*}	3.8±0.3^{b}	18.4±3.1^{a,b*}
	Rp	12.96±3.21^{A}	17.6±7.9^{A}	3.7±0.4^{C}	2.9±0.6^{A}	11.0±0.9^{A}
D	Rd	40.74±11.56^{c*}	8.1±1.4^{b}	2.2±0.1^{c*}	3.2±0.2^{c*}	21.6±4.1^{b*}
	Rp	12.96±8.49^{B}	13.5±7.3^{A}	3.4±0.6^{C}	2.4±0.5^{A}	8.8±0.8^{B}
E	Rd	18.52±3.20^{c}	12.5±1.2^{a}	2.3±0.1^{b,c*}	3.4±0.2^{b,c*}	13.8±6.4^{a,b}
	Rp	16.67±5.55^{B}	17.5±7.3^{A}	3.5±0.2^{C}	2.8±0.2^{A}	9.3±0.9^{A,B}

2.3.2.2. Physicochemical data and metal(loid)s contamination

Regarding temperature and salinity, area A was characterized by lower values (22.00±1.20 ºC and 21.41±2.01, respectively) compared to the remaining areas (temperature 25.10±1.10 to 26.30 ºC and salinity 32.06±1.14 to 38.30±1.12), while no significant differences were found among areas B, C, D and E (Annex VI). The pH values varied between 7.30 and 7.87, but no significant differences were found among areas (Annex VI).

The areas located at the central body of the lagoon (areas A - C) were characterized by clean medium and fine sand, with a median values ranging from 1.66 to 2.24 Φ (Annex VI). These areas were characterized by low fines particles (1.8-4.4%) and TOM (1.13-1.58%) content. The areas located at the Bom Sucesso arm (areas D and E) were classified as mud, with the fine particles content higher than 66% and TOM content higher than 9% (Annex VI).

Total Metal(loid)s concentrations in sediments are present in Fig. 2.14 and Annex VII, revealing significantly lower values in areas located in the central part of the lagoon (area A - C; 9.66-14.72 mg/kg DW) when compared with the concentrations found in areas D and E, located at the Bom Sucesso arm (74.15-98.01 mg/kg DW). When correlating the total metal(loid)s concentration found in sediments with the physicochemical parameters (temperature, pH, salinity, fine particles and TOM content) a strong correlation of total metal(loid)s concentrations with TOM content (0.73) and with the percentage of fine particles (0.73) was obtained. When correlating the physicochemical parameters, and metal(loid)s found in sediments from the Óbidos lagoon with the

density of *R. decussatus* and *R. philippinarum* results revealed a poor correlation (< 0.5) for *R. decussatus* in all parameters analyzed, while for *R. philippinarum*, density was moderately correlated with the salinity (0.63) and temperature (0.63).

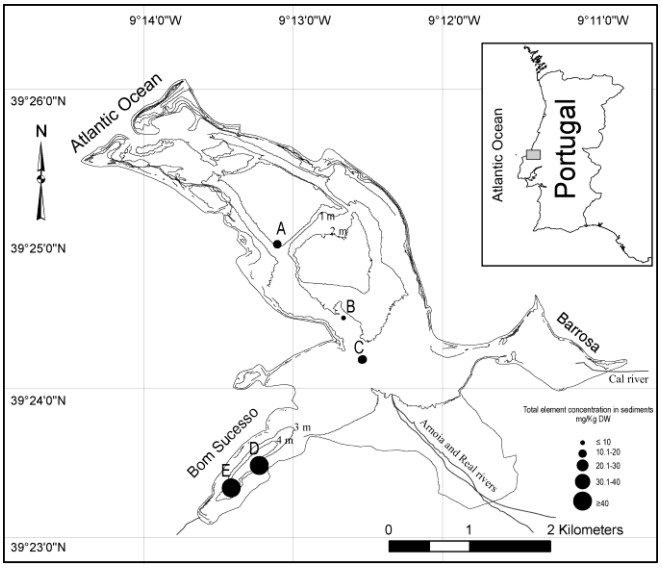

Figure 2.14 – Total element concentrations (mg/kg DW) in sediments from the Óbidos lagoon (A-E).

Regarding the concentration of each element (As, Cr, Ni, Pb and Cu) in sediments results demonstrated that, in all areas, the elements with higher concentrations were Cr (2.03±0.24 to 29.46±10.48 mg/kg DW), Cu (1.36±0.25 to 27.74±11.63 mg/kg DW) and Pb (1.90±0.10 to 15.86±5.03 mg/kg DW) (Annex VII), while Cd and Hg were below the detection limit (<0.03 mg/kg DW). As was the element with the lowest concentration in all the areas (0.74±0.10 to 9.36±2.99 mg/kg DW) (Annex VII). Nevertheless, the As concentrations in areas D (7.36±1.99 mg/kg DW) and E (9.36±2.99 mg/kg DW) reached the TEL (7.24 mg/kg DW) and ERL (8.20 mg/kg DW) values, while Cu concentrations in the same areas (area D 19.71±7.91 mg/kg DW and area E 21.74±11.63 mg/kg DW) reached the TEL values (Annex VII). For the remaining elements, the PEL and ERM values were not exceeded.

2.3.2.3. Metal(loid)s concentrations in organisms and BSAF values

The results regarding the total metal(loid)s concentrations in both species showed that the total metal(loid)s concentrations were lower in both species from areas B and C (31.25±5.01 - 47.39±9.62 mg/kg DW) than in species from areas A, D and E (43.95±3.15 - 57.42±15.54 mg/kg DW) (Fig. 2.15).

When comparing both species, in the same area, the results of total element concentrations showed that *R. decussatus* presented similar values than *R. philippinarum* (areas B, C, D and E) (Fig. 2.15).

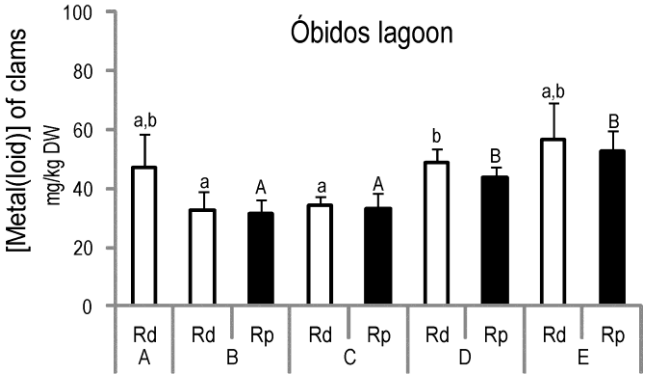

Figure 2.15 - Total metal(loid)s concentrations (mg/kg DW) in *Ruditapes decussatus* (Rd) and *R. philippinarum* among areas (mean±SD). Areas are presented according to the increase of sediment contamination gradient. For each species, significant differences ($p \leq 0.05$) among areas are represented with different letters (a-b for Rd and A-B for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with an asterisk.

Additionally, when correlated sediment metal(loid)s contamination with metal(loid)s concentrations in both species, a very strong correlation was observed for both species (0.9).

Regarding the BSAF values for total metal(loid)s concentration in both species and sediment, it is possible to observe that values were higher than 1 in the areas A to C for *R. decussatus* and in the areas B and C for *R. philippinarum*, while for both species in the areas D and E the BSAF values were lower than 1 (Fig. 2.16). Furthermore, it is possible to observe that species from the least contaminated areas (especially areas A and B) presented the highest BSAF values (4.91±0.17) (Fig. 2.16).

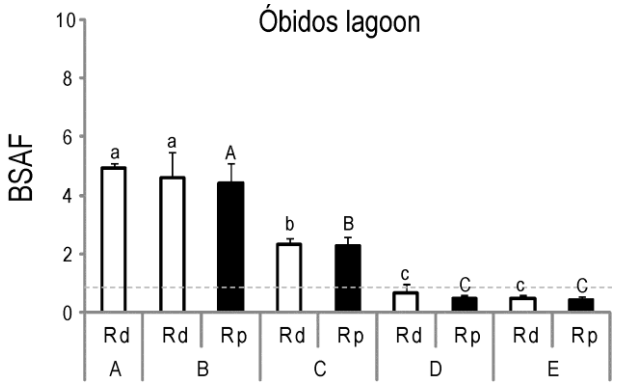

Figure 2.16 - Biota-Sediment Accumulation Factor (BSAF) in *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp) among areas (mean±SD). Areas are presented according to the increase of sediment contamination gradient. For each species, significant differences ($p \leq 0.05$) among areas are represented with different letters (a-c for Rd and A-C for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with an asterisk.

The native and introduced species, in general, presented higher concentrations of As (9.19±1.42 - 12.92±1.92 mg/kg DW) and Cr (8.33±2.10 - 17.92±0.80 mg/kg DW) than the reaming elements, except in both species from area D where the element with highest concentration was Cu (14.14±11.63 - 15.22±4.73 mg/kg DW) and in area E where the element with highest concentration in *R. philippinarum* was Ni (12.00±2.28 mg/kg DW) (Annex VIII).

In both species from all areas, Pb (1.72±0.16 - 3.42±1.74 mg/kg DW) Cd (<0.03 mg/kg DW) and Hg (<0.03 mg/kg DW) were the elements with the lowest concentrations (Annex VIII).

Taking into account BSAF values, both species showed high capacity (>1) to bioaccumulate Ni and As in all areas (Annex VIII). Furthermore, both species from the least contaminated areas (A to C) presented higher Cr and Cu BSAF (>1) values than organisms from the most contaminated areas (D to E) (Annex VIII). In general, Pb was the lowest bioaccumulated element by both species in all areas.

2.3.2.4. Subcellular partitioning

Regarding the soluble and insoluble concentration of each element in both species (Annex IX), results showed that both species tended to bioaccumulate the majority of the elements in the insoluble fraction. However, both species accumulated the majority of As in the soluble fraction (Annex IX).

2.3.2.5. Human health risks from clams consumption

In all the study areas, element concentrations found in *R. decussatus* and *R. philippinarum* were below the MLs defined by international organizations (USFDA; EFSA; FSANZ) (Table 2.5).

Taking into account that according to USFDA, inorganic As is approximately 10% of total As it is possible to estimate that inorganic As concentration in both species from the Óbidos Lagoon (≈0.1 mg/Kg) were above the MLs for inorganic As established by FSANZ (1 mg/Kg) (Table 2.5). Despite, the PTWI values established by JECFA and FSANZ for As, only refer its inorganic form in seafood, the results in the present thesis are mentioned as total As concentrations for both species. But assuming the inorganic arsenic estimate in clams from the Óbidos Lagonn (0.2-1.4 mg/Kg), results showed low health risks concerning the contamination by As, since, the consumption of high amount of clams during one week by an adult of 70 kg is needed to exceed the PTWI. For the remaining elements, larger amounts (>1.60 Kg) of both species is needed to be consumed in one week to exceed the PTWI values.

Table 2.5 - Concentrations of different elements (mg/kg fresh weight, FW) in *Ruditapes decussatus* and *R. philippinarum* from the Óbidos lagoon and the amount of clams that a 70 kg adult needs to consume to exceed PTWI (Provisional Tolerable Week Intake). max corresponds to the highest concentration found for each element among the sampling areas. min corresponds to the lowest concentration found for each element among the sampling areas. Highlighted values are the lowest values needed to exceed the PTWI. nd- corresponds to not detected concentration (lower than the detection limit).*** inorganic arsenic.

			As	Cr	Ni	Pb	Cu	Cd	Hg
MLs (mg/Kg)									
	EFSA					1.50		1.00	0.50
	USFDA		86.00	13.00	80.00	1.70		4.00	1.00
	FSANZ		1.00***			2.00		2.00	0.50
PTWI (mg/Kg/week)	JECFA		0.015***		0.035	0.025	3.5	0.007	0.005
	FSANZ		0.015***			0.025		0.007	0.005
Metal concentration in clams (mg/ Kg FW)									
R. decussatus		max	1.53	2.15	1.53	0.42	1.72	nd	nd
		min	1.1	1.10	0.52	0.24	0.54	nd	nd
R. philippinarum		max	1.53	1.26	1.53	0.29	1.83	nd	nd
		min	1.3	1.05	0.45	0.21	0.56	nd	nd
Amount of clams consumed per week to exceed PTWI (Kg)									
R. decussatus		max	6.90#		1.60	4.17	142.44	nd	nd
		min	9.50#		4.71	7.29	453.70	nd	nd
R. philippinarum		max	6.90#		1.60	6.03	133.88	nd	nd
		min	8.10#		5.44	8.33	437.50	nd	nd

Current consumption guidelines for elements set by different organizations are also presented: EFSA - European Food Safe Authorities; USFDA - United States Food and Drug Administration; FSANZ - Food Standards Australia and New Zealand; JECFA - Joint FAO/WHO Expert Committee on Food Additives. #inorganic arsenic estimative.

2.3.2.6. Biomarkers

Protein (PROT) content

The results of PROT content for *R. decussatus* and *R. philippinarum* are present in Fig. 2.17. The results obtained showed that the total PROT content present in *R. decussatus* and *R. philippinarum* was significantly higher in both species from areas with the lowest contamination levels (areas A to C) than in organisms from areas with the highest levels of contamination (areas D and E). When comparing both species in each area, significant differences were only found at area C, with *R. decussatus* presenting higher protein content than *R. philippinarum* (Fig. 2.17).

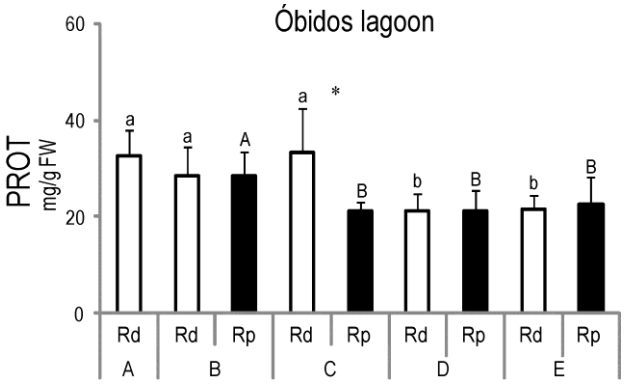

Figure 2.17 - Total protein content (PROT, mg/g FW) in *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp) among areas (mean±SD). Areas are presented according to the increase of sediment contamination gradient. For each species, significant differences ($p \leq 0.05$) among areas are represented with different letters (a-b for Rd and A-B for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with an asterisk.

Lipid Peroxidation (LPO) levels

The results of LPO showed for *R. decussatus* higher values in areas A and B than in remaining areas (Fig. 2.18), while for *R. philippinarum*, except in D area, no significant differences were found in LPO among areas. When comparing both species in the same area, the results showed significant differences between both species in area D, where *R. decussatus* showed higher LPO than *R. philippinarum* (Fig. 2.18).

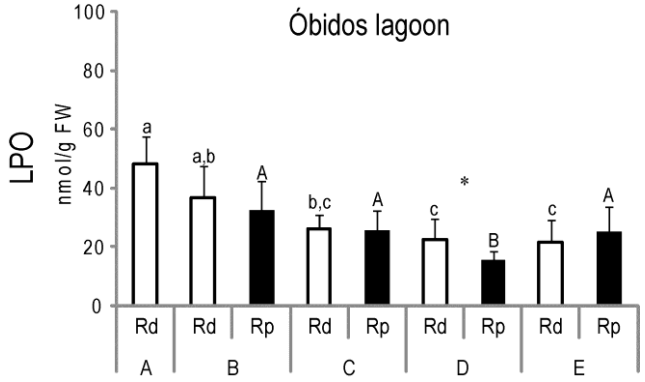

Figure 2.18 - Lipid peroxidation levels (LPO, nmol/g FW) in *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp) among areas (mean±SD). Areas are presented according to the increase of sediment contamination gradient. For each species, significant differences ($p \leq 0.05$) among areas are represented with different letters (a-c for Rd and A-B for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with an asterisk.

Superoxide dismutase (SOD) activity

The SOD activity in *R. decussatus* was lower in organisms from areas A, D and E than in organisms from areas B and C (Fig. 2.19). For *R. philippinarum* a significant higher SOD activity was observed in clams from area B comparing with the remaining areas. When comparing both species, in the same area, results revealed no significant differences (Fig. 2.19).

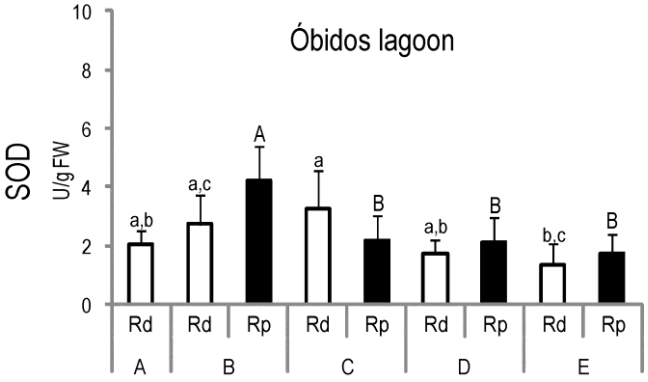

Figure 2.19 - Superoxide dismutase activity (SOD, U/g FW) in *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp) among areas (mean±SD). Areas are presented according to the increase of sediment contamination gradient. For each species, significant differences ($p \leq 0.05$) among areas are presented with different letters (a-c for Rd and A-B for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with an asterisk.

Catalase (CAT) activity

The activity of CAT showed no significant differences among areas for *R. decussatus* and *R. philippinarum* and no significant differences were noticed between species in each area (Fig. 2.20).

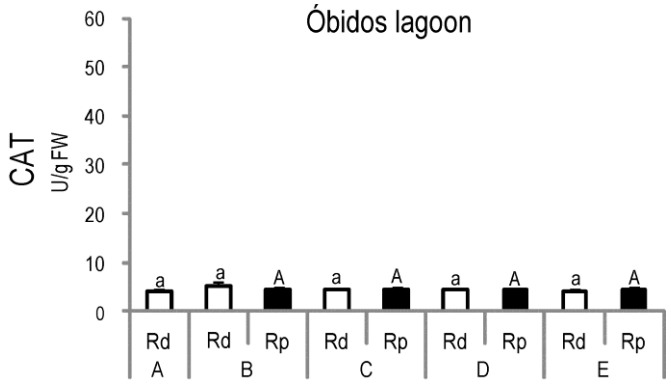

Figure 2.20 - Catalase activity (CAT, U/g FW) in *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp) among areas (mean±SD). Areas are presented according to the increase of sediment contamination gradient. For each species, significant differences ($p \leq 0.05$) among areas are presented with different letters (a-a for Rd and A-A for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with an asterisk.

Glutathione-S-transferases (GSTs) activity

Regarding GSTs activity, *R. decussatus* showed no significant differences in the GSTs values among areas A to D, but in area E significant lower values were found compared to areas B and C (Fig. 2.21). The introduced species showed no significant differences among areas in the GSTs activity, except in area D, where GSTs presented the highest activity. In general, in each area, *R. philippinarum* exhibited significantly higher GSTs activity than *R. decussatus* (Fig. 2.21).

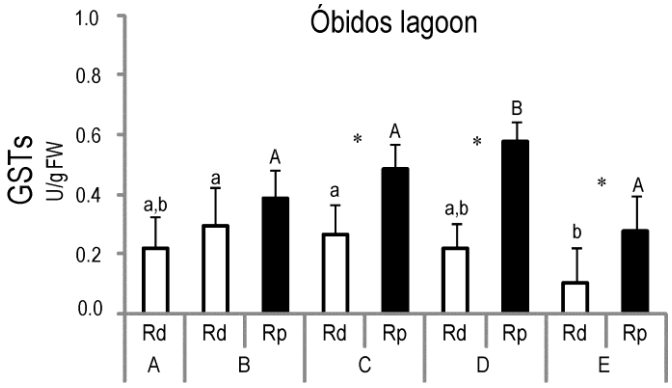

Figure 2.21 – Glutathione *S*-transferases activity (GSTs, U/g FW) in *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp) among areas (mean±SD). Areas are presented according to the increase of sediment contamination gradient. For each species, significant differences ($p \leq 0.05$) among areas are presented with different letters (a-b for Rd and A-B for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with an asterisk.

2.3.2.7. Relation between physicochemical, metal(loid)s concentrations and biochemical performance - multivariate analysis

The BIOENV procedure showed that salinity, TOM content, fines content, and Cu concentration in sediments and Cr concentrations in organisms were the variables best correlated with biochemical parameters for *R. decussatus* (p=0.964). For *R. philippinarum,* salinity, temperature, and Pb concentration in tissues were considered the best-correlated variables with biochemical responses (p=0.829).

The results from PCO analysis (Fig. 2.22A,B) were based on the biochemical parameters of *R. decussatus* and *R. philippinarum* from the Óbidos lagoon and the superimposed vectors were the environmental and sediment and species contamination data. For *R. decussatus*, PCO axis 1 revealed that the first principal component (PCO1), which accounted for 59.9% of the total variability, showed a clear distinction between areas A to C (on the negative side of the axis) and areas D and E (on the positive site of the axis) (Fig. 2.22A). The PCO axis 2 explained 25.9% of total variance, with just areas A on the positive side of the axis and the remaining areas on the negative side of the axis. *R. decussatus* from area A was characterized by higher LPO values, while organisms from area B were characterized by higher antioxidant and biotransformation activity than organisms from the remaining areas. Similar results were observed for SOD and GSTs activity in *R. decussatus* from area C. Total metal(loid)s concentration and the concentration of each metal, as well as, the TOM content and the percentage of fine content were the main factors related to the biochemical performance of organisms from areas D and E (Fig. 2.22A).

Regarding the PCO analysis for *R. philippinarum* from the Óbidos lagoon, the PCO axis 1 explained 74.4% of the total variation, with a clear distinction of area B on the negative side of the axis and the remaining areas on the positive side of the axis (Fig. 2.22B). The PCO axis 2 explained 21.8% of total variance, with just areas E on the positive side of the axis and the remaining areas on the negative side of the axis. The introduced species from area B were characterized by higher PROT content and antioxidant activity than organisms from the remaining areas. Temperature and salinity were the main environmental factors relate to the biochemical performance of *R. philippinarum* from area B, while Pb and Cr concentrations in *R. philippinarum* were the main factor related to the introduce species from area C.

In area D, *R. philippinarum* presented the highest GSTs activity, and the Pb concentration in tissues was the main factor related to the biochemical responses of this species in this area (Fig.2.22B).

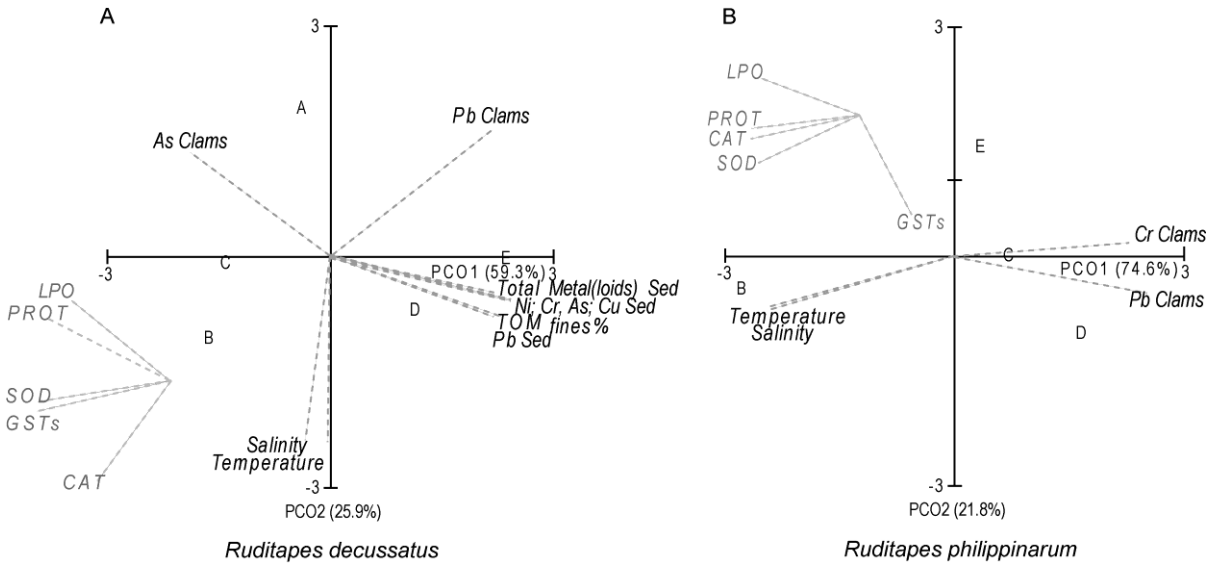

Figure 2.22 - Centroid ordination diagram (PCO). The matrix was based on biochemical parameters for *Ruditapes decussatus* (A, $r > 0.85$) and for *R. philippinarum* (B, $r > 0.85$) and spearman correlation vectors are superimposed as supplementary variables, namely physicochemical parameters, contamination in sediments and organisms.

2.3.3. Sediment data: Ria de Aveiro versus Óbidos lagoon

Results from PCO analysis (Fig. 2.23) were based on physicochemical and contamination sediment values and the superimposed vectors were physicochemical and contamination sediment values. This analysis revealed that in the first principal component (PCO1), which accounted for 53.1% of the total variability, it was possible to separate areas D and E from the Óbidos lagoon and areas M, K, L and F from the Ria de Aveiro present on positive side of the axis and the remaining areas of both ecosystems on the negative side of the axis. The PCO axis 2 represents 14.4% of the total variability, separating the majority of the Ria de Aveiro areas (on the positive side of the axis) and Óbidos lagoon areas (on the negative side of the axis). It was possible to distinguish two groups according to contamination and abiotic factors: the first group corresponded to all areas from Ria de Aveiro and three areas from the Óbidos lagoon (A, B, C) and the second group corresponded to areas D and E from the Óbidos lagoon. Fines percentage, Ni and Cr contamination were the factors that characterized areas D and E.

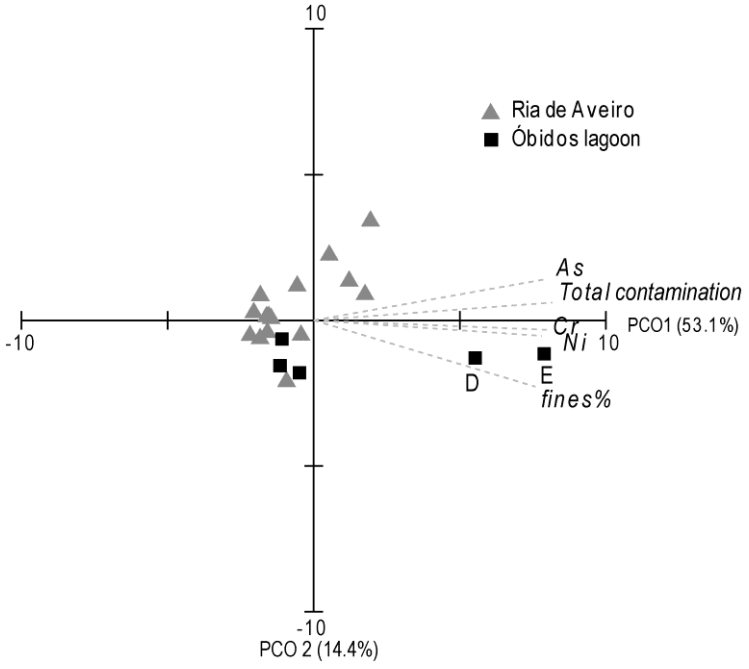

Figure 2.23 - Centroid ordination diagram (PCO). The matrix was based on physicochemical sediment parameters and sediment contamination and spearman correlation vectors are superimposed as supplementary variables, namely physicochemical sediment parameters and sediment contamination ($r > 0.75$). ▲ - represent areas from the Ria de Aveiro (A-O) and ■ - represent areas from the Óbidos lagoon (A-E).

2.4. Discussion

Coastal lagoons are generally shallow, and often characterized by restricted water exchange with the open sea (Carvalho et al., 2011), forming complex natural habitats situated between the fresh water coastal ecosystems and the subtidal zone, with high biodiversity (Sobrino et al., 2014). However, these environments can often be impacted by the effects of numerous sources of contamination, such as metals and As (e.g. Alkarkhi et al., 2008; Beltrame et al., 2009; Kucuksezgin et al., 2010). Several authors have studied marine ecosystems with high levels of contamination due to the threat that they represent to biota (Di Leonardo et al., 2014; Maanan et al., 2015). However, ecosystems with low and moderate levels of contamination have been poorly studied (e.g. Buruaem et al., 2012; Jena et al., 2009), being scarce the information on the performance of different species coexisting in the same areas. Therefore, the present study will contribute with relevant information relative to the impact of metals and As on the clams *R. decussatus* and *R. philippinarum*, two species with high ecological and economic role living in sympatry in the Ria de Aveiro and the Óbidos lagoon.

2.4.1. Relative abundance and density

Results showed that in the Ria de Aveiro, the introduced species presented a wider spatial distribution on this lagoon than the native one. Along the sampling areas, the introduced species appeared associated with native species in seven of the nine areas but when coexisting, *R. decussatus* presented just a higher density than introduced one in three of seven areas. According to Maia and Gaspar (2014), the biomass and abundance of *R. decussatus* declined 67 and 76.6%, respectively, since 2006/2007 up to 2013, while *R. philippinarum* became the second species most commercialized in the Ria de Aveiro after 2 years of their introduction. Since 2013 until nowadays there are no studies that provide more information on the status of *R. decussatus* and *R. philippinarum* stocks in the Ria de Aveiro.

Regarding the Óbidos lagoon, the present results revealed that the native species presented a wider distribution than the introduced one. When coexisting in the same area, the introduced species presented low density and abundance when compared to the native one. Present results also revealed that this is the first report on the presence of *R. philippinarum* at the Óbidos lagoon.

Taking into account the comparison of both ecosystems was possible revealed that *R. decussatus* and *R. philippinarum* from the Ria de Aveiro presented lower density than both species from the Óbidos lagoon. The present results from both marine ecosystems further suggested that the introduced species has not yet supplanted the native one. However, there is an urgent need to

develop new conservation and management strategies for the native species from the Ria de Aveiro to avoid the overfishing on natural banks, severe clam mortalities, and recruitment failures (Matias et al., 2013). According to several authors, the conservation of *R. decussatus* is an important priority, since the reduction of this species can consequently pose major threats to the biodiversity of this ecosystem, affecting the provision of goods and services (Bidegain et al., 2013; 2015; Juanes et al., 2012). Maia and Gaspar (2014) suggested the creation of a fishing exclusion zone in the Ria de Aveiro and artificial restocking of this species as an alternative way to manage *R. decussatus* stocks, taking into account the slow growth of this species and its poor resilience to unfavorable environmental conditions when compared to *R. philippinarum* (Usero et al., 1997). Despite high abundance of native species in the Óbidos lagoon, the introduction of *R. philippinarum* may pose an unbalance between both co-existing species. Therefore, it is of most importance to implement a responsible monitoring program in order to provide accurate information in the Óbidos lagoon that can help maintaining the conservation and the stock management of these commercially exploited resources.

The co-existence of native and introduced species was also reported by Juanes et al. (2012) in the Bay of Santander (Spain). Recently, for the same Bay, Bidegain and Juanes, (2013) and Bidegain et al. (2015) confirmed that *R. philippinarum* cannot be defined as an ecological threat to *R. decussatus*. However, in other European ecosystems (Arcachon Bay, France and the Lagoon of Venice, Italy), *R. philippinarum* supplanted *R. decussatus*, being considered an invasive species (Blanchet et al., 2004; Dang et al., 2010).

2.4.2. Environmental and sediment data

2.4.2.1. Physicochemical characteristics and total metal(loid)s concentrations

The Ria de Aveiro and the Óbidos lagoon have long been subjected to high anthropogenic pressure (Coelho et al., 2014; Oliveira et al., 2006; Pereira et al., 2008a,b). It is known that multiple environmental factors (pH, salinity and temperature), sediment type and TOM content may affect the concentration of elements present in sediments from marine ecosystems, their bioavailability and consequently their toxicity (Allen and Janssen, 2006; Duarte et al., 2011; Eggleton and Thomas, 2004). Physicochemical parameters showed that the Ria de Aveiro presented salinity and temperature ranges lower than the Óbidos lagoon (Table 2.6). However, the Óbidos lagoon showed the lowest pH changes among areas when compared with the Ria de Aveiro (Table 2.6).

For the Ria de Aveiro, the results obtained also revealed that areas with the lowest contamination levels presented the lowest percentage of TOM and fine particles, while areas with

the highest contamination levels were associated with high fines and TOM content (Table 2.6). Similar results were obtained in the Óbidos lagoon, allowing to identify two distinct regions in this system: sediments from the Bom Sucesso arm, characterized by high TOM content, high percentage of fine particles and high contamination levels; and sediments from the central part of the lagoon with low TOM content, low percentage of fine particles and low contamination levels (Table 2.6). In this way, the present study supported the idea that the highest contamination levels recorded in areas of Ria de Aveiro (E, G, F, K, and M) and Óbidos lagoon (Bom Sucesso arm) is related to elements affinity to TOM and fine particles. In accordance with present findings, other studies have shown that the finest particle fraction of sediments tends to retain a higher proportion of metals (Selim and Amacher, 1996; Tarradellas et al., 1996), since fine particles have a higher surface area allowing a greater adsorption than in larger particles (Eggleton and Thomas, 2004; Yu et al., 2012). Company et al. (2011) also showed the capacity of the fine sediments to retain metal contaminants. Furthermore, it is well accepted that natural organic matter, such as humic acid and fulvic acid, has a strong binding affinity for trace metals in the aquatic environment (Zhong and Wang, 2009).

Taking into account the comparison of total sediments metal(loid)s concentrations in the Ria de Aveiro and the Óbidos lagoon, present results revealed that sediments from the Ria de Aveiro (4.3-49.7 mg/kg DW) showed 2 times lower total metal(loid)s concentrations than sediments from the Óbidos lagoon (9.7-98.0 mg/kg DW) (Table 2.6), being considered Óbidos lagoon the most polluted one. However, the contamination levels in both marine ecosystems presented contamination values similar or even lower values than sediment concentrations found in other marine ecosystems, such as Venice lagoon, Italy (48-280 mg/kg DW, Moschino et al., 2012), Augusta Bay, Italy (31-116 mg/kg DW, Di Leonardo et al., 2014) and Santander Bay, Spain (205-488 mg/kg DW, Viguri et al., 2007), Gulf and Gulf of Oman, Bahrain, Oman, Qatar, and the United Arab Emirates (14.0-2335.5 mg/kg DW, Mora et al., 2004).

Relating the density of both species, the physicochemical parameters and metal(loid)s concentrations in areas where both species were collected, the present study revealed that the density of *R. decussatus* in the Ria de Aveiro and the Óbidos lagoon was not dependent on environmental conditions and metal(loid)s concentrations in sediments. However, the density of *R. philippinarum* from the Ria de Aveiro can depend on the contamination and/or water temperature. Present results further revealed that the density of *R. philippinarum* from the Óbidos lagoon may be dependent on salinity and temperature of seawater. Juanes et al. (2012) showed that *R. decussatus* and *R. philippinarum* from the Santander Bay (Spain) presented high abundance in areas with high freshwater influence and low levels of pollutants.

Table 2.6 – Physicochemical characteristics (salinity, pH, temperature (ºC), total organic matter (TOM) and fine particles content), sediments metal(loid)s contamination in the Ria de Aveiro and the Óbidos lagoon.

	Ria de Aveiro	Óbidos lagoon
Salinity	28.00-40.00	21.41-38.30
pH	6.82-8.53	7.30-7.87
Temperature	19.3-22.6 ºC	22.0-26.0 ºC
TOM	↑ in the most contaminated areas ↓in the least contaminated areas	↑ in the most contaminated areas ↓in the least contaminated areas
Fine particles content	↑ in the most contaminated areas ↓in the least contaminated areas	↑ in the most contaminated areas ↓in the least contaminated areas
Range of total elements concentrations (mg/kg DW)	4.31-49.73	7.10 – 98.01

2.4.2.2. Concentration of each element

Regarding the concentration of each element, the present study showed that Cr and Pb were the elements with the highest concentrations in sediments from the Ria de Aveiro and the Óbidos lagoon (Table 2.7). It was possible also to observe high As concentrations in sediments from the Ria de Aveiro and high Cu concentrations in sediments from the Óbidos lagoon (Table 2.7). Moreover, in both ecosystems, Cd and Hg were the elements with the least concentrations in sediments. Although both ecosystems presented similar tendency for the elements with the highest and the lowest concentrations, it was also possible demonstrated that the Óbidos lagoon presented higher As (9.36 mg/kg DW), Cr (29 mg/kg DW) and Ni (15.5 mg/kg DW) concentrations than the same elements in the Ria de Aveiro (6.14, 16, 8.61 mg/kg DW, respectively) (Table 2.7).

In the present study, the concentration of each element found in sediments from the Ria de Aveiro was below of the metal(loid)s concentrations previous reported by others authors for the same systems (Martins et al., 2011; Monterroso et al., 2003; Monterroso et al., 2007). However, it is not possible to assess the recovery capacity of this ecosystem since Martins et al. (2013) reported higher metal(loid)s concentrations than concentration reported in the present study. Due to these, the sediments metal(loid)s concentrations need to be monitored to provide more information about the ecosystem health.

Previous studies conducted in the Óbidos lagoon reported that this ecosystem presents a moderate contamination by metals (Carvalho et al., 2006; Carvalho et al., 2011; Pereira et al., 2008b). However, when comparing the present data with previous studies in the Óbidos lagoon, it is possible to conclude that the metal(loid)s contamination found in this ecosystem in 2006 was higher than concentrations found in the present data (Carvalho et al., 2006; Carvalho et al., 2011; Pereira et al., 2008b). These results suggested that the metal(loid)s contamination is decreasing,

possibly due to ecosystems recovery, the increase of water renewal and/or due to the maintenance of sediment dredging. Due to these factors, new studies need to be carried out in order to assess the health of this ecosystem.

Taking into account the both set of Saltwater Sediment quality guidelines (TEL/PEL; ERL/ERM) and the concentration of each element in sediments from the Ria de Aveiro and the Lagos de Óbidos, it is possible to reveal that sediments from both systems presented low risk to aquatic life, except for Cu and Hg in the Ria de Aveiro and for As and Cu in the Óbidos lagoon (Table 2.7). Regarding Cu and Hg sediment concentrations in the Ria de Aveiro and for As and Cu in the Óbidos lagoon, the sediments are classified as slightly to moderately contaminated and additional testing is required to evaluate the potential risks to aquatic life, since above PEL and ERL values adverse effects on biota are more likely to occur.

Table 2.7 – The elements with the highest and the lowest concentrations and elements that research TEL and ERL values in sediments of the Ria de Aveiro and the Óbidos lagoon.

	Ria de Aveiro	Óbidos lagoon
The elements with the highest concentrations	Cr, Pb, As	Cr, Pb, Cu
The elements with the lowest concentrations	Cd, Hg	Cd, Hg
Elements that reached TEL	Cu, Hg	As, Cu
Elements that reached ERL	-	As

When comparing the concentration of each element, present in the Ria de Aveiro and the Óbidos lagoon with other marine ecosystems, results revealed that the concentrations of each element in the present data was lower than values found in several marine ecosystems around the world (Cheggour et al., 2005; Hatje et al., 2010; Mora et al., 2004; Moschino et al., 2012; Ramos-Gómez et al., 2011; Sfriso et al., 2008; Trocine and Trefry, 1996).

2.4.3. Organisms metal(loid)s concentrations

2.4.3.1. Total metal(loid)s concentration

Results revealed that, in general, when in the same area, the native and introduced species from the Ria de Aveiro and the Óbidos lagoon tend to present similar total metal(loid)s concentrations. In both marine ecosystems, *R. decussatus* and *R. philippinarum* presented high total metal(loid)s concentrations in areas with the lowest and highest levels of sediment contamination. This tendency was more pronounced in the Óbidos lagoon, indicating that *R. decussatus* and *R. philippinarum* may be considered a sentinel species. When comparing both species in both ecosystems results revealed lower metal(loid)s concentrations in *R. decussatus* from the Ria de Aveiro than in *R. decussatus* from the Óbidos lagoon, while an opposite tendency was observed in the introduced species (Table 2.8).

The present study further revealed that *R. decussatus* and *R. philippinarum* from the Ria de Aveiro and the Óbidos lagoon presented total metal(loid)s concentrations similar or even lower when compared to values obtained for marine bivalves, by other authors, in other marine ecosystems (Mora et al., 2004; Sfriso et al., 2008, Trefry and Trocine, 2011).

Relatively to the BSAF values, the present results demonstrated that, in general, both species collected in areas with the least contaminated areas from the Ria de Aveiro and the Óbidos lagoon tend to bioaccumulate higher proportion of elements (BSAF>1) than both organisms from the most contaminated areas (BSAF<1). These results may be explained by the influence of TOM and fines particles content in the bioavailability of metal(loid)s concentrations in contaminated and non-contaminated areas, limiting or not their accumulation in both species. Previous studies identified the organic matter and fine particles content as two of the major factors that influence metals uptake, being responsible for their reduced mobility and toxicity (Eggleton and Thomas, 2004; Hyun et al., 2006; Yu et al., 2012). Recent studies conducted by Freitas et al. (2012) and Figueira et al. (2011) also found, for *Cerastoderma edule*, higher BSAF values at the least contaminated areas.

2.4.3.2. Concentration of each element

Regarding the concentration of each element in *R. decussatus* and *R. philippinarum* from the Ria de Aveiro and the Óbidos lagoon, results revealed that both species tend to accumulate similar concentrations of the same element. These results may, therefore, indicate that metal(loid)s tolerance/accumulation probably will not be the mechanisms responsible for the replacement of native species.

The present study further revealed that As, Ni and Cu were the elements with the highest concentrations in both species from the Ria de Aveiro (Table 2.8). The BSAF values of these elements were higher in *R. decussatus* and *R. philippinarum* from areas with the lowest contamination values than in both species from areas with the highest contamination. In both species from the Óbidos lagoon As and Cr were the elements with the highest concentrations. The BSAF values of these elements were, as in the Ria de Aveiro results, higher in *R. decussatus* and *R. philippinarum* from areas with the lowest contamination values than in both species from areas with the highest contamination levels. The native and introduced species from the most areas of both marine ecosystems presented Hg and Cd as the elements with the lowest concentrations (Table 2.8). Even at low concentrations, MeHg results showed a decrease of MeHg concentrations in both species from the Ria de Aveiro according to the distance of historically contaminated source. Concerning the most abundant elements in sediments from the Ria de Aveiro and the Óbidos lagoon, the present results suggest differences in bioavailability of each element, since the most abundant elements in sediments (Cr and Pb, Ria de Aveiro and Pb and Cu, Óbidos lagoon) were not the most abundant elements in the *R. decussatus* and *R. philippinarum* (Ni and Cu, Ria de Aveiro; As, Óbidos lagoon). In accordance with present findings, other authors found low As concentrations in sediments from Gulf and Gulf of Oman, while the bivalves, *Pinctada radiate*, *Crassostrea virginica*, *Saccostrea cucullata*, *Mytilus edulis*, presented high concentrations of this metalloid in tissues (Mora et al., 2004). Other studies reported similar results with higher Cd concentrations in scallops from clean sites compared to the contaminated ones (Uthe and Chou, 1987; Bustamante and Miramand, 2005).

When comparing the concentrations of each element in *R. decussatus* and *R. philippinarum* from the Ria de Aveiro and the Óbidos lagoon, the results obtained showed that both species from the Ria de Aveiro presented lower Cr, Ni and Pb concentrations than same species from the Óbidos lagoon and an opposite tendency was observed for As and Cu. According to Yang et al. (2013), *R. philippinarum* from China presented similar or high values of Cr, Ni, Cu and Pb (0.94-4.75, 4.76-14.32, 14.0-38.0 and 0.32-2.59 mg/kg DW) when compared with the present study. Similar results for As, Cr, Ni, Pb, and Cu were previously reported by Usero et al. (1997), in *R. philippinarum* from the Atlantic coast, Spain (18.9-64.0, 1.9-5.7, 0.15-8.7, 0.5-2.6 and 8.2-29.0 mg/kg DW).

Table 2.8 - Total metal(loid)s concentrations, Biota-Sediment Accumulation factor (BSAF), the highest and the lowest concentration of elements and elements concentration that reached maximum permissible limits (MLs) in *Ruditapes decussatus* and *R. philippinarum* from both Ria de Aveiro and Óbidos lagoon.

	Ria de Aveiro		Óbidos lagoon	
	R. decussatus	*R. philippinarum*	*R. decussatus*	*R. philippinarum*
Range of total metal(loid)s concentrations (mg/Kg)	20.17-48.13	17.72-84.67	32.62-56.69	31.25-52.53
BSAF of total metal(loid)s >1	Low contaminated areas	Low contaminated areas	Low contaminated areas	Low contaminated areas
BSAF of total metal(loid)s <1	High contaminated areas	High contaminated areas	High contaminated areas	High contaminated areas
The elements with the highest concentrations	As, Ni, Cu	As, Ni, Cu	As, Cr	As, Cr
The elements with the lowest concentrations	Cd, Hg	Cd, Hg	Cd, Hg	Cd, Hg
Elements concentration that reached MLs (EFSA/USFDA)	-	-	-	-

2.4.4. Subcellular partitioning

The toxicity of an element is not only dependent on the total amount accumulated but on its partition as well (Figueira et al., 2013). When in the organism, elements may be allocated in the soluble and insoluble fractions (Wallace et al., 2003). While the soluble fraction provides information about the metal(loid)s trophically available, the absorption of the insoluble fraction is dependent on the digestive capacity of consumers (Metian et al., 2009; Rainbow and Smith, 2010). Thus, bivalve species that have a higher proportion of elements in solution potentially constitute a higher risk to consumers than species accumulating most of the element burden in insoluble form. In *R. decussatus* and *R. philippinarum* from the Ria de Aveiro and the Óbidos lagoon, except for As, most of the elements were not trophically available, being present in the insoluble fraction. These results suggested that most of the elements should be bound to metal-rich granules, cellular debris, and organelles (Geffard et al., 2010; Wallace and Luoma, 2003).

Present results are in agreement with findings from several authors (Figueira et al., 2011; Pellerin and Amiard, 2009; Rainbow and Smith, 2010) which demonstrated that marine bivalves accumulated predominantly higher concentrations of elements in the insoluble fraction than at the soluble fraction, being these metals less available for predators and less toxic to organisms. However, the present study also revealed that both species from all areas accumulated the majority of the As content in the soluble fraction, suggesting that this element is in the free form in the cytosol and consequently more trophically available for predators, which is in agreement with a

previous study reported by Velez et al. (2014). Also, Freitas et al. (2012) reported that in the *C. edule* most of the Cd and As was found in the soluble fraction, showing that these elements (Cd and As) are more trophically available than other elements.

2.4.5. Human health risks from clams consumption

The present work demonstrated that both species from the Ria de Aveiro and the Óbidos lagoon had elements concentrations below the MLs defined by international organizations (EFSA and USFDA). However, taking into account the USFDA, inorganic As is approximately 10% of total As. When applied this ratio to the present data for total As concentrations in both ecosystems, it is possible to estimate that only inorganic As concentration in both species from the Ria de Aveiro (0.2-1.4 mg/Kg) were above the MLs for inorganic As established by FSANZ (1 mg/Kg). These results showed that even in areas with the lowest contamination levels the MLs for some elements can easily be achieved, prohibiting marketing and invalidating clams harvesting and culture for commercial purposes in these areas. These findings reveal that clams consumption can constitute a health risk even when these bivalves are collected in areas from the Ria de Aveiro.

For As, the PTWI can be exceeded when more than 0.80 (inorganic arsenic estimation) kg of *R. philippinarum* from the Ria de Aveiro is consumed per week by an adult (70 Kg) (Table 2.9). Furthermore, when comparing both species from the Ria de Aveiro, *R. philippinarum* seems to present a higher risk to human consumption than *R. decussatus* since for most of the elements a lower amount of this species is sufficient to exceed the PTWI. This may be related to higher capacity shown by this species to remove particles from seawater when comparing to the native one (Gosling, 2015; Nagasoe et al., 2011). Nevetheless, the present results must be taken into consideration since, in general, coastal population regularly consumes large amounts of bivalves. In Portugal around 58 kg/cap/year of seafood is consumed, being the clams among the most consumed species (DGRM, 2014; INE, 2013; Willemsen, 2003). Comparing the present results with the coast of China, consumers need to eat similar or less amount (0.13 kg and 0.20 kg, respectively) of clams from the coast of China in one week to exceed PTWI for As (Yang et al., 2013). It is important to highlight that, although in the present study the As values measured in clams are total and the PTWI in FSANZ and JECFA standards are for inorganic As, according to the National Status and Trends (NST) Mussel Watch Project oyster contamination is considered ‘high’ when total arsenic levels are above 14.5 mg/kg DW (approx. 2.15 mg/kg wet weight) (Valette-Silver et al., 1999). If the same level are considered for clams, both species from the Ria de Aveiro are also highly contaminated with As.

Table 2.9 – The minimum and the maximum amount of both species from the Ria de Aveiro and the Óbidos lagoon that an adult with 70 kg needs to consume to exceed PTWI. Highlighted values are the lowest values needed to exceed the PTWI. - correspond to not detected concentration (lower than the detection limit). # inorganic As estimation.

	Cu	As	Cd	Pb	Hg	Ni	Cr
Amount of clams consumed per week to exceed PTWI (Kg)							
Ria de Aveiro	137.6-444.1	0.8-6.5[#]	5.2-10.9	4.3-10.9	9.0-27.6	2.1-6.1	-
Óbidos lagoon	133.8- 453-7	6.9-9.5[#]	-	6.0-8.3	-	1.6-5.4	-

2.4.6. Biomarkers

The accumulation of elements in organisms may induce biochemical changes, being the toxicity of each element often dependent on the capacity of each species to repair the damage caused (Bergayou et al., 2009; Jena et al., 2009; Zhang et al., 2010). In fact, depending on the capability of each species, the biochemical processes that lead to the induction of some enzymes may be activated, such as antioxidant enzymes (SOD, CAT) and biotransformation (GSTs) enzymes, preventing the damages caused by oxidative stress (Alves de Almeida et al., 2007; Regoli and Giuliani, 2014).

In general, results from the Ria de Aveiro revealed that *R. decussatus* from areas N, E, K and M, (N - low; E- medium; K and M high levels of contamination in sediments) and *R. philippinarum* from areas N, E, K presented higher and similar levels of PROT, LPO, and antioxidant enzymes than both species from the remaining areas (O, B – low; C- medium; F high – levels of contamination in sediments). These results suggested that both species from areas N, E and K presented an induction of detoxification mechanisms against ROS production to prevent the damages. However, these antioxidant mechanisms were not sufficient to prevent damages in membrane lipids, leading to an increase of LPO levels in both species from areas N, E, and K. On the other hand in remaining areas (O, B – low; C- medium; F high – metal(loid)s contamination in sediments) probably both species were able to regulate the production/elimination of ROS, avoiding an increase in LPO levels. Present results also suggest that possibly, in general, both species from all areas were not responding specifically to total metal(loid)s contamination in sediments and the majority of elements present in tissues may not induce oxidative stress. But both of these species may have been responding to combined effects of contaminants and environmental factors. Other works also demonstrated that it is possible to find another types of contaminants in the Ria de Aveiro, such as polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), hexachlorobenzene (HCB), polycyclic aromatic hydrocarbon (PAH), pharmaceutical drugs (carbamazepine) (Antunes and Gil, 2004; Calisto et al.,

2011; Grilo et al., 2013; Oliveira et al., 2009), and possible these contaminants may also contribute to the biochemical performance obtained in the present study in both species. Additionally, results from the Ria de Aveiro did not demonstrate a clear response to the GSTs activity to contaminations levels in sediments, suggesting that these mechanisms were able to regulate the ROS production and elimination by forming conjugates with reduced glutathione (Wang et al., 2012). However, other authors showed a clear GSTs response since in *Perna viridis*, *C. farreri*, and *R. philippinarum* higher GSTs levels were found in organisms from polluted areas than in organisms from the non-polluted areas (Jena et al., 2009; Wang et al., 2012; Zhang et al., 2010).

Regarding the biochemical results for *R. decussatus* in the Óbidos lagoon, it was possible to observe that in the least contaminated areas this species presented higher LPO levels when compared with remains areas, while the detoxification mechanisms were similar among areas (except area E). These results suggested that possibly several environmental characteristics (namely salinity that was significantly lower in area A) may act as confounding factors and may induce biochemical alterations in specific areas masking the effects caused by metals and As contamination. Similar results were observed for *R. philippinarum*. When comparing species at each area, the biochemical responses were similar, which could be explained by the fact that the majority of elements present in the organisms was allocated to the insoluble fraction, i.e., in a precipitated form or bound to membranes (Wallace and Luoma. 2003; Wallace et al., 2003) and, therefore, inducing low damage to the cells. Similar findings were reported in *C. edule* from the Ria de Aveiro by Freitas et al. (2012). Also, Torres et al. (2002) showed that the activity of SOD was not induced in the mussel *Mytella guya* nensis from polluted areas. However, the induction of GSTs and SOD activity was clearly reported by Fernández et al. (2010) in *M. galloprovincialis* from metal-polluted sites. Also, Wang et al. (2012) reported that *R. philippinarum* transplanted to polluted areas in Maluan Bay (China) presented an increase of SOD, CAT, and GSTs activity, suggesting the presence of redox active chemical compounds such as metals. Jena et al. (2009) reported that higher LPO, SOD, CAT, and GSTs levels in *P. viridis* from polluted areas than in organism from areas with low pollution levels.

In general, since the metal(loid)s concentrations in sediments and accumulation in organisms appear not to be enough to increase the oxidative stress in native and introduced species from the Ria de Aveiro and the Óbidos lagoon, it was difficult to identify a relevant set of biomarkers useful to relate the sediment and/or organisms contamination in the present study. In this way, the used of *R. decussatus* and *R. philippinarum* as a bioindicator species should be furtherly investigated. Furthermore, since the presence of other types of contaminants in both marine ecosystems, the low contamination levels, and the environmental conditions that may act

as a confounding factor in the study areas, it is difficult to assume that any of the biomarkers used was responding to contamination.

When comparing *R. decussatus* and *R. philippinarum* from the Ria de Aveiro and the Óbidos lagoon, in general, results revealed higher levels of antioxidant enzymes (SOD and CAT) in both species from the Ria de Aveiro than both species from the Óbidos lagoon (Table 2.9). On the other hand, both species from the Óbidos lagoon presented higher PROT and LPO values than both species from the Ria de Aveiro (Table 2.10).

Table 2.10 – Biomarkers values (min and max) in *Ruditapes decussatus* and *R. philippinarum* from the Ria de Aveiro and the Óbidos lagoon. Highlighted values represent the highest content of protein (PROT), lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT) and glutathione *S*-transferase (GSTs) in both species from the Ria de Aveiro and the Óbidos lagoon.

	Ria de Aveiro		Óbidos lagoon	
	R. decussatus	*R. philippinarum*	*R. decussatus*	*R. philippinarum*
PROT (mg/g FW)	15.3-38.2	13.6-36.8	21.1-32.5	21.1-28.6
LPO (nmol/g FW)	12.8-32.2	12.1-27.4	21.8-48.4	14.9-31.7
SOD (U/g FW)	3.5-6.2	3.1-5.4	1.4-3.2	1.7-4.2
CAT (U/g FW)	15.7-34.1	15.1-33.8	4.0-5.2	4.4-4.6
GSTs (U/g FW)	0.2-0.4	0.2-0.3	0.1-0.3	0.3-0.5

The present results suggest a specific adaptation of each species to the environmental conditions. However, further studies should be conducted to investigate if the introduced species was able to change the biochemical performance when introduced in these marine ecosystems or, on the other hand, if the characteristics of the introduced species are similar to the ones found in other systems. Pellerin and Amiard (2009) demonstrated that *M. edulis* and *M. arenaria* present in the same areas showed different Cd detoxification/storage mechanisms and different induction of metallothioneins mechanisms. According to Bergayou et al. (2009), different CAT activity and LPO levels were also observed in *Scrobicularia plana* and *C. edule* from the same areas in Oued Souss estuary (Morocco), being the induction of CAT higher in *C. edule*, while higher LPO levels were observed in *S. plana*.

2.5. Conclusion

Overall, the present study revealed that *R. decussatus* and *R. philippinarum* co-exist in both marine ecosystems and the introduced species has not yet supplanted the native species. However, there is an important need to monitor the native clam *R. decussatus* and the introduced species *R. philippinarum* in the Ria de Aveiro and the Óbidos lagoon to prevent the replacement of native species by introduced ones, not only due to the ecological value but also due to the socio-economic importance of *R. decussatus*. Since clams are amongst the most relevant bivalve species for Portuguese consumers (Willemsen. 2003; INE. 2013; DGRM. 2014). Due to that additional studies about the distribution of both species are strongly suggested to better understand the coexistence of these species, and to predict the replacement of the native species.

The Ria de Aveiro presented lower levels of contamination when compared to Óbidos lagoon. The results further demonstrated that, in general, both species from the same area in the Ria de Aveiro and the Óbidos lagoon presented similar metal(loid)s concentrations, similar cellular metal(loid) partition, and similar biochemical performance, independently of the contamination level of each area. In general, both species from both ecosystems accumulated higher metal(loid)s concentrations in the least contaminated areas (BSAF > 1) than in the most contaminated areas. According to the present results, it is also suggested additional studies concerning the bioavailability of each element in the sediments and its speciation to better understand the element accumulation and its partition in each species.

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Chapter 3

Organisms' response to single stress

This chapter is based on the following manuscripts:

Velez, C., Freitas R., Antunes S.C., Soares A.M.V.M., Figueira E., 2016. Clams sensitivity towards As and Hg: A comprehensive assessment of native and exotic species. Ecotoxicology And Environmental Safety. 125, 43-54.

Velez, C., Figueira E., Soares, A. M.V.M, Freitas R., 2017. Effects of seawater temperature increase on economically relevant native and introduced clam species. Marine Environmental Research.123, 62-70.

Velez, C., Figueira E., Soares, A. M.V.M, Freitas R., 2016. Native and introduced clams biochemical responses to salinity and pH changes. Science of the Total Environment. 566-567, 260-268.

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3.1. Introduction

Marine systems are exposed to a large amount of contaminants from natural and anthropogenic sources, including erosion, volcanic eruptions, industry, agriculture, urbanization, and sewage discharges (Bergayou et al., 2009; Moschino et al., 2012; Spada et al., 2012). Among contaminants, metals and metalloids, even at low concentration, may contribute to estuaries deterioration affecting the organisms inhabiting these areas (among others, Coelho et al., 2006; El-Nemr et al., 2012). These compounds, especially those not required for metabolic activity and presenting toxicity, such as arsenic (As), can be accumulated by aquatic species (e.g. Ventura-Lima et al., 2009 and Ventura-Lima et al., 2011). Arsenic is an ubiquitously distributed element, identified in different aquatic systems (e.g Freitas et al., 2014; Hédouin et al., 2009; Mamindy-Pajany et al., 2013). Although As is released into the environment from natural sources, the major contributors to environmental As contamination are from anthropogenic activities (Bissen and Frimmel, 2003; Smedley and Kinniburgh, 2002). This metalloid is one of the most toxic elements in the environment being extremely harmful when in its inorganic form (Akter et al., 2005). The bioaccumulation and toxicity of As have been the focus of research, revealing impacts of this metalloid on marine and estuarine organisms (Fattorini et al., 2004) and implications for human health (Kapaj et al., 2006). Arsenic toxicity in different marine species, namely bivalves, can negatively affect their metabolic activity, oxidative status, cellular integrity leading to apoptosis (Ahmad et al., 2013; Chen, 2014; Rajkumar and Rajkumar, 2013; Wu et al., 2013). In fact, different bivalve species have been used as sentinel species to assess and monitor As contamination, due to their sessile nature, filter-feeding capacity, wide distribution and high abundance, bioaccumulation capacity and ecological and economic role in several aquatic systems (Kucuksezgin et al., 2010; Wang et al., 2012).

Besides metal(loid)s contamination, anthropogenic activities also release greenhouse gas (namely CO_2, a heat-trapping gas) into the atmosphere, which is the main reason why the climate is changing (IPCC, 2014). Climate change are a worldwide problem and have been studied by several authors during the last years (among others; Berge et al., 2006; Dickson et al., 2012; Parker et al., 2013; Schiedek et al., 2007). According to the International Panel on Climate Change (IPCC, 2007; 2014), the consequences of climate change may occur at several levels at the end of the 21st century, leading to global warming, extreme weather events, and ocean acidification.

Due to global warming, oceans and coastal systems (e.g. lagoons, estuaries) will absorb more heat and seawater mean temperatures will increase (EPA, 2016; IPCC, 2007; 2014). Changes in seawater temperature can alter marine ecosystems in different ways (EPA, 2016). Among marine environments, coastal areas may be especially affected by this phenomenon, possibly leading to changes in ecosystems structure and function, including loss of breeding areas and aquatic biodiversity (Eissa and Zaki, 2011). Due to that, several authors have studied the responses of marine organisms under temperature changes, showing that variation in seawater

temperature may alter their tolerance limits, acclimation capacity and biological performance (Eissa and Zaki, 2011; EPA 2016). In the specific case of marine bivalves, temperature is one of the limiting factors that may reduce their geographic distribution (Verdelhos et al., 2015). Recent studies reported that temperature changes may influence internal biological processes, reproduction patterns, growth and population abundance (e.g. Pörtner, 2007; Santos et al., 2011; Verdelhos et al., 2011; 2015).

It is known that global warming may also contribute to the occurrence of extreme weather events, leading to salinity shifts in the ocean and coastal areas (Coughlan et al., 2009), which may affect marine biota (Hamer et al., 2008). Recently, different authors used marine bivalves to assess the impacts of salinity changes, namely on their metabolic, physiological and biochemical performance (Carregosa et al., 2014; Coughlan et al., 2009; Sarà et al., 2008).

In marine ecosystems, in addition to temperature and salinity fluctuations, with the increase of atmospheric CO_2, oceans have absorbed almost half of the CO_2 emissions produced from burning fossil fuels and cement manufacture, leading to changes in the carbon cycle and consequently ocean acidification (Branch et al., 2013; Canadell et al., 2007; Duarte et al., 2013 Eriander et al., 2016; Hofmann et al., 2011). The decrease of pH expected by the end of the century (0.3 to 0.5 units) may affect different organisms, namely bivalves (Berge et al., 2006; Duarte et al., 2014; Dickinson et al., 2012; Kleypas et al., 2006; Orr et al., 2009). Studies assessing the impacts of ocean acidification on the biochemical performance of bivalves are scarce, but recent studies have demonstrated that pH decrease induced oxidative status in oysters (Moreira et al., 2016a; Tomanek et al., 2011), clams (Freitas et al., 2016; Matozzo et al., 2013) and mussels (Matozzo et al., 2013).

Most of the marine organisms are able to survive a broad range of abiotic factors, although the tolerance of each species is species-specific and depends on acclimation capacity (Compton et al., 2007; Gosling, 2004; Schiedek et al., 2007). However, it is known that climate change and high tolerance to contaminants may also facilitate the spread of invasive species, creating new habitats with suitable conditions for introduced species that can become invasive (FAO, 2016; Otero et al., 2013; Stylianos et a., 2014; Sorte et al., 2013). According to Sorte et al. (2013), non-native species living in aquatic systems have a strong performance advantage associated with increases in temperatures and CO_2 levels. In addition, studies conducted by Byers et al. (2014) support the idea that climate change can influence the interaction between native oyster *Crassostrea virginica* and invasive crab *Petrolisthes armatus*, especially competitive and predatory effects over the native ones. Recently, Bielen et al. (2016) demonstrated that the invasive clam *Sinanodonta woodiana*, presented higher tolerance to warm water temperature than the native *Anodonta anatine*, contributing to the replacement of the native species. Nevertheless, few studies have assessed and compared the performance of native and introduced bivalves species under the predicted temperature increase, salinity shifts, pH decrease and As contamination (Allen and Horn, 2006; Carregosa et al., 2014.; Dukes and Mooney, 1999; Gestoso et al., 2016; Hamer et al., 2008; Pfeifer

et al., 2005; Matozzo et al., 2013; Miththapala, 2013). However, there is still an urgent need to investigate how these factors will affect native and introduced species. Among the most widespread marine invasive/introduced bivalve species is the clam *Ruditapes philippinarum* (Adams and Reeve, 1850) (FAO, 2016). It is known that this species has successfully occupied several areas (Otero et al., 2013), cohabiting in some areas with close related species, namely *R. decussatus* and *Venerupis corrugata,* namely in the Ria de Aveiro (chapter 2) or replacing the native species (Venice lagoon, Otero et al., 2013). The success of *R. philippinarum* has been associated with its faster growth (FAO, 2016), immune response to bacteria (Moreira et al., 2012) and greater reproductive ability than the native species, namely *R. decussatus* (Delgado et al., 2007).

Thus, the objectives of the present study were to assess and to compare the native clam, *R. decussatus*, and the introduced clam, *R. philippinarum* induced responses, when exposed to single stressors, namely As contamination, temperature, salinity and pH changes (Fig. 3.1.1). For both species, different conditions were evaluated:

- **Arsenic:** acute exposure (96 hours) to a range of As concentrations (0, 4, 8.2, 17, 34 and 70 mg/L), being determined the As concentration in each specimen, the Bioconcentration Factor (BCF), glycogen (GLY) content, lipid peroxidation (LPO) levels, antioxidant (superoxide dismutase (SOD) and catalase (CAT) activity) and biotransformation (glutathione S-transferases, GSTs and Ω-Glutathione-S-transferase, GSTΩ) enzymes activity, as well as the metallothioneins (MTs) content (section 3.2).

- **Temperature:** chronic exposure (28 days) was carried out with both species under different warming scenarios (17, 21 and 25 ºC), being a relevant set of biomarkers analyzed: electron transport system (ETS) activity, antioxidant enzyme (SOD) activity and LPO levels, as well as the gene expression of BCL2-associated X protein (*Bax*), cytochrome C oxidase subunit I gene (*Cox-1*), 16 S, Heat shock protein 70 mRNA (*Hsp70*), Copper/zinc superoxide dismutase (*Cu-ZnSOD*), Manganese superoxide dismutase (*MgSOD*), Growth Arrest and DNA Damages (*gadd45*) (section 3.3);

- **Salinity:** chronic exposure (28 days) was carried out with both species exposed to different salinities (14, 28, 35) and a relevant set of biomarkers was analyzed: GLY content, ETS activity, osmoregulation (carbonic anhydrase, CA) activity, LPO levels, and the activity of antioxidant (SOD and CAT) and biotransformation (glutathione *S*-transferases, GSTs) enzymes (section 3.4);

pH: chronic exposure (28 days) to different pH levels (7.8 and 7.3) was carried out with both species and the following biomarkers were analyzed: GLY content, ETS activity, osmoregulation (CA) activity, LPO levels, and the activity of antioxidant (SOD and CAT) and biotransformation (GSTs) enzymes (section 3.4);

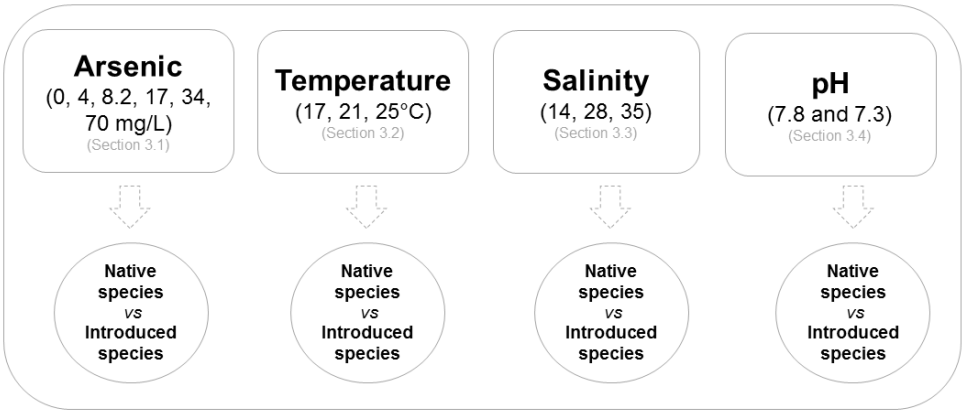

Figure 3.1.1 - Native *Ruditapes decussatus* and *R. philippinarum* species exposed to single stressors (arsenic, temperature, salinity and pH).

3.2. Arsenic exposure

3.2.1. Aim

The aim of the present study was to evaluate the performance of the native clam *R. decussatus* and the introduced clam *R. philippinarum* under As exposure (0, 4, 8.2, 17, 34 and 70 mg/L), through the evaluation of different biomarkers (mortality, energy sources, cellular damages and detoxification mechanisms).

3.2.2. Materials and methods

3.2.2.1. Sampling strategy and experimental conditions

Clams *R. decussatus* and *R. philippinarum* were collected in the Ria de Aveiro (northwest Atlantic coast of Portugal), in Spring 2013. The sampling site was selected taking into account the low As contamination levels (Freitas et al., 2012) and the coexistence of both species (Chapter 2). Individuals of similar weight and length were selected in order to minimize the effect of body size on element uptake and biochemical response. The mean length and weight of organisms were, respectively, 50.0±2 mm and 28.0±2 g for *R. decussatus* and 60.0±3 mm and 41.0±2 g for *R. philippinarum*. After sampling, organisms were transported to the laboratory where they were depurated, in artificial seawater (salinity 28.0±1.50 g/L), under continuous aeration, at 18.0±1 °C and 12L:12D photoperiod (Fig. 3.2.1).

Exposure experiment was conducted under continuous aeration, in 1 L of seawater (salinity 28.0±1.5). During the experimental period (96 h), organisms were not fed and temperature (18.0±1 °C), salinity (28.0±1.5), photoperiod (12L:12D) and aeration were monitored. For each As concentration, and for each species, 12 organisms were used (4 replicates per concentration, with 3 organisms per replicate). Organisms of both species were exposed to a range of As (0.0, 4.0, 8.2, 17.0, 34.0 and 70.0 mg/L) concentrations (Fig 3.2.1), selected taking into account the effects range low (ERL, 8.2 mg/L) and effects range median (ERM, 70 mg/L) values for As.

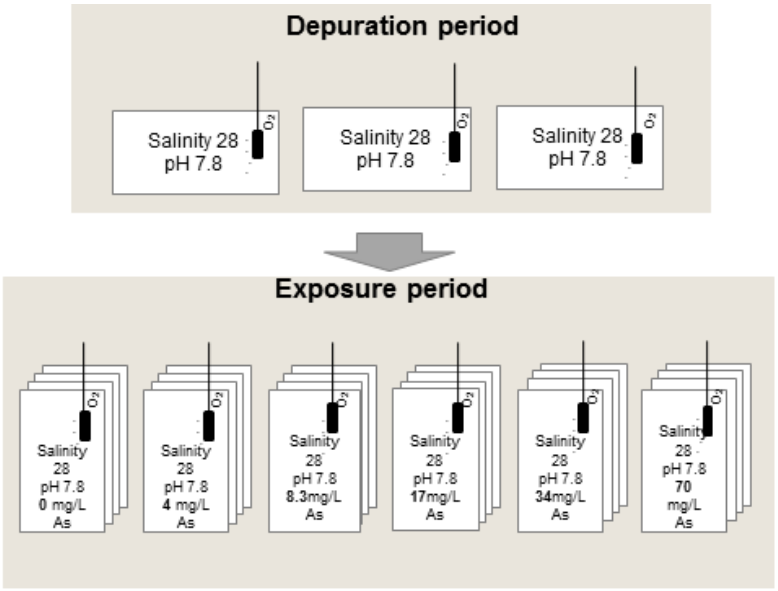

Figure 3.2.1 – Depuration and experimental setup used for *Ruditapes decussatus* and *R. philippinarum* exposure to different As concentrations (0, 4, 8.2, 17, 34 and 70 mg/L).

Seawater from each container was renewed daily and dead organisms removed. Organisms were considered dead when their shells gaped and failed to shut again after external stimulus. After the exposure period, the surviving organisms were frozen at - 80°C until As quantification and biochemical analyses.

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3.2.2.2. Arsenic quantification and BCF values

Quantification of As was determined in both species (3 replicates/ condition; 1 individuals/replicate) from five different conditions (0, 4, 8.2 17, and 34 mg/L As). The total As concentration, and the soluble and insoluble fractions accumulated in whole soft tissues were determined following Freitas et al. (2012) procedure. Soluble fraction includes the fraction present in cytosol, while the insoluble fraction comprises elements present in organelles, metal-rich granules (MRG) and cellular debris (Wallace and Luoma, 2003). The quantification of As was performed by ICP-MS (Inductively Coupled Plasma-Mass Spectroscopy), in a certified laboratory at the University of Aveiro. The calibration curve of the apparatus was made with dilutions of multi-element standard ICP 71A IV (Inorganic Venture, Christiansburg, VA, USA). The whole procedure was verified with standard certificated reference materials (TORT-2), from NRCC (National Research Council of Canada), with the recovery from 90% to 110%. All samples below this accuracy level were rejected and the analyses repeated. Concentration of elements was expressed in mg per kg of fresh weight (FW).

In order to assess the ability of each species to accumulate As under experimental conditions, the Bioconcentration Factor (BCF) was calculated, for each condition, as the ratio

between As concentrations in the organisms and the respective nominal exposure concentration (McGeer et al., 2003).

3.2.2.3. Biomarkers

Biomarkers were determined when there were at least three replicates (1 replicates/ condition; 1 individuals/replicate). For each analysis, frozen organisms were mechanically pulverized under liquid nitrogen. Extraction was performed with specific buffers for each biochemical analysis and samples centrifuged for 10 min at 10,000*g* and 4 °C. Supernatants were stored at - 80 °C or immediately used to determine: glycogen (GLY) content, lipid peroxidation (LPO) levels, activity of antioxidant enzymes (superoxide dismutase, SOD, catalase CAT) and biotransformation enzymes (glutathione S-transferase, GSTs; glutathione S-transferase omega, GSTΩ), metallothioneins (MTs) synthesis, and total soluble protein (PROT).

Glycogen (GLY) content

The GLY content was measured based on phenol-sulphuric acid method, as described by Yoshikawa (1959). GLY content was performed with phosphate buffer 50 mM, pH 7.0, with Triton 100 (0.1%). GLY extraction was performed with 0.1 M Tris–HCl pH 8.5, 15% (*w*/*v*) PVP, 153 mM magnesium sulfate ($MgSO_4$) and 0.2% (*v*/*v*) Triton X-100. For GLY determination, 10 µL of extracted supernatant of each organism was used. To every sample, 100 µL of phenol (5%) and 600 µL of sulphuric acid (H_2SO_4, 96%) were added. 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/g fresh weight (FW).

Lipid peroxidation (LPO) levels

Levels of LPO were measured based on Buege and Aust (1978) method described in the session 2.2.2.6 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. The levels of LPO was determined at 532 nm (1.56 × 105 M^{-1} cm^{-1} extinction coefficient). The results were expressed in nmol of MDA equivalents per mg of protein.

Superoxide dismutase (SOD) activity

The activity of SOD was measured according to Beauchamp and Fridovich (1971) as described in the session 2.2.2.6, at 560 nm, after 20 min of incubation at room temperature. Superoxide Dismutase from bovine liver (Sigma) was used as a standard (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.

Catalase (CAT) activity

The activity of CAT was determined by the reaction of this enzyme with methanol (EMSURE®) in the presence of H_2O_2 (Johansson and Borg, 1988, see section 2.2.2.6), with some modification according to Freitas et al. (2012). Sample extraction was performed with phosphate buffer at pH=7.0. The standard curve was determined using formaldehyde standards (0–150 mM) (Sigma®, purity level 99%). The incubation was for 20 min in a shaker, at room temperature. The formaldehyde formation with purpald was measured at 540 nm. Results were expressed in U per mg of protein. One unit (U) of enzyme activity represents the formation of 1.0 nmol formaldehyde, per min.

Glutathione S-transferase (GSTs) activity

The activity of GSTs was determined following an adaptation of the method described by Habig et al. (1974), see section 2.2.2.6. The cell extraction was performed with potassium phosphate, pH 7.0. The activity of GSTs was determined at 340 nm (ε = 9.6 mM^{-1} cm^{-1}) and the absorbance values were read in intervals of 10 s during 5 min. Results were expressed in U per mg of protein. One unit (U) of enzyme was defined as the amount of enzyme that caused the formation of 1 µmol thioether, per min.

Glutathione-S-transferase omega (GSTΩ) activity

The ativity of GSTΩ was determined based on DSMA (V) (disodium methylarsenate) reductase activity according to Sampayo-Reyes and Zakharyan (2006), with some modifications. Samples were incubated with and without DSMA (V). After sample incubation of samples (10 min) with 5 mM GSH, 18 mM DSMA (V), 0.25 mM NADPH and 0.8 U glutathione reductase the DSMA (V) reduction was determined. The reduction of DSMA (V) was measured at 340 nm. Absorbance values were read in intervals of 2 min during 30 min. The signal of samples without MMA(V) was subtracted to that of samples with DSMA (V). Activities were calculated using an extinction coefficient of 6.22 per mM cm^{-1}. Results were expressed in U per mg protein.

Metallothioneins (MTs) content

For MTs content, 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). Proteins absorbance presented in each band were determined using Quantity One program software (Bio-Rad) and the protein concentration measured according to the Robinson and Hogden (1940). After this, each band was cut and extraction performed according to Shevchenko et al. (2006). Confirmation of MTs was done through quantification of thiol groups, according to Moron et al. (1979). The results were expressed in mg per g of FW.

The concentration of PROT was quantified using the Biuret method (Robinson and Hogden, 1940, see section 2.2.2.6), at 540 nm. The cell extraction was performed with potassium phosphate buffer, pH 7.0. Bovine serum albumin (BSA) was used as the standard (0-40 mg/ml). The quantification of PROT was used to express the above biomarkers per mg of protein, except MTs.

3.2.2.4. Statistical analysis

Probit analysis (Minitab statistical software) was used to estimate LC_{50} values that correspond to the concentration of As that caused lethal effects in 50% of tested organisms (Finney, 1971).

For both species, data from As accumulation (total, insoluble and soluble fractions) and biomarkers were submitted to hypothesis testing using permutational multivariate analysis of variance with the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008), following the calculation of Euclidean distance matrices among samples. A one-way hierarchical design, with As exposure concentration as the main fixed factor, was followed in this analysis. The pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance. When the main test revealed statistical significant differences ($p \leq 0.05$), pairwise comparisons were performed.

The null hypotheses tested were: i) for As (soluble and insoluble fraction; total element concentration), and for each exposure concentration, no significant differences exist between species; ii) for As (soluble and insoluble fractions; total element concentration), and for each species, no significant differences exist among exposure concentrations; iii) for each biomarker (GLY, LPO, CAT, SOD, GSTs, GSTΩ and MTs) and for each exposure concentration, no significant differences exist between species; iv) for each biomarker (GLY, LPO, CAT, SOD, GSTs, GSTΩ and MTs) and for each species, no significant differences exist among exposure concentrations. Significant differences ($p \leq 0.05$) among exposure concentrations, for each species, are represented with distinct letters for *R. decussatus* (lowercase letters) and for *R. philippinarum* (uppercase letters). Significance levels ($p \leq 0.05$) between species, at each exposure concentration, are represented with asterisks.

3.2.3. Results

3.2.3.1. Mortality

When submitted to As both species presented a similar pattern with no (*R. philippinarum*) or very low (*R. decussatus;* 20%) mortality up to 17 mg/L. When exposed to higher As concentrations (34.0 and 70.0 mg/L), both species revealed significantly higher mortality rates, between 67 and 100%, with higher mortality observed in the introduced species than in the native one.

The results obtained further revealed that when under the same As concentrations, both species presented similar LC_{50} values (29.0 mg/L in *R. decussatus*, and 31.0 mg/L in *R. philippinarum*).

3.2.3.2. As accumulation in organisms

Total As concentrations in both species are presented in Table 3.2.1. In both species, the total As concentration increased with As exposure and no significant differences between species were noticed, except for 17 mg/ L (Table 3.2.1). *R. decussatus and R. philippinarum* showed low capacity to accumulate As (BCF≤1), except in *R. decussatus* exposed to 4.0 mg/L (Table 3.2.1).

Table 3.2.1 - Total As concentration (mg/Kg fresh weight) and Bioconcentration Factor (BCF) in *Ruditapes decussatus* and *R. philippinarum* (mean±SD). For each species, significant differences ($p \leq 0.05$) As concentrations are represented with different letters (a-e for Rd and A-E for Rp). Significant differences ($p \leq 0.05$) between species, at each As concentration, are represented with asterisks. n.a. - not applicable.

	Total		BCF	
As (mg/L)	*R. decussatus*	*R. philippinarum*	*R. decussatus*	*R. philippinarum*
0	1.73 ± 0.18^{a}	2.06 ± 0.23^{A}	n.a.	n.a.
4	4.36 ± 0.77^{b}	3.46 ± 0.53^{B}	1.09	0.87
8.2	5.83 ± 0.28^{c}	7.58 ± 1.81^{C}	0.72	0.92
17	$10.47 \pm 1.59^{d*}$	14.76 ± 2.96^{D}	0.62	0.87
34	20.10 ± 5.46^{e}	30.15 ± 5.27^{E}	0.59	0.89

3.2.3.3. Cellular partitioning

Regarding the concentration of As in the soluble and insoluble fractions (Fig. 3.2.2), both species presented similar partitioning of this metalloid in both fractions, which increased with the increase of the exposure concentration.

Although *R. philippinarum* showed higher As concentrations in both fractions, no significant differences were found between species at each As concentration, except in the soluble fraction at the highest exposure concentration (34.0 mg/L, Fig. 3.2.2).

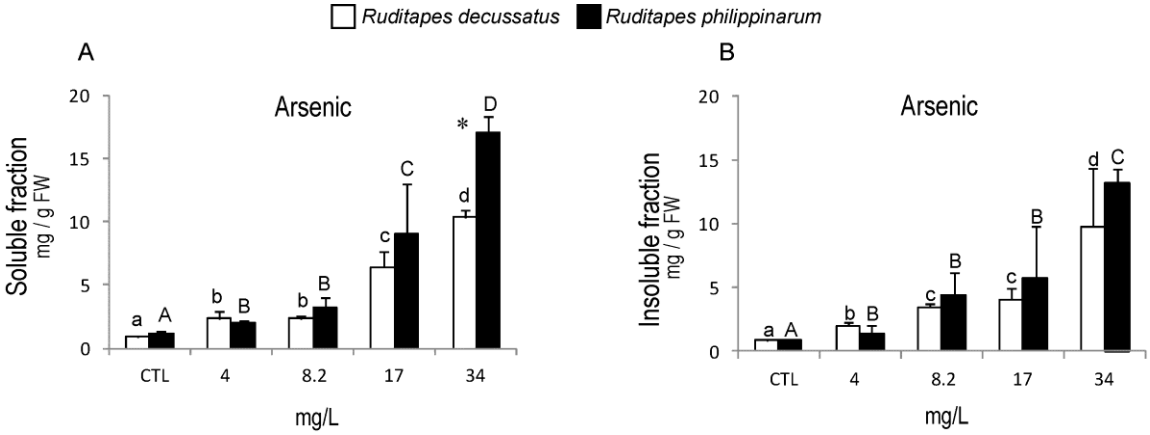

Figure 3.2.2 - Arsenic concentrations (mg/kg fresh weight, FW) in the soluble (A) and insoluble (B) fractions of *Ruditapes decussatus,* and *R. philippinarum*, exposed to different As concentrations (mean±SD). For each species, significant differences ($p \leq 0.05$) among As conditions are represented with different letters (a-d for Rd and A-D for Rp). Significant differences ($p \leq 0.05$) between species, at each As exposure, are represented with an asterisk.

3.2.3.4. Biomarkers

Glycogen (GLY) content

The results obtained showed that under an As exposure both species decreased the GLY content, especially noticed in *R. decussatus* (45.3–19.8 mg/g FW). Comparing both species, results demonstrated that *R. decussatus* presented higher GLY content than *R. philippinarum* at all As tested concentrations (Fig. 3.2.3).

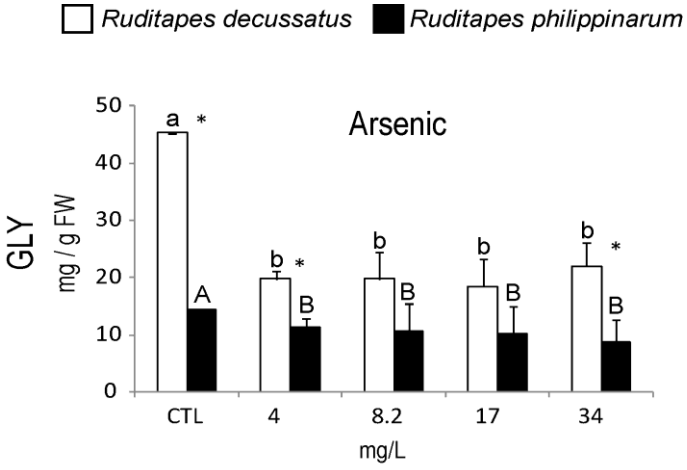

Figure 3.2.3 - Glycogen content (GLY, mg/g FW) in *Ruditapes decussatus,* and in *R. philippinarum*, exposed to different As concentrations (mean±SD). For each species, significant differences ($p \leq 0.05$) among As conditions are represented with different letters (a-b for Rd and A-B for Rp). Significant differences ($p \leq 0.05$) between species, at each As exposure, are represented with an asterisk.

Lipid peroxidation (LPO) levels

Under As exposure LPO levels significantly increased in both species at the highest exposure concentrations (As 17.0 and 34.0 mg/L). Significant differences between species were observed at 4.0, 8.2 and 17.0 mg/L, where the native species presented higher LPO levels than the introduced one, except at 17 mg/L.

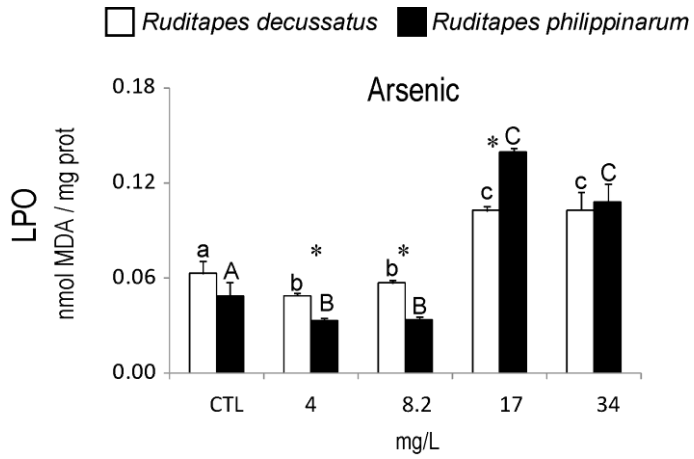

Figure 3.2.4 - Lipid peroxidation values (LPO, nmol MDA/ mg prot) in *Ruditapes decussatus,* and in *R. philippinarum*, exposed to different As concentrations (mean±SD). For each species, significant differences ($p \leq 0.05$) among As conditions are represented with different letters (a-c for Rd and A-C for Rp). Significant differences ($p \leq 0.05$) between species, at each As exposure, are represented with an asterisk.

Superoxide dismutase (SOD) activity

For *R. decussatus* a strong increase of the SOD activity was specially noticed at 8.2 mg/L of As, whereas for the highest exposure concentrations the activity of SOD significantly decreased (Fig. 3.2.5A). Along the increasing of As exposure concentrations, *R. philippinarum* increased SOD activity (Fig.3.2.5A). No significant differences between species were noticed when exposed to As, except at 8.2 mg/L.

Catalase (CAT) activity

The native species (*R. decussatus*) increased the activity of CAT up to 8.2 mg/L of As, followed by a significant decreased of this enzyme activity to values similar to the control (Fig. 3.2.5B). Although significant, *R. philippinarum* revealed a small increase in the activity of CAT with the increase of As exposure (Fig. 3.2.5B). When exposed to As, for most of the tested concentrations, *R. decussatus* showed significantly higher CAT activity than *R. philippinarum* (Fig. 3.2.5B).

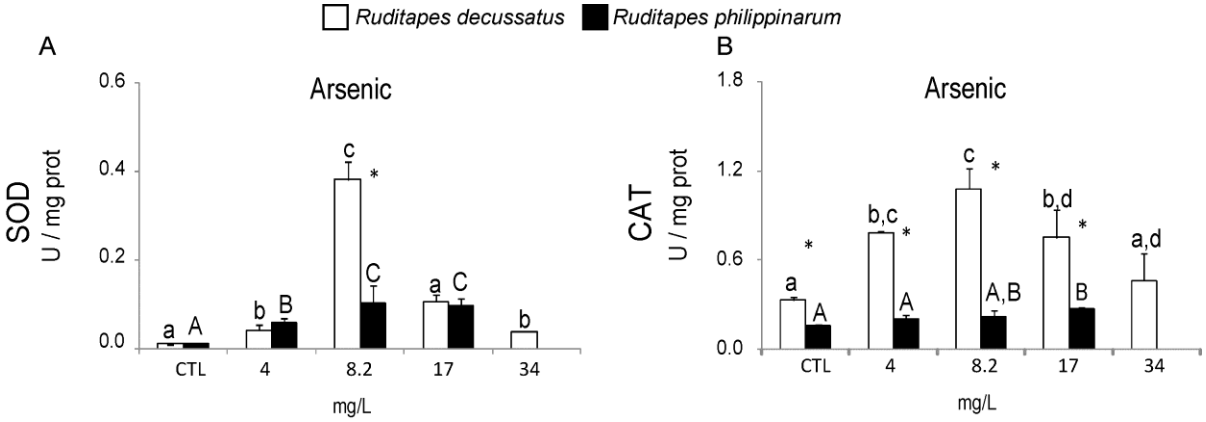

Figure 3.2.5 - Superoxide dismutase and catalase activity (SOD, A; CAT, B, U/mg prot) activity in *Ruditapes decussatus,* and in *R. philippinarum*, exposed to different As concentrations (mean±SD). For each species, significant differences ($p \leq 0.05$) among As conditions are represented with different letters (a-d for Rd and A-C for Rp). Significant differences ($p \leq 0.05$) between species, at each As exposure, are represented with an asterisk.

Glutathione S-transferases (GSTs) activity

The activity of GSTs in *R. decussatus* was significantly lower at 4 and 8.2 mg/L of As, when compared with remaining conditions, while *R. philippinarum* mantained GSTs activity along increasing As exposure (Fig. 3.2.6A). GSTs activity was significantly higher for *R. decussatus* than *R. philippinarum*, in species under control and exposed to As (Fig. 3.2.6A).

Glutathione-S-transferase omega (GSTΩ) activity

The activity of GSTΩ in *R. decussatus* and *R. philippinarum* for As exposure is presented in Fig. 3.2.6B. Results showed lower GSTΩ activity in *R. decussatus* at control and at 4 and 8.2 mg/L of As than in clams exposed at the highest As concentration. In *R. philippinarum* a significant increase in the activity of this enzyme was observed when compared to the control. In general, GSTΩ activity was higher in the introduced (*R. philippinarum*) than in the native species (*R. decussatus*) (Fig. 3.2.6B).

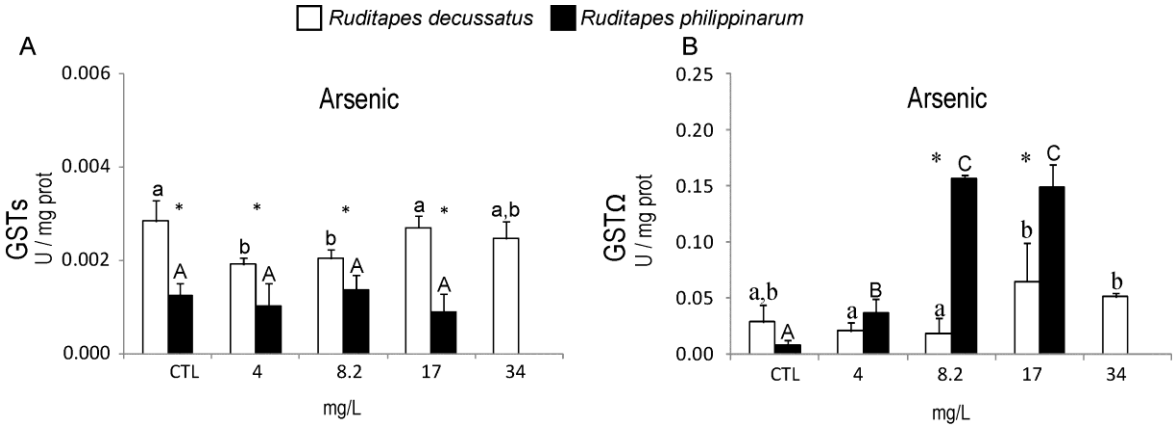

Figure 3.2.6 **-** Glutathione *S*-transferases and Glutathione *S*-transferase omega activity (GSTs, A; GSTΩ, B, U/mg prot) in *R. decussatus,* and in *R. philippinarum*, exposed to different As concentrations (mean±SD). For each species, significant differences ($p \leq 0.05$) among As conditions are represented with different letters (a-b for Rd and A-C for Rp). Significant differences ($p \leq 0.05$) between species, at each As exposure, are represented with an asterisk.

Metallothioneins (MTs)

For *R. decussatus* a strong increase of MTs synthesis was noticed up to 8.2 mg/L of As (Fig. 3.2.7), and a significant decrease was observed at higher concentrations (17.0 and 34.0 mg/L). The synthesis of MTs in *R. philippinarum* was not significantly different among As exposure concentrations (Fig. 3.2.7). No significant differences between species were reported in all tested conditions.

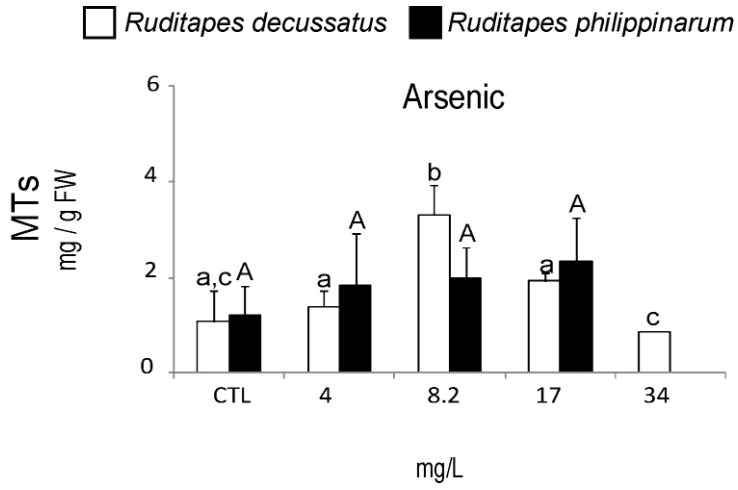

Figure 3.2.7 - Metallothioneins content (MTs, mg/g FW) in *Ruditapes decussatus,* and in *R. philippinarum*, exposed to different As concentrations (mean±SD). For each species, significant differences ($p \leq 0.05$) among As conditions are represented with different letters (a-c for Rd and A-A for Rp). Significant differences ($p \leq 0.05$) between species, at each As exposure, are represented with an asterisk.

3.2.4. Discussion

In the present study, total As concentrations and BCF values present in the native and introduced species provided information about the accumulation and the bioconcentration patterns of both species. However, the subcellular partitioning of As can provide information about internal processes occurring after element uptake, which can partially explain tolerance and detoxification differences between both clam species. Analyzing As partitioning the results showed similar partitioning between the soluble and insoluble fractions in both species. However, the native species tended to accumulate lower As concentrations in both fractions than the introduced species. Velez et al. (2016) exposed *V. corrugata* to a range of As concentrations (0 to 25 mg/L) and revealed that this species accumulated the majority of As in the soluble fraction (MTs, organelles, and enzymes). Under environmental conditions, Velez et al. 2015 (Chapter 2) demonstrated that in *R. decussatus* and *R. philippinarum* collected from the Ria de Aveiro and the Óbidos lagoon (Portugal), the majority of As was found in the soluble fraction.

When subjected to stress conditions, as As exposure, organisms can induce changes in energy metabolism through the increase of energy expenses, associated with detoxification and excretion processes (Holmstrup et al., 2011). Energy sources are mainly accumulated as GLY and consumed during reproduction or stress conditions (Brown and Luoma, 1999; Moussa et al., 2014). The present findings showed that under As exposure both species presented lower GLY content compared to control, evidencing higher energy consumption due to As contamination. GLY content was higher in *R. decussatus* than in *R. philippinarum* in As exposures. These results suggest that *R. philippinarum* consumed more energy to tolerate As than *R. decussatus*. According to Yu et al. (2016) As (V) exposure caused disturbances in energy metabolism of *Mytilus galloprovincialis* marked by different metabolic responses, including betaine, taurine, glucose, and GLY. Similar results were observed by Wu et al. (2013) in *R. philippinarum* exposed to As. Duquesne et al. (2004) showed that the clam *Macoma balthica* presented the lowest GLY content when exposed to the highest Cd concentration (300 mg/L), suggesting the use of GLY consumption by organisms to fight stressful environmental conditions. Also, Devi (1996) reported lower levels of GLY when *Mytilopsis sallei* was exposed to Hg.

The results obtained further revealed induction of different response mechanisms to As between both species, namely biotransformation (GSTΩ), sequestration (MTs) and antioxidant (SOD and CAT) mechanisms. However, at the highest As concentrations (17 and 34 mg/L), none of these species were able to prevent oxidative damage caused by this metalloid and mortality was observed in both species.

Arsenic is known to induce oxidative stress by triggering the formation of reactive oxygen species through mitochondrial electron transport chain in organisms (Birben et al., 2012). Several studies showed that under element contamination, namely As, bivalves increase the formation of reactive oxygen species causing LPO (e.g. Wu et al., 2013; Yu et al., 2016). The present study

showed that when exposed to the highest As concentrations both species significantly increased LPO levels and cells were not able to prevent membrane damage. Different studies found similar results when bivalves were submitted to metal contamination. In *R. decussatus* the exposure to Cu, Pb and Zn caused LPO (Freitas et al., 2014; Geret et al., 2003; Geret and Bebianno, 2004). Kamel et al. (2012) showed an increase in LPO when *R. decussatus* was exposed to treated municipal effluents containing metals (Cu, Cd, and Zn). Figueira et al. (2012) also reported higher LPO in *R. philippinarum* and *R. decussatus* exposed to Cd, with higher values observed in *R. philippinarum*. Ahmad et al. (2011) also showed a direct relation between LPO levels and Hg concentrations in *Scrobicularia plana*.

Relatively to antioxidant enzymes, the present results showed that *R. decussatus* increased antioxidant defences (SOD and CAT) up to 8.2 mg/L of As. These results support the capacity of cells to detoxify superoxide anion through the antioxidant enzymatic system. However, at the highest As concentrations these enzymes were inhibited, impairing the efficiency of this reactive oxygen species scavenging system and boosting oxidative stress. On the other hand, the introduced species increased SOD and CAT activity along the increasing exposure concentrations of As. SOD activity also increased when *R. philippinarum* was exposed to highly metal contaminated areas (Wang et al., 2012). Figueira et al. (2012) showed that in *R. decussatus* and *R. philippinarum* both antioxidant enzymes (CAT and SOD) tend to increase their activity when exposed to increasing Cd concentrations. Kamel et al. (2012) reported a significant increase of CAT in gills and digestive gland of *R. decussatus*, exposed to treated municipal effluents containing metals. Works conducted by Ahmad et al. (2011) showed an increase in CAT activity in the clam *Scrobicularia plana* from a highly Hg-contaminated area and Freitas et al. (2014) showed an increase in CAT activity and no induction of SOD activity when *R. decussatus* was exposed to Pb. Geret et al. (2003) demonstrated that in *R. decussatus* both CAT and SOD activities were enhanced in contaminated sites.

It is known that As has a strong affinity for sulfhydryl, phosphoryl and carboxyl groups that can result in the inactivation of a wide range of enzymes and also that the inorganic arsenate (molecular analogue of phosphate, HPO_4^{2-}), can compete for phosphate anion transporters and replace phosphate in some biochemical reactions (Shen et al., 2013). This can explain why in the present study the GSTs activity for both species did not change or was inhibited under As exposure. GSTs belong to susceptibility biomarkers, used to assess the sensitivity of organisms to a compound or a group of compounds. However, Dafre et al. (2004) also showed no differences in GSTs activity when exposing *Perna perna* mussels to Pb. Studies conducted by Figueira et al. (2012) did not find a clear pattern between Cd concentration and GSTs activity in both *R. decussatus* and *R. philippinarum*. On the other hand, Zhang et al. (2010) showed a strong increase in GSTs activity when *Chlamys farreri* was exposed to Hg. Also, Wang et al. (2012) revealed a strong increase in GSTs activity in *R. philippinarum* from highly metal polluted sites. However, Kamel et al. (2012) showed that GSTs activity decreased in gills of *R. decussatus*

exposed to increasing concentrations of treated municipal effluents, which may imply different responses between different organs.

Regarding GSTΩ results, it was possible to observe that the activity of this enzyme was induced along the increasing exposure concentrations of As especially noticed for *R. philippinarum*.

In the present study, at lower exposure concentrations, both species increased MTs synthesis with As increasing concentrations, avoiding the deleterious effects of intracellular free As ions. Similar findings were observed by Figueira et al. (2012) when *R. decussatus* were exposed to Cd, demonstrating that, under an increasing Cd gradient in *R. decussatus* MTs increased near three times in the presence of Cd, while *R. philippinarum* did not significantly increase the synthesis of these metal-binding proteins. Earlier studies by Bebianno and Serafim (2003) reported that the amount of MTs in the gills and digestive gland of *R. decussatus* can be used as a warning signal for Cd exposure. On the other hand, Moschino et al. (2012) observed that MTs amount in *R. philippinarum* was generally quite similar in polluted and uncontaminated areas, not reflecting the spatial variability of metals in sediments and clam tissues. Nevertheless, at higher As exposure concentrations, the present results showed that neither *R. philippinarum* nor *R. decussatus* were able to maintain MTs synthesis and together with antioxidant response decline, oxidative stress increased and toxicity, evidenced by LPO levels, overcame.

3.2.5. Conclusion

The introduced species presented slightly higher mortality and As accumulation than the native species at the highest exposure concentrations. To prevent damage originated by As, the native species (*R. decussatus*) was more efficient in the induction of protein synthesis, such as antioxidant enzymes (SOD and CAT) and MTs and the introduced species (*R. philippinarum*) relied on biotransformation enzymes (GSTΩ), rendering inorganic As in a less toxic form. The two defense mechanisms did not induce measurable differences in tolerance to As between the two clam species as proved by similar $LC_{50,}$ similar accumulation, and LPO. The energetic and material demands to maintain tolerance mechanisms effectively are too high and at a certain point impossible to withstand, collapsing the resistance strategy and death overcomes.

Overall, comparing both species under As contamination, it was possible to conclude that both species were tolerant to As contamination up to 8.2 mg/L of As, while exposure to the highest As concentrations (17, 34 and 70) induced oxidative stress.

3.3. Temperature exposure

3.3.1. Aims

The aim of the present study was to evaluate and compare the response of native (*R. decussatus*) and introduced (*R. philippinarum)* clams species under warming scenarios (17, 21 and 25 ºC), through the evaluation of different biomarkers (mortality, metabolic activity, cellular damage, detoxification mechanisms and gene expression).

3.3.2. Materials and methods

3.3.2.1. Experimental conditions

Clams *R. decussatus* and *R. philippinarum* (80 of each species) were collected in the Ria de Aveiro (northwest Atlantic coast of Portugal), in October 2015. In order to minimize the effect of body weight, organisms with similar weight (15-22 g) were selected.

Individuals of both species were maintained in the laboratory for 7 days before testing, in separate aquaria, to release metals and microorganisms (Freitas et al., 2012; Maffei et al., 2009). Both species were maintained at salinity 28±1, 17.0±1.0 ºC; pH 7.80±0.10, 12 light: 12 dark photoperiod and continuous aeration, in artificial seawater (salinity 28) (Tropic Marin® SEA SALT from Tropic Marine Center) (Fig. 3.2.1). Seawater was renewed every two days.

After this period, 60 specimens of each species were acclimated to experimental conditions (21 and 25 ºC; 20 specimens/temperature/species). The seawater temperature was increased one or two degrees per day until 21 and 25 ºC. The water temperatures were increased and maintained in different aquaria through electronic thermostats. The acclimatization period finished when all tested temperatures were reached in all testing groups. Higher temperatures (21 and 25 ºC) were selected taking into account the range of annual mean temperature (13.4 to 22.9 ºC) for *R. decussatus* and *R. philippinarum* habitats in the Ria de Aveiro (Coelho et al., 2014; Santos et al., 2009; Velez et al., 2015), the mean temperature in October in the sampling area (16-19 ºC, IPMA, 2016) and the predicted temperature increase from 1.0 to 4.0 ºC (IPCC 2007).

After acclimation, both species were submitted to different temperatures (17, 21 and 25 ºC), for 28 days. For each temperature and for each species, 18 organisms were used per condition (17, 21 and 25 ºC) (3 replicates/ condition, with 6 organisms/ replicate). Six containers, 3 for each species, with 2.5 L of seawater (salinity 28) were placed in different water baths at 17, 21 and 25 ºC. Posteriorly, 6 specimens of each species were added to the respective container (Fig. 3.3.1). The seawater in each water bath was maintained at constant values using electronic thermostats. Containers were continuously aerated. Temperature and salinity were daily checked with a thermometer and refractometer. Temperature and salinity values were adjusted, adding water to

the containers, according to the above conditions, whenever necessary. Mortality was daily checked and organisms were considered dead when their shells gaped and failed to shut again after external stimulus.

Animals were fed with Algamac protein plus (150.000 cells/animal) twice a week. During the experiment, seawater was removed from the containers once a week.

At the end of the exposure, the gills from 27 organisms (3 pairs of gills of 3 organisms/ replicate/species) of both species were frozen separately for biochemical analyses. For gene analysis, the gills of 9 organisms (one pair of gills/replicate; three replicates/condition) of both species were removed and conserved separately in RNA later® at -80 ºC.

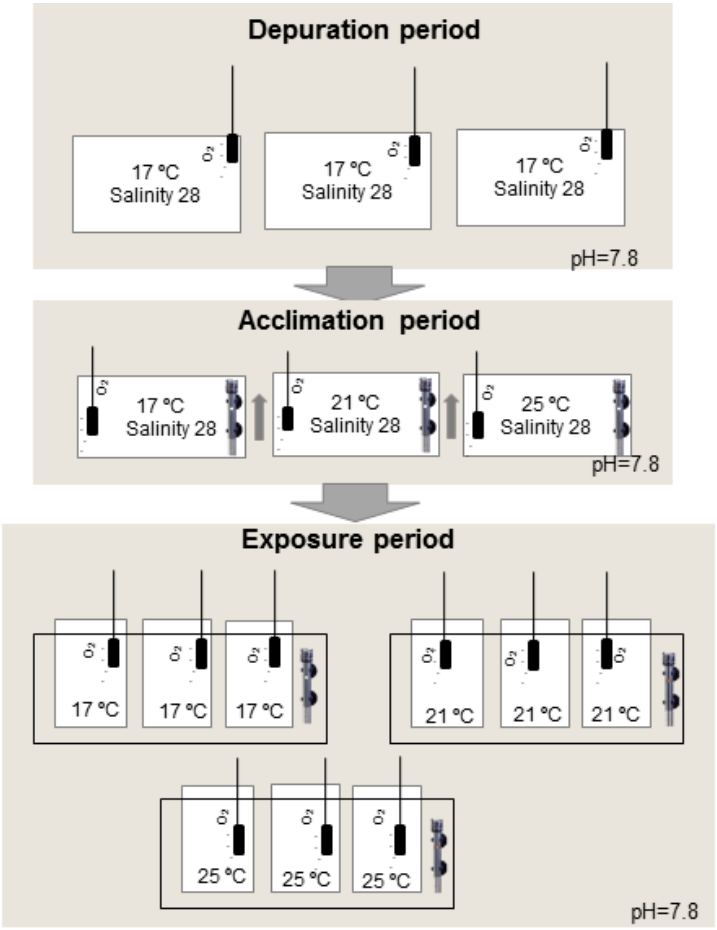

Figure 3.3.1 – Depuration, aclimation and experimental setup used for *Ruditapes decussatus* and *R. philippinarum* clams under different temperatures (17, 21 and 25 ºC).

3.3.2.2. Biomarkers

Before analysis, the gills of each organism were manually homogenized with a mortar and a pestle under liquid nitrogen.

Biomarkers were determined in the gills of both species from 3 different conditions (17, 21 and 25 ºC). For each biochemical determination, 0.1 g of gills tissue per organism was used. The extraction for each biomarker was performed with specific buffers. For this, samples were homogenized for 15 s at 4 ºC and centrifuged for 10 min at 10,000*g* for lipid peroxidation (LPO) levels, superoxide dismutase (SOD) activity and total soluble protein (PROT) content and at 3,000*g* for electron transport system (ETS). Supernatants were stored at -80 ºC or immediately used. All biomarkers measurements were performed using a microplate reader (Biotek).

Electron transport system (ETS) activity

The activity of ETS was measured based on King and Packard (1975) and modifications performed by Coen and Janssen (1997). The extractions were made in 0.1 M Tris–HCl buffer (pH 8.5), 15% (w/v) PVP, 153 mM magnesium sulfate ($MgSO_4$) and 0.2% (v/v) Triton X-100. Supernatant (35.7 µL) was incubated on a microplate with 107 µL of buffered substrate solution (0.13 M tris-HCl, 0.3% (v/v) triton X-100, pH 8.5), 35.7 µL of NAD(P)H (1.7 mM NADH and 250 µM NADPH). The reaction was started by adding 71.4 µL of 8 mM p-iodonitrotetrazolium. ETS was determined at 490 nm during 10 min with intervals of 25 s (15.900 M^{-1} cm^{-1} extinction coefficient). Results were expressed in nmol per min per mg protein.

Lipid Peroxidation (LPO) levels

The levels of LPO were measured by the quantification of TBARS (thiobarbituric acid reactive substances), according to the protocol described by Buege and Aust (1978) in the session 2.2.2.6. Results were expressed in nmol of MDA equivalents per mg protein.

Superoxide dismutase (SOD) activity

For SOD activity, the method of Beauchamp and Fridovich (1971) was followed with slight modifications, as described in the session 2.2.2.6. Results were expressed in U per mg protein. One unit of enzyme activity corresponds to a reduction of 50% of nitroblue tetrazolium (NBT), per min.

Protein (PROT) content

Total soluble PROT content was determined according to Robinson and Hogden (1940), following the Biuret method, as previously described in the session 2.2.2.6. PROT quantification was used to express the above biomarkers per mg of protein.

3.3.2.3. RNA extraction and quantitative real-time PCR

The total RNA collected from the gills of both species (one pair of gills/ species/ replicate) was extracted through SV Total RNA isolation system (Promega), according to manufacturer´s protocol. RNA was quantified using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc) and the RNA concentrations adjusted to 3 mg of total RNA.

The reverse transcription reaction was performed using GoScript Reverse Transcription System (Promega) and real-time PCR was performed using GoTaq®qPCR Master Mix (Promega). The CFX Connect™ Real-Time PCR Detection System was used for amplification with SYBR® Green I (Promega). Primer sets used are in Table 3.3.1, which were designed from sequences available in GenBank for introduced *R. philippinarum* and native *R. decussatus* clam species, except for the *gadd45* and *Bax* sequence for *R. philippinarum*, that were designed by Binias et al. (2014). Primers were designed through PRIMER3 program and confirmed through Beacon Designer TM-Free edition (Thornton and Basu, 2011). Efficiency and specificity of each primer was determined by observing the obtained standard and melting curves, respectively, for all primer sets. cDNA was 10x diluted and 1 µL was used in a 20 µL PCR reaction with 2 µL of forward and reverse primers mix of specific primer (final concentration 300 nM for each primer) and 17 µL of master mix with SYBR® Green I (Promega). Quantitative real-time PCR (qPCR) was performed with five biological replicates of each condition, applied in triplicate on a 96-well optical plate (CFX Connect™ Real-Time PCR Detection System). The standard cycling conditions consisted of an initial cycle at 95 ºC for 3 min, followed by 40 cycles at 95 ºC for 10 s, 55 ºC for 10 s and 72 ºC for 30 s.

The housekeeping gene *18S* was selected as reference for the calculation of relative expression levels of the genes for both species. The relative genes expression was calculated according to the methods described by Livak and Schmittgen (2001) ($2^{-\Delta\Delta C_T}$).

The mRNA inductor factor (IF) of each gene in comparison with control corresponds to the following equation (Paul-Pont et al., 2010):

$$IF = \frac{2^{-\Delta C_T}\text{ (temperature exposure)}}{2^{-\Delta C_T}\text{ (control)}}$$

To determine the gene expression at 25 ºC relative to gene expression at 21 ºC, the same equation depicted above was used.

Table 3.3.1 - Genes selected, specific function and the nucleotide sequences for each species and gene (F -forward primer, R- reverse primer). * Binias et al. (2014)

Gene target	Function	*Ruditapes* species		Primer DNA sequence 5´-3´
18 S	Housekeeping gene	*R. philippinarum*	F	ATAACGGGTAACGGGGAATC
			R	TGTCGGGAGTGGGTAATTTG
		R. decussatus	F	TCCGGCCTGCTAAATAGTTC
			R	GCTCAATCTCGTGTGGCTAAG
BCL2-associated X protein (*Bax*)	Apoptosis regulation	*R. philippinarum*	F	AGTTTTAAGGATGACATCATTGGTCAC*
			R	GCACCTTGGATTATAGAGAGAGGC*
Cytochrome oxidase subunit I (*Cox*-I)	Mitochondrial metabolism	*R. philippinarum*	F	GTACCCTCCGTTGTCGTCA
			R	CCTGTTACTCCTAAACACCAAG
		R. decussatus	F	GACTGCTCATGGGTTAGTGATG
			R	ACGAGGGAATGCCATATCAG
16 S	Mitochondrial metabolism	*R. philippinarum*	F	AGAAGACCCTGTCGAG
			R	TTACGGCTGTTATCCCT
		R. decussatus	F	AGCTCAATAGGGTCTTCTCGTC
			R	TCTGTCGCAAGTTGCTTGTC
Heat shock protein 70 mRNA (*Hsp70*)	Molecular chaperone	*R. philippinarum*	F	CGAAGCGAACACTTTCAAGC
			R	TCCACGGAAAAGGTCAGAAC
		R. decussatus	F	TCTCGGAGGTGAGGATTTTG
			R	TTCTCTTTGCTCGCTCACAC
Copper/zinc superoxide dismutase (*Cu-ZnSOD*)	Antioxidant response	*R. philippinarum*	F	CGCACTTCCTCACGCCCATCAT
			R	CATTTCTTGTTCAAAGTCCAAG
Manganese superoxide dismutase (*MnSOD*)	Antioxidant response	*R. philippinarum*	F	TGTGTGCTGCCAAAGGTAAG
			R	GATGACTGGTTCCAATGCTG
Growth Arrest and DNA Damages (*gadd45*)	DNA repair	*R. philippinarum*	F	CCTCAATCAGCTTGTGTTGG*
			R	ATGTCAGGCGATGAGTGC*

3.3.2.4. Statistical analysis

Biochemical parameters obtained from the gills of each species and tested condition were submitted to a statistical hypothesis testing using permutational analysis of variance, employing the PERMANOVA (permutational multivariate analysis of variance) -+add-on in PRIMER v6 (Anderson et al., 2008).

The null hypotheses tested were: i) for each biochemical or gene expression biomarker and for each species, no significant differences exist among temperatures; ii) for each biochemical biomarker and temperature tested, no significant differences exist between species.

In figures, lowercase letters represent differences among temperature for *R. decussatus*, while uppercase letters represent differences among temperatures for *R. philippinarum* and significant differences between species at each temperature are presented with asterisks.

3.3.3. Results

3.3.3.1. Mortality

No mortality was recorded at temperatures 17 and 21 ºC in either species. At 25 ºC, mortality was 7% for *R. decussatus* and 13% for *R. philippinarum*.

3.3.3.2. Biomarkers

Electron transport system (ETS) activity

In *R. decussatus* the ETS activity in gills increased at 21 and 25 ºC when compared with organisms at control conditions (17 ºC), but significant differences were only observed between 17 and 21 ºC (Fig. 3.3.2). On the other hand, the activity of ETS was only significantly higher in *R. philippinarum* gills at 25 ºC compared with the remaining temperatures (Fig. 3.3.2).

Significant differences between both species were only noticed at 21 ºC, with the native species presenting higher ETS activity than the introduced one (Fig. 3.3.2).

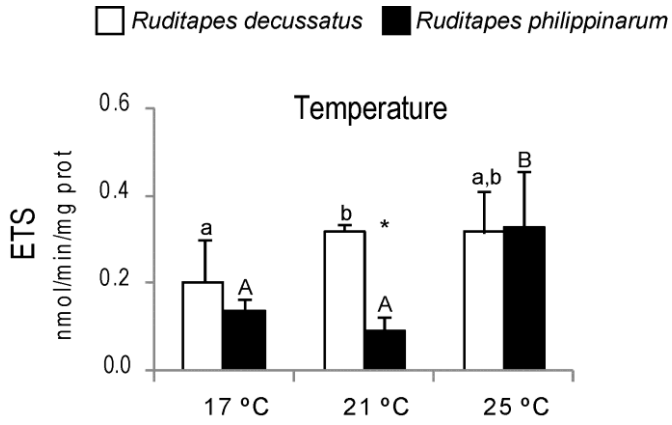

Figure 3.3.2 - Electron transport system activity (ETS, nmol/min/mg prot) in *Ruditapes decussatus,* and in *R. philippinarum,* exposed to different temperatures (17, 21 and 25 ºC, mean±SD). For each species, significant differences ($p \leq 0.05$) among temperatures are represented with different letters (a-b for Rd and A-B for Rp). Significant differences ($p \leq 0.05$) between species, at each temperature, are represented with an asterisk.

Lipid Peroxidation (LPO) levels

The levels of LPO presented no significant differences in *R. decussatus* gills among the exposure temperatures (Fig. 3.3.3). In contrast, the LPO levels in *R. philippinarum* increased at 21 and 25 ºC when compared with control condition (17 ºC), but significant differences were only observed between 17 and 21 ºC (Fig. 3.3.3).

When comparing both species at the same temperature, results showed significantly higher LPO in the introduced than in the native species at all tested conditions (Fig. 3.3.3).

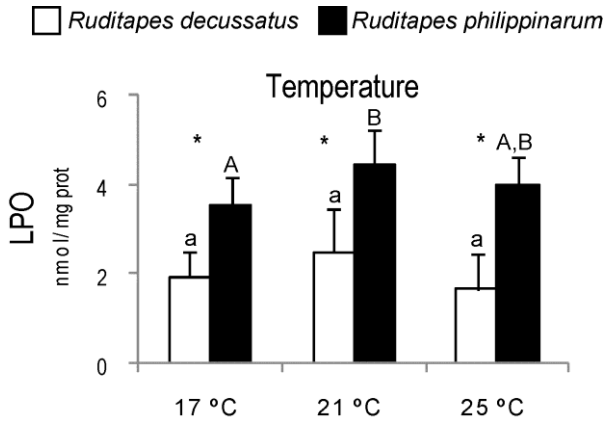

Figure 3.3.3 - Lipid peroxidation levels (LPO, nmol/mg prot) in *Ruditapes decussatus,* and in *R. philippinarum* exposed to different temperatures (17, 21 and 25 ºC, mean±SD). For each species, significant differences ($p \leq 0.05$) among temperatures are represented with different letters (a for Rd and A-B for Rp). Significant differences ($p \leq 0.05$) between species, at each temperature, are represented with an asterisk.

Superoxide dismutase (SOD) activity

SOD activity was significantly higher in *R. decussatus* exposed at 21 ºC than in organisms at 17 and 25 ºC (Fig. 3.3.4). In *R. philippinarum*, SOD activity was higher at 21 and 25 ºC compared with control condition (17 ºC), but significant differences were only observed between 17 and 25 ºC (Fig. 3.3.4).

When comparing both species under the same temperature, results demonstrated that independently on the temperature *R. philippinarum* presented higher SOD activity than *R. decussatus*, with significant differences at temperatures 17 and 25 ºC (Fig. 3.3.4).

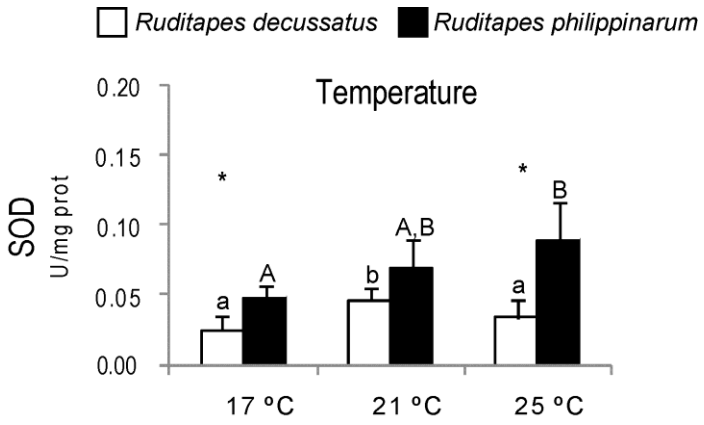

Figure 3.3.4 - Superoxide dismutase activity (SOD, U/mg prot) in *Ruditapes. decussatus,* and in *R. philippinarum,* exposed to different temperatures (17, 21 and 25 ºC, mean±SD). For each species, significant differences ($p \leq 0.05$) among temperatures are represented with different letters (a-b for Rd and A-B for Rp). Significant differences ($p \leq 0.05$) between species, at each temperature, are represented with an asterisk.

Gene expression

For *R. decussatus*, the *Hsp70* expression showed an up-regulation (>2) at 21 ºC relativety to the control (Table 3.3.2). Similar results were observed for *R. philippinarum*, as well as, the up-regulation of *Cu-ZnSOD* (>2) genes at 21 ºC, while the *MnSOD, 16S* and *gadd45* expression at 21 ºC were down-regulated when compared to individuals at 17 ºC (Table 3.3.2). The expression of the remaining genes (*Bax* and *Cox-1*) were unchanged (>0.5 and < 2) (21/17 and 25/17).

At 25 ºC, the expression of *Cox*-1, *16S,* and *Hsp70* in gills of *R. decussatus* were down-regulated relatively to the control (<0.5) (Table 3.3.2). For *R. philippinarum* the gene expression of the *Bax* (>2) and *Hsp70* (>2) genes was up-regulated, while a down-regulation of *MnSOD*, *16S* and *gadd45* expression was reported when compared to individuals at 17 ºC (Table 3.3.2). The remaing genes expression where unchanged (*Cox-1* and *Cu-ZnSOD*) (>0.5 and < 2).

When comparing the gene expression at 25 and 21 ºC, for the native species, results showed that at the highest temperature, *Hsp70,* and *16S* expression were down-regulated, while the expression of *Cox-1* has not changed (Table 3.3.2). For *R. philippinarum* results demonstrated an up-regulation of *Bax* at 25 ºC, while *16S*, *Hsp70, MnSOD and Cu-ZnSOD* genes were down-regulated (Table 3.3.2). The expression of the genes *gadd45* and *Cox-1* was unchanged (>0.5 and < 2). No significant differences were observed in gene expression of gills samples for both species, among the temperature conditions employed (Table 3.3.2).

Table 3.3.2 - Relative mRNA expression of Cytochrome oxidase subunit I (*Cox*-1), *16S*, Heat shock protein 70 mRNA (*Hsp70*), in gills of *Ruditapes decussatus* and BCL2-associated X protein (*Bax*), *Cox-1*, *16S*, *Hsp70*, copper/zinc superoxide dismutase (*Cu-ZnSOD*), manganese superoxide dismutase (*MgSOD*) and growth arrest and DNA damage (*gadd45*) in gills of *R. philippinarum,* exposed to different temperatures (17, 22 and 25 ºC). Values indicating up-regulation (>2 -fold)(↑) or down-regulation (<0.5-fold)(↓) between organisms under 17 ºC and higher exposure temperatures (25 and 21 ºC) are represented in bold and in highlighted values, respectively.

	Genes	21/17	25/17	25/21
R. decussatus	*Cox-1*	0.55	0.32(↓)	0.57
	16S	0.54	0.17(↓)	0.32(↓)
	Hsp70	**7.06**(↑)	0.37(↓)	0.05(↓)
R. philippinarum	*Bax*	0.98	**16.97**(↑)	**17.38**(↑)
	Cox-1	0.61	0.72	1.17
	16S	0.08(↓)	0.01(↓)	0.14(↓)
	Hsp70	**161.76**(↑)	**6.51**(↑)	0.04(↓)
	MnSOD	0.00(↓)	0.00	0.03(↓)
	Cu-ZnSOD	**71.78**(↑)	1.15	0.02(↓)
	gadd45	0.25(↓)	0.47(↓)	1.92

3.3.4. Discussion

At the end of the 21st century, an increase of global temperature is predicted to occur (IPCC, 2014). One of the direct consequences will be warming of oceans and estuarine systems (IPCC, 2014). Therefore, it is important to predict the impact of temperature increase on marine bivalve species, evaluating the capacity of native and introduced species to respond to temperature fluctuations. To our knowledge, few published studies have assessed (Munari et al., 2011; Paillard et al., 2004) and even less have compared (Anacleto et al., 2014a,b) the effects of temperature in the native *R. decussatus* and introduced/invasive *R. philippinarum* species. Furthermore, no studies evaluated mRNA expression and biochemical biomarkers in *R. decussatus* and *R. philippinarum* under seawater warming scenarios. Therefore, the present study provided information on how temperature changes may affect *R. decussatus* and *R. philippinarum* clams, using multiple biomarker approaches (biochemical and mRNA expression tools).

The increase of seawater temperature can enhance organisms metabolic capacity and oxidative stress (Solan and Whiteley, 2016). Oxidative stress may result from ROS overproduction due to thermal stress but also due to increased mitochondrial respiratory activity. To prevent the accumulation of these molecules, organisms produce and/or activate antioxidant enzymes. If antioxidant enzymes are not able to detoxify ROS caused by thermal stress, lipid peroxidation occurs (Solan and Whiteley, 2016). It is known that organisms present different mechanisms to regulate cellular pathways in response to external or internal environment changes (Liu et al.,

2012). Frequently these responses are behavioral or metabolic adjusted by gene expression. The expression of genes may be up or down-regulated to maintain homeostasis in organisms under environmental stress (Liu et al., 2012; Todgham and Hofmann, 2009). Recently, different authors studied the expression of genes regulation in marine bivalves under different environmental stressors (e.g. Hamdoun et al., 2003; Liu et al., 2012; Park et al., 2009). The expression of *Hsp* gene is related to thermo tolerance at the organism level (Buckley et al., 2001; Hamdoun et al., 2003). Temperature may affect *Hsp70* expression in bivalves since due to low temperature these organisms increased the expression of *Hsp70* (Liu et al., 2012). Park et al. (2009) and Pil et al. (2008) showed an up-regulation of genes responsible for antioxidant defence in bivalves under increase of temperature.

Regarding temperature exposure, results obtained in the present study for *R. decussatus* at 21 ºC showed increased metabolic activity compared to control condition (17 ºC), which may be possibly related to the activation of the respiratory chain in organisms exposed to this temperature. According to Solan and Whiteley (2016), estuarine and costal bivalves under temperature increase are able to enhance their metabolic rates. Similar results were observed in the bivalves *Sinanodonta woodiana*, *Anodonta anatine* (10 to 26 ºC) and *Crassostrea gigas* (12 to 20 ºC) exposed to increasing temperatures (10 to 26 ºC) (Bielen et al., 2016; Le Moullac et al., 2007). The present results further revealed an induction of antioxidant enzymes (SOD) by native species at 21 ºC, as a defence mechanism to detoxify superoxide anion and prevent damages (LPO) induced by temperature and that may also result from higher ETS activity. Similar results were observed by Abele et al. (2002) that revealed an increase in SOD activity with the increase of temperature (0 to 22 ºC) in gill tissues of the clam *Mya arenaria.* In addition, the present results showed that 21 ºC did not induce damages in membranes, possibly due to detoxification mechanisms present in cells, such as antioxidant enzymes (as evidenced by higher SOD activity). These findings may indicate that *R. decussatus* may control cellular damages after long exposure periods to increased temperature. Anacleto et al. (2014b) observed a similar response in *R. decussatus* exposed to thermal shock (22 to 24 ºC), with induced detoxification mechanisms (SOD, catalase and glutathione S-transferases) to avoid damage by temperature increase. Relatively to the introduced species, the present study further demonstrated that metabolic activity (ETS) was maintained at 21 ºC when compared with control conditions, suggesting that this temperature was not sufficient to induce an increase in respiration and energy production rates. The introduced species induced detoxification mechanisms (SOD) as a protection against superoxide anion toxicity generated at 21 ºC. However, this antioxidant mechanism and the remaining detoxification mechanisms were possibly not able to prevent damages caused by this temperature since LPO increased. According to Abele et al. (1998; 2002) in different marine molluscs, species thermal stress may result in oxidative stress.

Temperature changes may also up- or down-regulate genes responsible for thermoprotection (Anestis et al., 2008). The present study showed that at 21 ºC, native and

introduced species up-regulated the *Hsp70* gene when compared with organisms at 17 ºC. These results suggested an induction of this gene as a stress response of temperature to protect cells against denaturation, possibly contributing to the increase of organism's tolerance at 21 ºC. It is known that the *Hsp70* induction promotes thermoprotection, preventing the aggregation of heat-damaged proteins and repairing denatured proteins after heat shock (Anestis et al., 2008). The effect of temperature in the *Hsp70* expression in marine bivalves has already reported in previous studies. Liu et al. (2012) revealed an increase of *Hsp70* expression in the Pearl Oyster *Pinctada fucata* up to 24 hours exposed to 27 and 30 ºC, and a decrease up to 96 h. Hamdoun et al. (2003) reported that the threshold temperature for the Pacific Oyster (*C. gigas*) may be related to the level of environmental stress since oysters collected in January and exposed to 37 ºC up-regulated *Hsp70* expression, while oysters collected in August exposed to the same temperature did not induce the *Hsp70* expression. The present results also revealed that at 21 ºC *R. philippinarum* was also able to induce the expression of the gene that encodes the antioxidant enzyme *Cu-ZnSOD* used as defence mechanisms to prevent the damage caused by ROS. Other authors demonstrated that *C. gigas* acutely and chronically exposed to stress temperatures increased ROS production, and induced *Cu/Zn SOD* and *MnSOD* expression (Park et al., 2009; Pil et al., 2008). On the other hand, the present results demonstrated that the mitochondrial gene responsible by encoding antioxidant *MnSOD* enzymes and the gene *gadd45* responsible by DNA repair were down-regulated at 21 ºC. These results suggested that possibly the down-regulation of *MnSOD* gene may contribute to lower antioxidant capacity, resulting in higher LPO levels at this temperature compared to control.

Regarding data obtained at 25 ºC, the present results showed that both species increased their metabolic (ETS) activity compared with control temperature (17 ºC). Anacleto et al. (2014b) demonstrated a metabolic rate increase of *R. philippinarum* along with increasing temperatures (22 to 30 °C). Similar results were previously reported by several authors for ETS activity for other bivalves (*S. woodiana*, *Dreissena polymorpha* and *M. edulis*) under increasing temperatures, indicating that this factor may pose changes in the enzymatic complexes of the respiratory chain (Bielen et al., 2016; Doucet-Beaupré et al., 2010; Fanslow et al., 2001). Also, Le Moullac et al. (2007) showed an increase of the ETS activity with increasing temperatures (12 to 20 ºC) in *C. gigas*, suggesting that the increase may be linked to the up-regulation of some genes in the respiratory chain.

The present results further revealed that at 25 °C, *R. decussatus* organisms presented an increase of their metabolic activity (identified by higher ETS) compared with control temperature (17 ºC) but not higher than values at 21 ºC. The fact that at 25 ºC ETS activity was not higher than at 21 ºC may be related to clams behavioral strategies, namely valves closure, at temperatures higher than a certain threshold (in this case higher than 21 ºC). According to Anestis et al. (2007), the bivalve *Mytilus galloprovincialis* exposed to 25 ºC tended to close their valves compared with mussels held at 20 ºC, reducing their metabolic activity. In the present study, the combination of

strategies (valve closure and metabolic activity increase limitation) may increase clams survival. It is known that bivalves close their shells and reduce filtration rates as a strategy to isolate themselves from stressful conditions (namely temperature changes). This strategy in bivalves is accompanied by anaerobioses periods and consequently metabolic depression (e.g. Anestis et al., 2007; Ortmann and Grieshaber, 2003). This may explain why, in the present study, *R. decussatus* did not present increased metabolic activity at 25 ºC when compared with organisms at 21 ºC. The present findings further revealed that at 25 ºC, *R. decussatus* maintained similar LPO and SOD levels compared with control conditions. These results supported the hypothesis that *R. decussatus* keep their valves closed, preventing oxidative stress. The introduced species maintained LPO levels and induced the antioxidant mechanisms (SOD) when exposed to 25 ºC compared with control conditions, which may contribute to increasing species tolerance to high temperature. Similar results were also observed by Monari et al. (2007), demonstrating that the clam *Chamelea gallina* induced SOD activity at 25 and 30 ºC compared with 20 ºC. Also according to Matozzo et al. (2013), SOD activity was higher in the gills of *C. gallina* at 28 ºC than at 22 ºC. The present study further revealed that at 25 ºC damages in lipid membrane (LPO) were not increased compared to 21 ºC, possibly due to the activation of SOD but also due to other detoxification mechanisms not evaluated in this study. Also Matoo et al. (2013) showed that temperature increase (22 to 27 ºC) did not induce damages (LPO) in clams *Mercenaria mercenaria*. Other detoxification strategies such as the increase of catalase, glutathione peroxidase and glutathione *S*-transferases activity were developed by the mussel *Perna viridis* to avoid LPO by the increase of temperature (Verlecar et al., 2007). Overall, biochemical results for *R. philippinarum* showed that at 25 ºC metabolic activity was induced, while the antioxidant activity and LPO were maintained compared with levels obtained at 21 ºC.

Assuming that a consequence of high seawater temperature (25 ºC) exposure *R. decussatus* close their valves during short periods remaining in hypoxia, avoiding metabolic and biochemical changes, this could explain low mortality observed at these conditions (25 ºC) and the inhibition of *Cox*-1, *16S*, and *Hsp70* genes. These results suggested the responsiveness of native species at 25 ºC with the down-regulation of *Hsp70* genes as protection mechanism of cell integrity under this temperature. Woo et al. (2013) reported that the bivalve *M. galloprovincialis* exposed to hypoxia during 24 h and 48 h decreased the expression of *Cox*-1, indicating that the decrease of *Cox*-1 transcription consequently resulted in a decrease of mitochondrial *Cox*-1 protein synthesis. It is known that expression of *Cox*-1 subunits is regulated by the concentration of oxygen (Roemgens et al., 2011) and therefore when the oxygen concentration is low, the expression of this gene is down-regulated. This may explain why, in the present study, *Cox*-1 was down-regulated in the native species at 25 ºC. The present results also showed that comparing gene expression of *Hsp70* in *R. decussatus* at 25 and 21 ºC a down-regulation of this gene at the highest temperature was observed, supporting the behavioral strategy as the closure of valves in response to high temperatures. The present study further revealed a different strategy *for R. philippinarum* since at

25 ºC this species unregulated the gene expression of *Hsp70* when compared to organisms at 17 ºC, as a protective mechanism of cells exposed to such temperature. Meistertzheim et al. (2007) also reported an induction of *Hsps* synthesis in *C. gigas* chronically (24 days) exposed to 25 ºC. On the other hand, the introduced species at 25 ºC down-regulated *gadd45* expression compared with 17 ºC, suggesting that cells were not able to repair DNA damage due to cellular induction of the gene responsible for executing apoptotic pathways (*Bax*). It is known that *Bax* protein encoded by *Bax* gene induces cell cycle arrest or apoptosis in response to DNA damages, such mechanisms are crucial for life and death control of cells (Basu and Haldar, 1998). Although *R. philippinarum* presented increased metabolic activity as well as antioxidant enzymes to combat the increase in ROS production, the genes that encoded antioxidant mitochondrial enzymes (*MnSOD*) were down-regulated by temperature increase suggesting that these mechanisms were limited to prevent cellular damages. Nevertheless, it is known that the increase of ROS species in cells and a decrease in antioxidant defence mechanisms may result in oxidative stress and consequently, cells induce apoptotic signals (*Bax*). This may explain why organisms exposed to 25 ºC showed up-regulation of *Bax* expression when compared with organisms at 21 and 17 ºC. In general, gene expression for *R. philippinarum* showed that the highest temperature was responsible for down-regulation of genes responsible for antioxidant enzymes (*MnSOD* and *Cu-ZnSOD*) and the ones responsible for thermoprotection (*Hsp70*), while the apoptotic gene (*Bax*) was induced compared with organisms at 21 ºC

When comparing native and introduced clam species, the present results demonstrated different responses to temperature range selected. Native species exposed to 17 and 21 ºC presented higher ETS activity than the introduced one, suggesting higher metabolic activity of *R. decussatus* at these temperatures. Similar results were observed by Lockwood and Somero (2011) in different species, revealing that the native mussel *Mytilus trossulus* presented higher metabolic activity than the invasive mussel *M. galloprovincialis* when subjected to 14 and 21 ºC. Also, Bielen et al. (2016) showed differences between native *Anodonta anatina* and invasive *S. woodiana* species, but in this case, authors showed that the native species presented lower ETS than the invasive ones when exposed to 10 and 26 ºC. Nevertheless, our study demonstrated that at the highest temperature of exposure (25 ºC) the native and introduced species presented similar ETS activity values. Regarding LPO levels and SOD activity, in general, the present study revealed that the introduced species exposed to increasing temperatures presented a higher increase of detoxification mechanisms and lipid peroxidation than native species, indicating that species presented different biochemical strategies to overcome the stress induced by temperature.

The present findings also revealed that both species were able to induce the expression of *Hsp70* at 21 ºC, as a thermal protection strategy. Similar thermal protection capacity induction was observed in the *R. philippinarum* at 25 ºC while *R. decussatus* down-regulated the expression of C*ox*-1, *16S*, and *Hsp70* at 25 ºC. These results suggested different molecular strategies of both species to regulate the same genes under 25 ºC. While the introduced species was able to up-

regulate gene expression of *Hsp70* as a protection defence (at 25 ºC), the native species appeared to reduce this gene expression capacity, possibly as resulted of valve closure.

3.3.5. Conclusion

Overall, the present findings indicated that both species presented different biochemical responses and gene expression adjustment in an estuarine warming context. At 21 ºC, the present results revealed that the native species was more tolerant than the introduced one. These results may be explained by the capacity of native species, when exposed to 21 ºC, to induce ETS and SOD activities and the expression of heat shock genes to protect the cells against the effects of temperature. On the other hand introduced species although was able to induce SOD activity and the expression of heat shock protein and antioxidant genes, these mechanisms were not able to detoxify ROS efficiently, ultimately resulting in LPO increase and apoptosis.

The present study further revealed that both species were tolerant to 25 ºC, despite presenting different strategies against temperature exposure. At 25 ºC, the native species presented similar biochemical responses to organisms under control conditions. However, the gene expression of *Cox-1*, 1*6S* and *Hsp70* were down-regulated. These results suggest that the native species at 25 ºC may close their valves in order to reduce the effects of high temperature exposure, increasing their survival. On the contrary, the introduced species increased their ETS activity, antioxidant enzymes, the expression of genes associated with apoptosis regulation and thermal tolerance at 25 ºC, as response to warming.

In conclusion, our study provides evidence that global seawater temperature of 21 ºC may affect more the introduced species than the native one since in the introduced species detoxification mechanisms were not sufficient to avoid damages in the membrane. Nevertheless, our findings suggested that both species were tolerant to 25 ºC, presenting different strategies against temperature exposure.

Although the native species possibly adopted the behavior strategy to avoid temperature effects (21 and 25 ºC) (namely valves closure), in long-term warming exposures this strategy may influence clams heath status and, ultimately, their survival.

3.4. Salinity and pH exposure

3.4.1. Aim

The aim of the present study was to evaluate how the native clam *R. decussatus* and the introduced/invasive clam *R. philippinarum* respond to single exposure to different salinity and pH levels, through the evaluation of different biomarkers (mortality, energy sources, osmoregulation capacity, metabolic activity, cellular damages and detoxification mechanisms).

3.4.2. Materials and methods

3.4.2.1 Experimental conditions

Clams *R. decussatus* and *R. philippinarum* were collected in the Ria de Aveiro (northwest Atlantic coast of Portugal), in November 2015.

After sampling, individuals with similar weight (22.5 to 28.9 g) were selected to prevent differences in physiological and biochemical responses. Organisms were depurated in artificial seawater (salinity 28) made with artificial sea salt (Tropic Marin® SEA SALT from Tropic Marine Center) and deionized water, for 5 days, at 18.0 ± 1.0 °C, pH 7.80 ± 0.10, 12 light: 12 dark photoperiod and continuous aeration. During this period, organisms were not fed and water was renewed every other day.

After depuration, 50 organisms of each species were separated by salinity level. To reach salinities of 14 and 35, salinity was gradually (every 2–3 days) lowered or increased in 2–3 values to reach the testing levels in 3 separate groups of organisms of each species (Fig. 3.4.1). During this period, clams exposed to different salinity conditions were maintained under pH 7.8 (considered as control pH). After this period, 20 organisms of each species were submitted to a gradual pH decrease (0.2 units per day) to reach pH 7.3 and 20 individuals were maintained at pH 7.8. In both situations, salinity was maintained at 28 (Fig. 3.4.1). Acclimation to low pH was obtained by directly diffusing CO_2 into aquaria. Individual aquarium pH levels were continuously monitored and controlled using a pH Stat system (Aquamedic AT Controler). During this acclimation period, organisms were maintained under continuous aeration, at 17.0±1.0 °C and 12 light: 12 dark photoperiod. Daily dissolved oxygen concentration was monitored in all aquaria and animals were checked for mortality. All organisms were fed with Algamac protein plus (150,000 cells/organism).

Chronic exposure to salinities 14, 28 and 35 (under pH 7.8) and pH 7.8 and 7.3 (at salinity 28) were separately tested for 28 days under continuous aeration, at 17.0 ± 1.0 °C and 12 L:12D photoperiod (Fig. 3.4.1). For each condition, three replicates were used. Species were tested

separately. Each replicate consisted of five organisms per species in 15 L of artificial seawater. Two times per week and every week, animals were fed with Algamac protein plus (150.000 cells/organism), seawater was renewed, and pH and salinity levels re-established. Daily procedures included checking for mortality and dissolved oxygen concentrations monitoring.

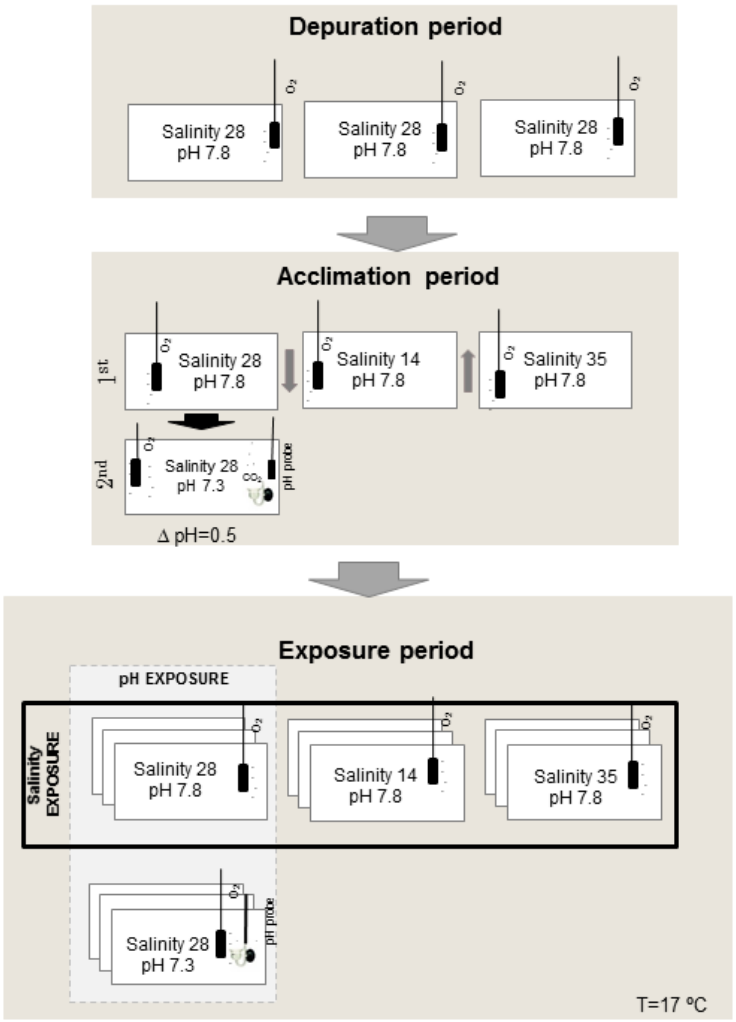

Figure 3.4.1 - Depuration, acclimation and experimental setup used for *Ruditapes decussatus* and *R. philippinarum* clams under different salinity (14, 28 and 35, pH 7.8) and pH (7.3, 7.8) levels.

Salinity 28 was used as the control taking into account the salinity at the sampling area. Salinities tested (14 and 35) were selected taking into account the range of salinities for *R. decussatus* (15–40) and *R. philippinarum* (4–40) habitats in European estuaries (Bidegain et al., 2015; Chapter 2; Cravo et al., 2012; Dang et al., 2010; Juanes et al., 2012; Smaoui-Damak et al., 2004).

For the pH experiment, the test pH levels were selected taking into account the worst predicted scenario of climate change for 2100 (ΔpH = −0.5) and the pH at the sampling area (7.8). Water was acidified by direct CO_2 diffusion into artificial seawater contained in each aquarium, to

reach pH test condition (7.3). Temperature and pH were continuously monitored, and pH was controlled by a pHstat system (Aquamedic AT Controler) and by specific probes (Hanna Instruments). pHstat system maintained targeted pH values automatically by adding CO_2 gas to individual tanks and the calibration was made using NIST buffers (NBS scale). During one week prior to exposure and the first week of exposure, seawater pH was daily crosschecked using an independent probe (Hanna Instruments). This procedure was repeated twice a week during the remaining experimental period, and the pH-Stat computer resets to match the independent probes pH, whenever needed.

During the exposure period, every week (before water renewal) water samples were collected from each aquarium to quantify total alkalinity (TA) by potentiometric titration, according to Gran (1952). TA, pH, temperature, and salinity were used to determine CO_2 partial pressure (pCO_2), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions concentrations, and the saturation states of calcite (ΩCal) and aragonite (ΩAg) for each aquarium. These concentrations were calculated using CO_2SYS software (Robbins et al., 2010; Mehrbach et al., 1973) refit by Dickson and Millero (1987) K1 and K2 carbonate dissociation constants, and KSO_4 from Dickson (1990). Physicochemical water parameters and the associated variation, for each condition, are presented in Table 3.4.1.

Table 3.4.1 - Carbonate system physicochemical parameters for each pH tested (7.8 and 7.3). Mean values (± SD) of measured pH and determined total alkalinity (TA) in water collected from each aquarium (temperature 19±1 °C and salinity 28 ± 1 g/L). Partial CO_2 pressure (pCO_2), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ion concentrations, saturation states of calcite (ΩCal) and aragonite (ΩAra) were calculated with CO_2SYS software (Robbins et al., 2010).

	pH	TA (µmol/Kg)	pCO_2 (µatm)	HCO_3^- (µmol/Kg)	CO_3^{2-} (µmol/Kg)	Ω Ara	Ω Cal
R. decussatus	7.8±0.08	2391.7±131.80	1192.0±162.35	2233.8±105.74	78.2±13.68	1.2±0.22	1.9±0.35
	7.3±0.05	2897.3±82.85	4062.6±742.39	2861.0±101.11	43.0±17.85	0.7±0.28	1.1±0.43
R. philippinarum	7.8±0.08	2350.7±47.34	1361.2±242.06	2217.0±35.01	66.2±12.44	1.1±0.20	1.7±0.32
	7.3±0.10	2801.7±139.54	4074.4±573.92	2733.6±131.24	33.9±6.82	0.7±0.32	0.7±0.09

After the exposure period (28 days), organisms of both species were frozen until analyses.

3.4.2.2 Biomarkers

Biomarkers were determined in both species (3 replicates/ condition; 4 individuals/species/replicate) from four different conditions (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). Frozen organisms (whole soft tissues) were individually pulverized with liquid nitrogen and separated into 0.5 g aliquots for physiological and biochemical analyses. For extraction, each sample (0.5 g of homogenized soft

tissues) was homogenized using ultrasounds during 15 s at 4 °C and centrifuged for 10 min at 10,000g (for all biomarkers except for electron transport system, 3,000g) and 4 °C with specific buffers for each physiological and biochemical analyses. Supernatants were stored at −80 °C or immediately used.

The biomarkers determined were: carbonic anhydrase (CA) activity, glycogen (GLY) content, electron transport system (ETS) activity, lipid peroxidation (LPO) levels, the activity superoxide dismutase (SOD) catalase (CAT) and glutathione S-transferases (GSTs), Glycogen (GLY) and protein (PROT) content.

Carbonic anhydrase (CA) activity

The activity of CA was quantified following an adaptation of the method described by Warrier et al. (2014) with modifications performed by Moreira et al. (2016a, 2016b). CA activity, extractions were made in 100 mM Tris-HCL buffer (pH 8.3): 0.1 mM EDTA 1% (w/v) 0.5% PVP (v/v) 2% Triton X-100 (v/v). For CA determination 20 µL of extracted supernatant in TRIS-HCL buffer (pH 8.3) was placed in microplate wells, with 80 µL TRIS buffer (0.1 M) and 20 ppm Bromothymol Blue. 200 µL of CO_2 saturated dH_2O (CO_2 gas was bubbled in dH_2O for 10 min, and considered saturated when pH < 3.5) was added to each sample. Bromothymol Blue conversion to yellow was immediately measured at 436 nm on a microplate reader during a min and the variation of absorbance per min (ΔABS/min) determined in triplicate for each sample. The non-enzymatic reaction rate was also determined in triplicate, following the same procedure, after denaturing samples at 100 °C for 15 min. Mean non-enzymatic reaction rate from each sample was subtracted to each sample mean. For this, a set of samples were analyzed by a standard pH assay described in Weis and Reynolds (1999). The same samples were also analyzed spectrophotometrically and a calibration curve between $\Delta pH \cdot min^{-1}$ and $\Delta ABS \cdot min^{-1}$ was established (r_2 = 0.989). The results were expressed in ΔpH·min/mg protein.

Glycogen (GLY) content

The content of GLY was quantified according to the phenol-sulfuric acid method (Yoshikawa, 1959), as described in the session 3.2.2.3. Results were expressed in mg per g of FW.

Electron transport system (ETS) activity

The activity of ETS was quantified according to King and Packard (1975) and the modifications performed by Coen and Janssen (1997) (see section 3.3.2.2). The absorbance was read at 490 nm during 10 min with intervals of 25 s (15.900 M^{-1} cm^{-1} extinction coefficient). Results were expressed in nmol per min per mg protein.

Lipid Peroxidation (LPO) levels

The levels of LPO were quantified according to the protocol described by Buege and Aust (1978) (see section 2.2.2.6). This method measures the amount of TBARS, based on the reaction of LPO by-products, such as malondialdehyde (MDA), with 2-thiobarbituric acid (TBA), forming TBARS. The absorbance used to quantify the amount of TBARS was 532 nm (1.56×10^5 M^{-1} cm^{-1} extinction coefficient), and results expressed in nmol of MDA equivalents per mg protein.

Superoxide dismutase (SOD) activity

The activity of SOD was determined based on the method of Beauchamp and Fridovich (1971) (see section 2.2.2.6). SOD activity was measured at 560 nm, after 20 min of incubation at room temperature. Results were expressed in U per mg protein. One unit (U) of enzyme activity corresponds to a reduction of 50% of nitro blue tetrazolium (NBT), per min.

Catalase (CAT) activity

The activity of CAT was determined according to Johansson and Borg (1988), with some modifications (Freitas et al., 2012), as described in the session 2.2.2.6. Results were expressed in U per mg protein. One unit (U) represents the formation of 1.0 nmol formaldehyde, per min.

Glutathione S-transferases (GSTs) activity

The acticity of GSTs were determined following the method described by Habig et al. (1974) in the session 2.2.2.6, at 340 nm (9.6 $mM^{-1}cm^{-1}$) during 5 min. Results were 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.

Protein (PROT) content

The concentration of PROT was assayed using the Biuret method Robinson and Hogden (1940), at 540 nm. Bovine serum albumin (BSA) was used as a standard. PROT quantification was used to express the above biomarkers per mg of protein.

3.4.2.3. Statistical analyses

Biochemical parameters obtained from each species and tested condition were submitted to hypothesis testing using permutational multivariate analysis of variance, employing the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008).

The null hypotheses tested were, for each parameter: i) for each species, no significant differences exist among salinities (pH 7.8); ii) for each salinity tested (pH 7.8), no significant differences exist between species; iii) for each species, no significant differences exist between pH conditions (salinity 28); iv) for each pH condition, no significant differences exist between species (salinity 28).

Significance levels ($p \leq 0.05$) among salinities (14, 28 and 35) and among pH conditions (7.8 and 7.3) were presented with different letters (lowercase letters for *R. decussatus* and uppercase letters for *R. philippinarum*). Significant differences ($p \leq 0.05$) between species, at each salinity and pH condition, were presented with asterisks.

3.4.3. Results

3.4.3.1 Mortality

Both species presented a similar mortality pattern when exposed to salinity 14 and 35, with higher percentage of mortality at salinity 14 (13% for *R. decussatus* and 30% for *R. philippinarum*) compared to salinity 35 (6% for *R. decussatus* and 5% for *R. philippinarum*). No mortality was observed at salinity 28 in organisms from both species exposed to both pH conditions (7.8 and 7.3).

3.4.3.2. Biomarkers

Carbonic anhydrase (CA) activity

In *R. decussatus*, the CA activity increased significantly at salinity 35, while at salinity 14 decreased when compared to salinity 28 (Fig. 3.4.2A). In *R. philippinarum* a significant induction of CA activity was observed at salinities 14 and 35 when compared to salinity 28 (control) (Fig. 3.4.2A).

Regarding pH results, *R. decussatus* showed no significant differences in CA activity when exposed to both pH conditions while *R. philippinarum* presented significantly higher CA activity at pH 7.3 than at pH 7.8 (Fig. 3.4.2B).

Comparing both species at each salinity and pH conditions, results showed lower CA activity in *R. decussatus* than in *R. philippinarum*, for all tested conditions (Fig. 3.4.2A and B).

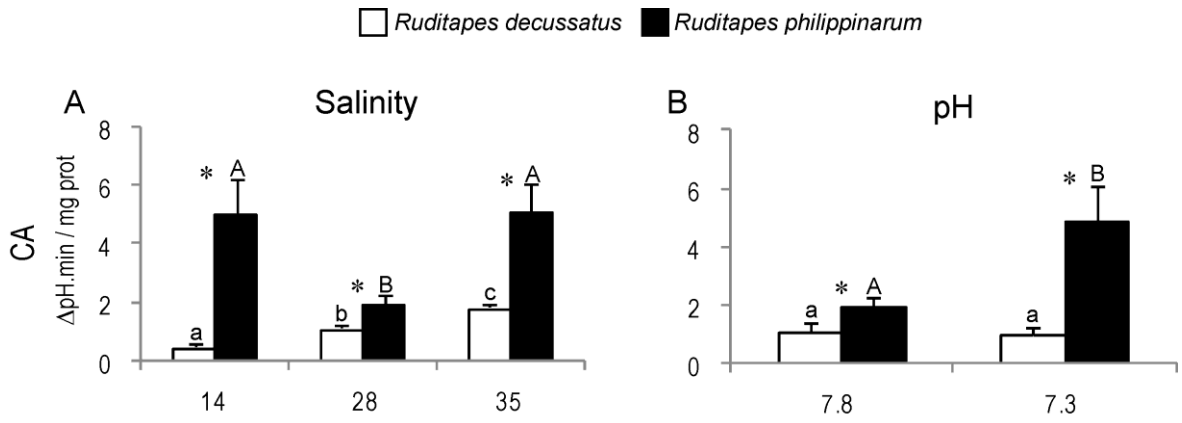

Figure 3.4.2 - Carbonic anhydrase activity (CA, ΔpH.min/mg prot) in *Ruditapes decussatus,* and *R. philippinarum*, exposed to different salinity (14, 28, 35) (A) and pH levels (7.8, 7.3) (B) (mean±SD). For each species, significant differences ($p \leq 0.05$) among different salinity and pH levels are represented with different letters (a-c for Rd and A-B for Rp). Significant differences ($p \leq 0.05$) between species, at each salinity and pH level, are represented with an asterisk.

Glycogen (GLY) content

The content of GLY was significantly higher in *R. decussatus* individuals at salinity 28 (control) than in organisms at salinities 14 and 35 (Fig. 3.4.3A). *R. philippinarum* showed a different pattern, with significantly higher glycogen content in organisms exposed to salinities 14 and 28 (control) compared to organisms under salinity 35 (Fig. 3.4.3A).

Results also demonstrated that *R. decussatus* and *R. philippinarum* exposed to both pH levels (7.8 and 7.3) showed no significant differences in terms of GLY content (Fig. 3.4.3B).

Comparing both species at each salinity and pH tested, in general, results showed that *R. decussatus* presented significantly higher GLY content than *R. philippinarum* at all tested conditions, except at salinity 14 (Fig. 3.4.3A and B).

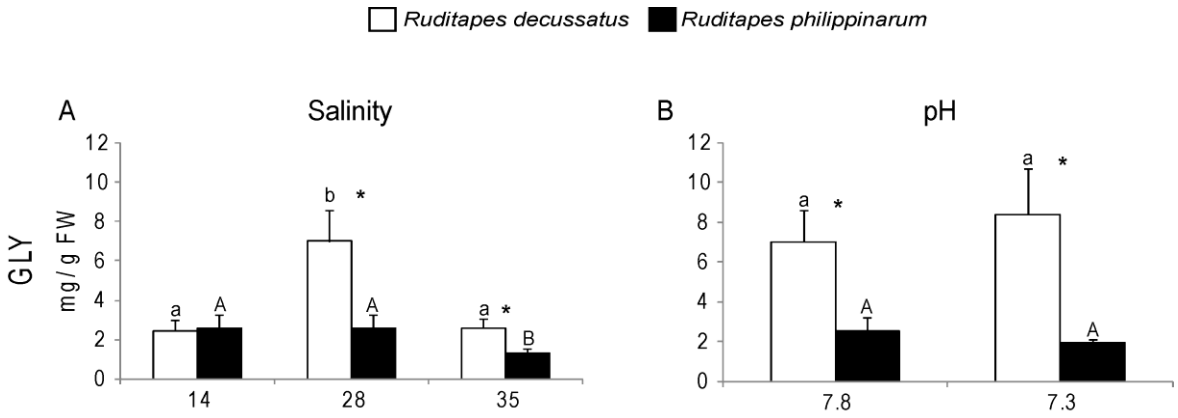

Figure 3.4.3 - Glycogen content (GLY, mg/g FW) in *Ruditapes decussatus,* and in *R. philippinarum,* exposed to different salinities (14, 28, 35) (A) and pH levels (7.8, 7.3) (B) (mean±SD). For each species, significant differences ($p \leq 0.05$) among different salinity and pH levels are represented with different letters (a-b for Rd and A-B for Rp). Significant differences ($p \leq 0.05$) between species, at each salinity and pH level, are represented with an asterisk.

Electron transport system (ETS) activity

The activity of ETS in *R. decussatus* was significantly higher at salinity 14, while at salinity 35 was significantly lower than at salinity 28 (Fig. 3.4.4A). On the contrary, *R. philippinarum* increased the ETS activity at salinity 35, while at salinity 14 decreased when compared to the control (Fig. 3.4.4A). Significant differences were reported between clams at salinity 14 and 35.

Relatively to pH exposure, results also showed that the ETS activity presented no significant variations for *R. decussatus* when exposed to both pH conditions while for *R. philippinarum* the ETS activity was significantly lower at pH 7.3 than at 7.8 (Fig. 3.4.4B).

Comparing both species, results demonstrated that at salinity 14 and pH 7.3 *R. decussatus* showed higher ETS activity than *R. philippinarum*, while in the remaining tested conditions the opposite was observed (Fig. 3.4.4A and B).

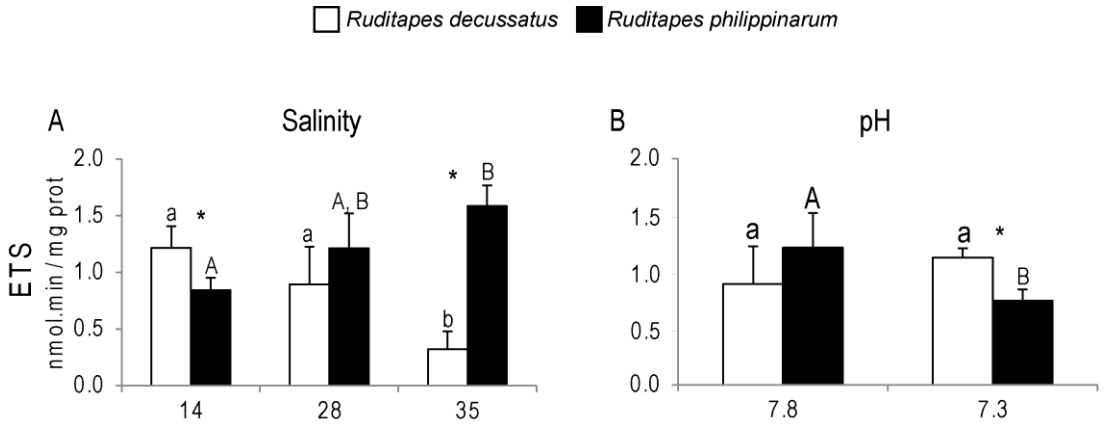

Figure 3.4.4 - Electron transport system activity (ETS, nmol.min/mg prot) in *Ruditapes decussatus,* and in *R. philippinarum,* exposed to different salinity (14, 28, 35) (A) and pH levels (7.8, 7.3) (B) (mean±SD). For each species, significant differences ($p \leq 0.05$) among different salinity and pH levels are represented with different letters (a-b for Rd and A-B for Rp). Significant differences ($p \leq 0.05$) between species, at each salinity and pH level, are represented with an asterisks.

Lipid peroxidation (LPO) levels

The levels of LPO were significantly higher in *R. decussatus* exposed to salinities 14 and 35 than to salinity 28 (control) (Fig. 3.3.5A). In *R. philippinarum* significantly higher LPO levels were observed in organisms at salinities 35 compared to salinities 14 and 28 (Fig. 3.4.5A).

The levels of LPO were similar for *R. decussatus* exposed to both pH conditions (7.8 and 7.3) (Fig. 3.3.5B), while *R. philippinarum* presented higher LPO levels at pH 7.3 than at 7.8 (control) (Fig. 3.4.5B).

At each salinity and pH tested, both species presented similar LPO levels at salinities 14 and 35 while at salinity 28 and both pH conditions *R. decussatus* presented lower LPO levels than *R. philippinarum* (Fig. 3.4.5A and 3.4.5B).

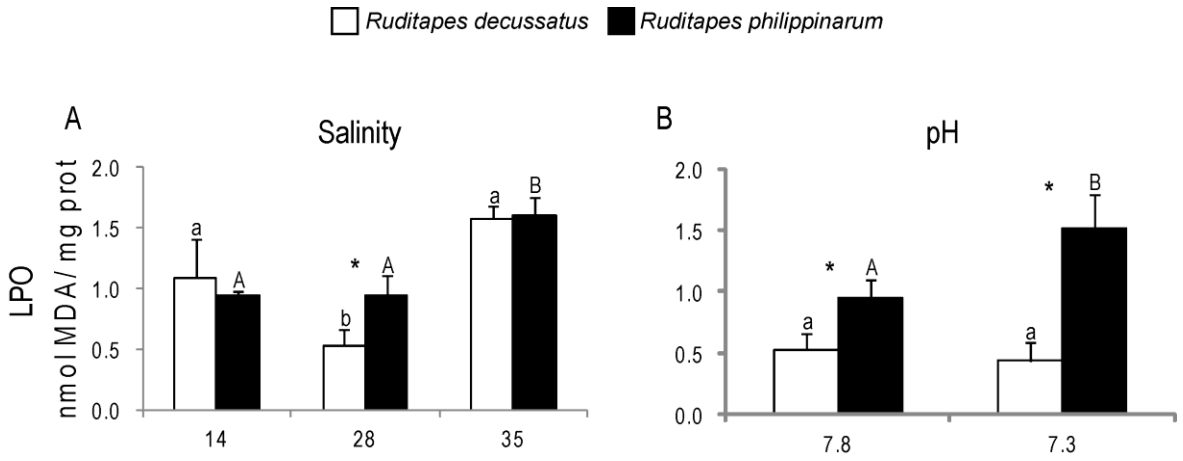

Figure 3.4.5 - Lipid peroxidation content (LPO, nmol MDA/mg prot) in *Ruditapes decussatus,* and in *R. philippinarum,* exposed to different salinities (14, 28, 35) (A) and pH levels (7.8, 7.3) (B) (mean±SD). For each species, significant differences ($p \leq 0.05$) among different salinity and pH levels are represented with different letters (a-b for Rd and A-B for Rp). Significant differences ($p \leq 0.05$) between species, at each salinity and pH level, are represented with an asterisk.

Superoxide dismutase (SOD) activity

The activity of SOD was significantly lower in individuals of both species exposed to salinity 28 compared with organisms under salinities 14 and 35 (Fig. 3.4.6A).

When under different pH levels *R. decussatus* presented no significant differences in SOD activity between pH conditions, while SOD activity was significantly induced at pH 7.3 for *R. philippinarum* (Fig. 3.4.6B).

Catalase (CAT) activity

In *R. decussatus*, CAT activity was higher in individuals exposed to salinity 28 than in organisms under salinities 14 and 35, with significant differences between salinities 28 and 35 (Fig. 3.3.6C). In *R. philippinarum* an induction of CAT activity was observed in organisms under salinities 14 and 35 compared to organisms under salinity 28 (Fig. 3.4.6C).

Results also showed that pH 7.3 significantly inhibited the activity of CAT in *R. decussatus* individuals comparing to values obtained in organisms under pH 7.8 (control) (Fig. 3.4.6D). In addition, *R. philippinarum* clams at pH 7.3 presented higher CAT activity than specimens at pH 7.8, but no significant differences were found between both pH conditions (Fig. 3.4.6D).

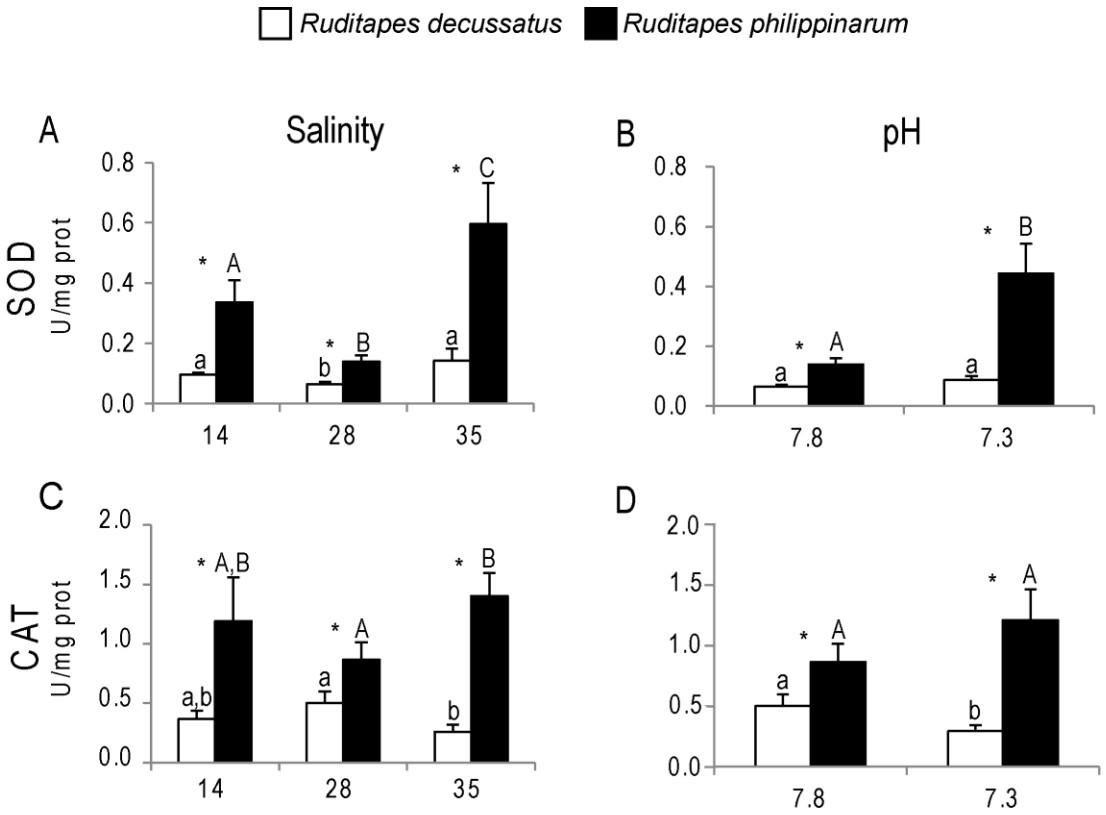

Figure 3.4.6 - Superoxide dismutase and catalase activity (SOD , CAT, U/mg prot) in *Ruditapes decussatus,* and in *R. philippinarum,* exposed to different salinities (14, 28, 35) (A,C) and pH levels (7.8, 7.3) (B,D) (mean±SD). For each species, significant differences ($p \leq 0.05$) among different salinity and pH levels are represented with different letters (a-b for Rd and A-C for Rp). Significant differences ($p \leq 0.05$) between species, at each salinity and pH level, are represented with an asterisk.

Glutathione-S-transferases (GSTs) activity

The activity of GSTs was significantly induced at salinity 14 compared with GSTs activity at salinities 28 and 35 in *R. decussatus*. GSTs activity in *R. philippinarum* showed no significant differences among all salinities tested (Fig. 3.4.7A).

Overall, both species presented higher GSTs activity at pH 7.3 than pH 7.8 (Fig. 3.4.7B).

The comparison of both species at each salinity and pH condition revealed that, in general, *R. decussatus* exhibited significantly lower SOD, CAT and GSTs activities than *R. philippinarum* (Fig. 3.4.6 and 3.4.7).

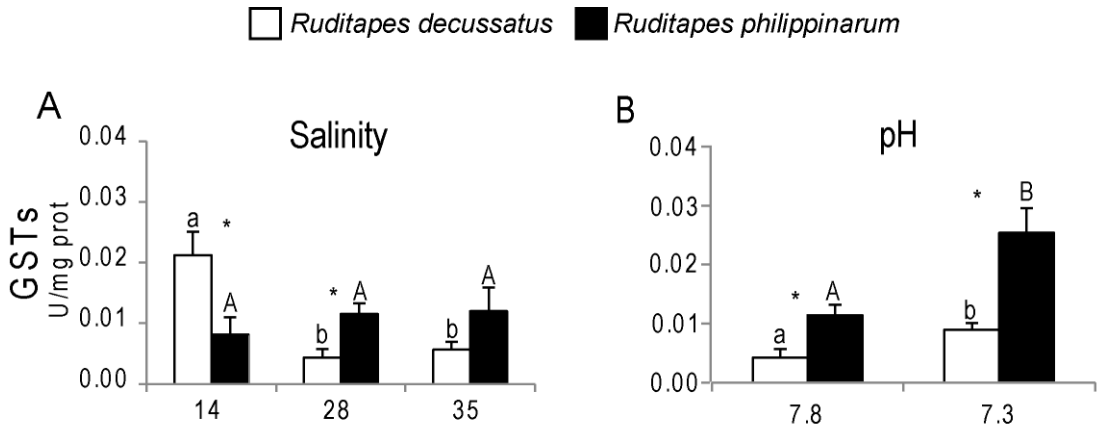

Figure 3.4.7 - Glutathione *S*-transferases (GSTs) activities in *Ruditapes decussatus,* and in *R. philippinarum,* exposed to different salinity (14, 28, 35) (A) and pH levels (7.8, 7.3) (B) (mean±SD). For each species, significant differences ($p \leq 0.05$) among different salinity and pH levels are represented with different letters (a-b for *R. decussatus* and A-B for *R. philippinarum*). Significant differences ($p \leq 0.05$) between species, at each salinity and pH level, are represented with an asterisk.

3.4.4. Discussion

In a near future, the frequency and intensity of salinity and pH fluctuations in aquatic ecosystems as a direct result of human activities are predicted to increase (Caldeira and Wickett, 2005). These salinity and pH shifts may induce strong impacts on aquatic organisms, which may favor the conditions for invasive species to spread into new ecosystem areas (Gestoso et al., 2016; Gosling, 2015). Gosling (2015) demonstrated that the native mussel *Mytilaster minimus* and the introduced mussel *Brachidontes pharaonis* revealed different heart beat rates at different salinities, revealing that the invasive species have the potential to invade most of the transitional environments across the entire Mediterranean basin. According to Gestoso et al. (2016), different responses to seawater acidification were also found for the native *Mytilus galloprovincialis* and non-indigenous *Xenostrobus securis* mussels, demonstrating that seawater acidification will pose a greater risk for survival and growth of the native mussels than in the invasive species. Nevertheless, up to now few studies reported differences between invasive and native species under predicted salinity shifts and seawater acidification conditions (Carregosa et al., 2014; Gestoso et al., 2016; Gosling, 2015; Matozzo et al., 2013). Therefore, the present study provides relevant information on the response of the native clam *R. decussatus* and the introduced clam *R. philippinarum* exposed to different salinity (14, 28 and 35) and pH (7.8 and 7.3) levels through the comparison of species physiological and biochemical responses.

3.4.4.1 Salinity exposure

Previous studies revealed the capability of *R. philippinarum* and *R. decussatus* to tolerate salinities from 14 to 42 and 7 to 42, respectively, during a short exposure period (96 h), with no mortality reported (Carregosa et al., 2014). The present study further demonstrated low-moderate mortality after chronic exposure to salinities 14 and 35 for both species, although at the lowest salinity the invasive species presented two times higher mortality (30%) than the native one (13%).

CA is one of the enzymes involved in the osmoregulation and acid-base balance of estuarine organisms (Sattin et al., 2010) and, therefore, this enzyme can be affected by salinity (Dickinson et al., 2012; Monserrat et al., 2007). The obtained results support the role of CA in osmoregulation since the native species was able to inhibit (14) or induce (35) CA activity according to the salinity in the surrounding environment. The inhibition of CA was also reported in the oysters *Crassostrea virginica* exposed to salinity 15, suggesting that CA activity reduction can negatively affect shell growth and/or lead to acidosis (Dickinson et al., 2012). Furthermore, the present study showed an induction of CA activity at salinity 14 and 35 in *R. philippinarum* when comparing with salinity 28, which may be related to species ability to balance ion homeostasis. It is known that CA can be unregulated in response to both hyper- and hypo-osmotic regulation in euryhaline marine organisms (Moreira et al., 2016b; Roy et al., 2007). Comparative analysis of both species, at all salinities tested, showed higher CA alterations in *R. philippinarum* than in *R. decussatus,* suggesting different species adaptation strategies to different salinities.

When exposed to stressful environmental conditions, organisms can increase their energy expenditure as a mechanism of cellular protection, allowing successful acclimation to stress conditions (Sokolova et al., 2012). The present findings showed that both stress salinities (14 and 35), affected the GLY storage in *R. decussatus*, indicating the mobilization of this source of energy towards cells protection mechanisms. *R. philippinarum* presented lower GLY content at salinity 35 than at salinities 14 and 28, suggesting the use of energy at higher salinity to activate defense mechanisms, while at salinity 14 clams may reduce energy expenditure by closing their valves. On the contrary, to our finding, Dickinson et al. (2012) demonstrated that *C. virginica* was able to maintain the GLY content when exposed to salinities 15 and 30, during a chronic exposure (11 weeks). The present study further revealed that the native species presented only higher GLY stores than introduced species at salinities 28 and 35.

ETS activity provides information about the potential metabolic activity and can be used as an indicator of organisms physiological status (Choi et al., 2001; Nahrgang et al., 2013). ETS activity can be affected by temperature, pH, food availability, hypoxia, hyperoxia and pharmaceutical drugs (Carbamazepine) (Choi et al., 2001; Freitas et al., 2016; Nahrgang et al., 2013). The present study also demonstrated that salinity affected the ETS activity in both clam species. *R. decussatus* decreased the ETS activity at salinity 35 when compared to the remaining conditions, suggesting partial damage to the inner mitochondrial membrane caused by LPO at

salinity 35. An opposite trend was observed in *R. philippinarum* at salinity 35. These results suggested that *R. philippinarum* induced the energy production by ETS, which may be related to the decrease of GLY possibly due to the cost of energy associated with antioxidant mechanisms (SOD). It is known that ETS activity increase can be related to the activation of the respiratory chain due to an increase in energy costs associated with detoxification processes (Choi et al., 2001). Comparing both species, results showed that at salinities 28 and 35 *R. philippinarum* presented higher ETS activity than native species, which is possibly associated to basal glycogen stores for each species at salinity 28 (control) and different use of energy reserves as mechanisms of stress defense by both clams at salinity 35.

The present study further demonstrated that salinities 14 and 35 induced oxidative damages in *R. decussatus*. These results suggested that salinities 14 and 35 lead to an unbalance in reactive oxygen species (ROS)/reactive nitrogen species production and an attack to lipid membranes by ROS causing, consequently, oxidative stress. On the other hand, in *R. philippinarum* LPO only increased at salinity 35, suggesting that this salinity induced higher oxidative damages than the remaining salinities, due to this fact cells increased energy production (ETS activity) to be used in mechanisms of stress defense at salinity 35. However, an increase of LPO may also be responsible for an increase of ETS activity, since this system is one of the major sites of ROS generation (Liu et al., 2012).

Results also demonstrated that, although *R. decussatus* induced SOD activity against superoxide radicals produced by extreme salinities, CAT was inhibited. These results indicated that hydrogen peroxide produced by SOD may have been detoxified by glutathione peroxidase. The present study showed an induction in GSTs activity only at salinity 14 in *R. decussatus*, protecting clams against oxidative stress. Changes in GSTs and CAT activity in the oyster species, *Crassostrea rhizophorae*, exposed to different salinities was also reported by Zaccaron da Silva et al. (2005). On the other hand, *R. philippinarum* exposed to salinities 14 and 35 induced antioxidant enzymes activity indicating that this species induced antioxidant enzymes as defense mechanisms to prevent and intercept ROS, as well as repair mechanisms for oxidized components. However, these mechanisms seemed not to be sufficient to avoid LPO. When analyzing antioxidant and biotransformation enzymes for both species, at each salinity, results showed that in general *R. philippinarum* presented higher activity of antioxidant enzymes than native species, while in the case of GSTs activity this pattern was only observed at salinity 28. Carregosa et al. (2014) demonstrated that different clam species presented different responses when exposed to a wide range of salinities for 96 h since at salinity 14 LPO and antioxidant enzymes increased while at salinity 35 antioxidant enzymes activity was inhibited. Matozzo et al. (2013) demonstrated that salinity can increase LPO and induce/inhibit detoxification mechanisms (CAT and GSTs), depending on bivalve species.

3.4.4.2. pH exposure

Regarding pH exposure, the results obtained showed that both clam species survived to all pH conditions. The present study also demonstrated that the native clam showed similar CA activity, GLY content, LPO levels, ETS, and SOD activity under pH 7.8 and 7.3, whereas low pH inhibited CAT activity and induced GSTs activity. These results suggest that native species conserved energy and maintained acid-base balance under low pH conditions, while increased GSTs to mediate oxidative stress and to maintain the integrity of lipid membranes. Marine organisms may tolerate high CO_2 levels (low pH) due to low activity lifestyle or pre-adaption to large fluctuations in environmental parameters (Gazeau et al., 2013; Pörtner et al., 2004). According to Fernández-Reiriz et al. (2011), when exposed to acidic conditions, *R. decussatus* is able to reduce its standard metabolic rate, through the reduction of clearance, ingestion, and respiration rates. This may also explain why, in the present study, *R. decussatus* generally maintained the physiological and biochemical responses at low pH. On the contrary, the present results demonstrated that the introduced *R. philippinarum*, when exposed to low pH, increased the activity of antioxidant and biotransformation enzymes. These results, in the introduced species, demonstrated an evident relationship between the increase of antioxidant enzymes and LPO levels since these antioxidant defense mechanisms were possibly overwhelmed by ROS produced in organism tissues, inducing LPO. In addition, the results also revealed an induction of CA activity at low pH to compensate acid-base disturbance in *R. philippinarum*, being the decrease of ETS activity a consequence of various processes, such as oxidative stress and damages in the lipid membrane. Similarly to the results obtained the clam *Scrobicularia plana* exposed to low pH (7.1) decreased the ETS activity, suggesting that decreased their metabolic activity as a defense mechanism (Freitas et al., 2016). These authors also observed that *S. plana* induced CAT activity while increasing the GSTs activity and LPO levels. Timmins-Schiffman et al. (2014) reported that low pH caused a decrease in electron supply from NADH to the electron transport chain in the oyster *Crassostrea gigas* and this response mechanism changed the cellular balance between resource supply and oxidative stress. Moreira et al. (2016b) reported that *Crassostrea angulata* and *C. gigas* chronically exposed to low pH (7.3) induced CAT activity while CA was inhibited. These authors also reported species-specific differences regarding LPO levels and CAT activity.

3.4.5. Conclusion

The present findings indicated that salinity changes may affect the native clam *R. decussatus* and the non-native clam *R. philippinarum*. Extreme salinities induced higher oxidative damages in the native species (*R. decussatus*) at both salinities 14 and 35 than in the introduced clam (*R. philippinarum*). Similar results were observed in the introduced species at salinity 35, while at salinity 14 *R. philippinarum* was able to induce antioxidant defense (SOD and CAT activity) and osmotic regulation (CA activity) mechanisms. On the other hand, ocean acidification proved to induce more oxidative stress in the introduced than in the native species, with *R. philippinarum* revealing higher LPO levels.

Overall, the native clam *R. decussatus* was most affected by salinity changes, while the introduced clam *R. philippinarum* showed to be especially vulnerable to low pH. Therefore, it is possible to state that salinity and pH will influence the spatial distribution of these species according to the predicted scenario of climatic changes. Thus, the present results suggested that predicted climate change scenarios will contribute for the replacement of the native species by the introduced one since salinity shifts will often occur while pH decrease will take longer time to be reached.

3.5. Arsenic, temperature, salinity and pH on native and introduced species

The summarized responses of the native clams *Ruditapes decussatus* and *Ruditapes philippinarum* exposed to a wide range of As concentrations, temperatures, salinity and pH are presented in Figure 3.5.1. Although this chapter compares the performance of both species under different stressors, it is important to emphasize that the temperature responses in both species were determined in a specific organ (gills), while the responses to the remaining stressors were performed in the whole soft tissues.

In marine systems, As contamination may have effects on marine and estuarine organisms (Ahmad et al., 2013; Chen, 2014; Rajkumar and Rajkumar, 2013; Wu et al., 2013). The acute exposure of both native and introduced species to different As concentrations revealed similar tolerance to As for both species, partially proven by similar LC_{50}, mortality and As accumulation. Although both species presented different detoxification mechanisms when exposed up to 8.2 mg/L of As, both were not able to prevent membrane damages induced by ROS at 17 and 34 mg/L of As, resulting in oxidative stress and death (Fig. 3.5.1).

Regarding temperature, the present results revealed that native species were more tolerant to 21 ºC than the introduced one (Fig. 3.5.1). These results may be explained by the capacity of *R. decussatus* to induce metabolic activity, heat shock protein expression, and detoxification mechanisms to avoid membrane damages, while, the introduced species, despite being able to induce these mechanisms, were not sufficient to avoid LPO, resulting in oxidative stress (Fig. 3.5.1). The present study further revealed that both species were tolerant to 25 ºC, despite presenting different strategies against temperature exposure.

Salinity is one of the most important environmental factors affecting bivalves (Hamer et al., 2008; Matozzo and Marin, 2011). The present results demonstrated higher tolerance of the introduced species to salinity 14 than the natives (Fig. 3.5.1). This fact may be justified by the capacity of antioxidant and biotransformation enzymes of the introduced species to avert LPO, whereas, in the native these mechanisms were not sufficient, resulting in damages at low salinity levels. The present findings revealed that salinity 35 induced oxidative stress in both species, presenting both species lower tolerance to salinity 35 when compared to salinity 28.

Besides salinity and temperature changes in marine systems, according to IPCC 2007, 2014, a decrease of pH levels is expected at the end of the 21st century, posing major threats to marine bivalves (e.g. Berge et al., 2006; Dickson et al., 2012; Moreira et al., 2016a). The present results showed higher tolerance to low pH of native species than the introduced, through efficient detoxification mechanisms, averting LPO, while on the other hand, the exposure of the introduced species resulted in oxidative stress and induction of acid-base mechanisms (Fig. 3.5.1).

Comparing the impacts caused by the different climate change related factors, the present findings further suggested that the introduced species was the most affected by water temperature of 21 ºC, salinity 35 and low pH (Fig. 3.5.1). Interestingly, the native species showed to be only affected by salinity changes (14 and 35), raising the concern on the acceleration of rainfall and evaporation cycle due to global warming, salinity shifts occur, which ultimately may lead to increasing pressure on the native species, contributing to the replacement or decrease in their abundance in marine ecosystems. The present results showed that, within certain parameters, it is possible to predict native and introduces species response to future climate change. However, their response needs to be further investigated, namely investigating the responses of *R. decussatus* and *R. philippinarum* populations from different geographical areas.

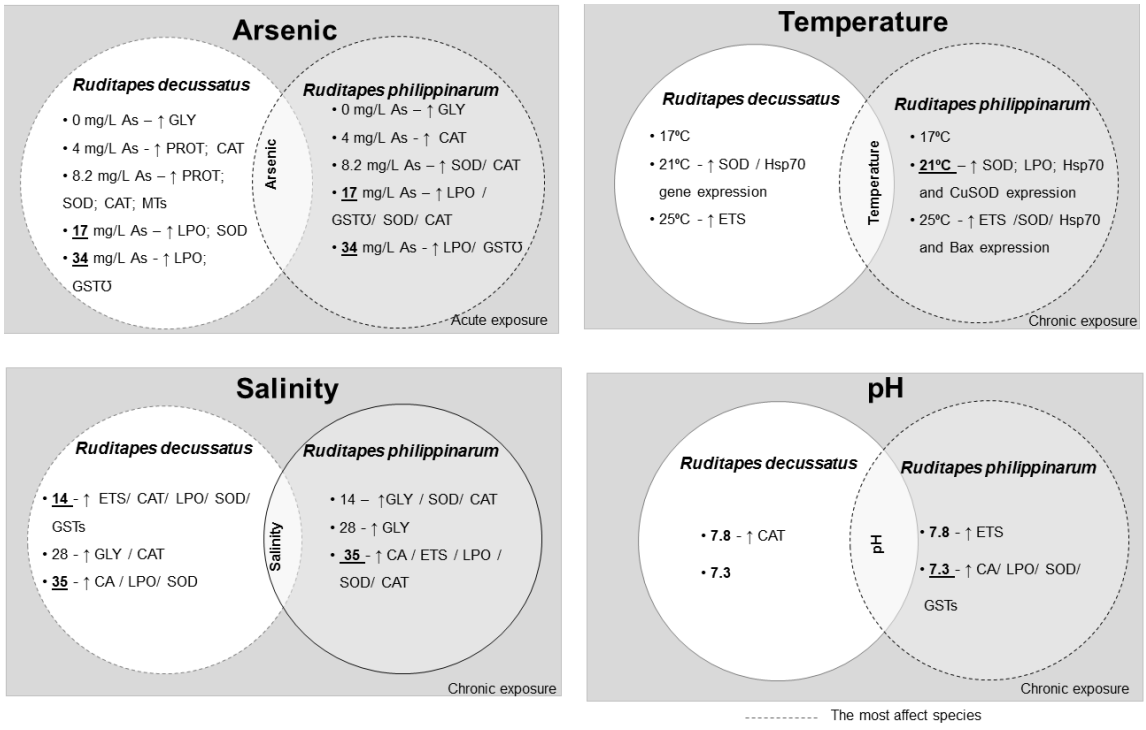

Figure 3.5.1 - Summarized response of *Ruditapes decussatus* and *R. philippinarum* under As exposure, temperature, salinity and pH.

The comparison of native and introduced species response under similar conditions is present in Figure 3.5.2. When comparing both species exposed to the same As concentration, the native clams presented higher GLY content, antioxidant and biotransformation (GSTs) activities than the introduced species (Fig. 3.5.2). Additionally, temperature, salinity, and pH, results showed that, in general, the native species presented lower levels of antioxidant, biotransformation enzymes and LPO than *R. philippinarum* (Fig. 3.5.2). On the other hand, an opposite tendency was observed for GLY content under salinity and pH shifts. These findings suggested that both species presented physiological and biochemical species-specific responses.

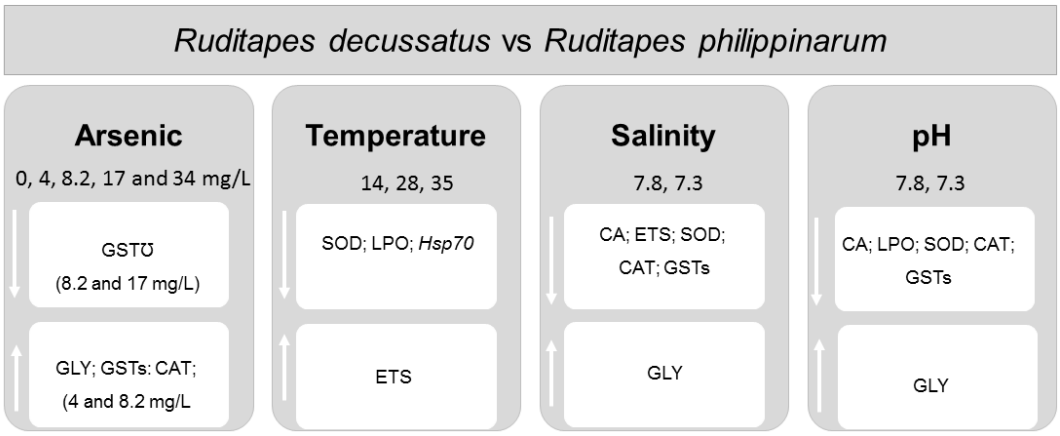

Figure 3.5.2 – Summarized comparison of *Ruditapes decussatus* and *R. philippinarum* response. ↑ represented higher levels of biomarkers in *R. decussatus* when compared to *R. philippinarum* and ↓ represented lower levels of biomarkers in *R. decussatus* than in *R. philippinarum.*

Overall, the present study was successful in increasing knowledge on predicted effects of As contamination, temperature, salinity and pH changes on relevant ecological and economical bivalves species, namely the native clam *R. decussatus* and the introduced clam *R. philippinarum*.

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Chapter 4

Organisms' response to combined stressors

This chapter is based on the following manuscript:

Velez C., Figueira E., Soares A. M.V.M, Freitas R. (2016) The impacts of As accumulation under different pH levels: comparing *Ruditapes decussatus* and *Ruditapes philippinarum* biochemical performance. Environmental Research. 151, 653-662.

4.1. 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 to 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 (0.5–8.8 µg/L of As) and sediment (0.82–220 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 (Fattorini et al., 2004; Flora, 2014). 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; Vernberg, 2012; 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) (Chenglong et al., 2013; Diniz et al., 2008; Freitas et al., 2016; Rajkumar and Rajkumar, 2013; Vernberg, 2012; Wu et al., 2013a; Yu et al., 2016).

Industrialization has also led to the increase of atmospheric CO_2 concentrations, consequently influencing CO_2 and pH levels in the aquatic environment (Billé et al., 2013), with 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 demonstrated the effects induced by seawater acidification in bivalves (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 CO_2, 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, 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; 2016b). Both species present high capacity to accumulate metals and As concentrations from water and sediment (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 contaminant, including Hg (Velez et al., 2016b), and Cd (Moraga et al., 2002).

Studies have already demonstrated that metal(loid)s may disturb energy metabolism and osmotic regulation as well as the increase of oxidative stress in Ruditapes species (Ji et al., 2015; Vernberg, 2012; Velez et al., 2016a, 2016b; Wu et al., 2013a, b). *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). Nevertheless, no studies have assessed the impacts generated by the combined effect of low pH and As contamination on *R. decussatus* and *R. philippinarum*, which is the focus of the present study. For this, both species were exposed to two pH levels (7.8, control; 7.3) combined with As exposure (0 and 4 mg/L) for 28 days, and clams bioaccumulation capacity, human health risks associated to clams consumption, and alterations on oxidative stress and energy reserves were evaluated.

4.2. Materials and Methods

4.2.1. Experimental conditions

Clams *R. decussatus* and *R. philippinarum* were collected in the Ria de Aveiro (northwest Atlantic coast of Portugal), in November 2014. In order to minimize the effect of body weight on organisms responses and accumulation, organisms with similar weight (17-20 g) were selected.

Organisms were maintained for 7 days at 19.0±1.0 °C, pH 7.80±0.10, 12 light (L):12 dark (D) 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 (Fig. 4.1). For this, in each aquarium, seawater was bubbled with CO_2. To reach low pH levels, in the corresponding aquaria, pH was gradually decreased (0.1-0.2 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 Controler). 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 (Fig 4.1). Arsenic concentration was prepared with arsenate (Na_2HAs^{4-}), the least toxic form of inorganic As (Hughes et al., 2011). The As concentration tested was selected taking into account the As tolerance of both species (Chapter 3.4), the As concentration present in tissues of both species collected from different ecosystems (0.6-64 dry weight, DW) (Sfriso et al., 2008; Velez et al., 2015a), as well as the As concentration found in seawater (0.5-8.8 mg/L) (Smedley and Kinniburgh, 2002) and sediments (0.82-220 mg/Kg DW) (Hédouin et al., 2009; Mamindy-Pajany 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 (ΔpH = - 0.5 units, Caldeira and Wickett, 2005; IPCC, 2013) and the pH range found in the Ria de Aveiro (6.82-7.8) (Velez et al., 2015a).

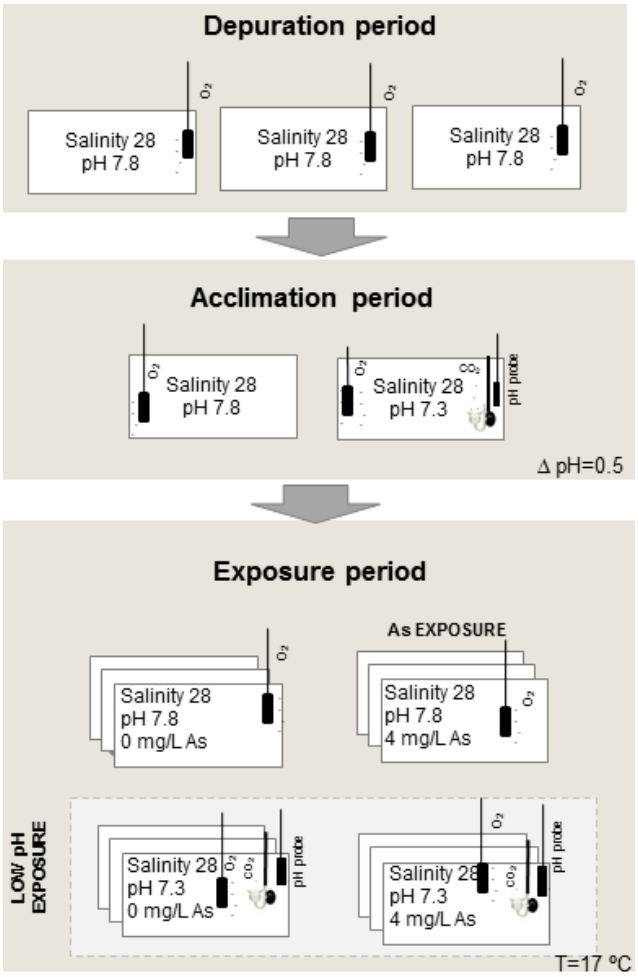

Figure 4.1 – Depuration, acclimation and experimental design of *Ruditapes decussatus* and *R. philippinarum* under different pH levels (7.8 and 7.3) and As concentrations (0 and 4 mg/L).

During the exposure period (28 days), organisms were maintained in 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 Controler) was used to control automatically pH in each aquarium, through the addition of CO_2 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 (TA) by potentiometric titration (Gran, 1952). TA, pH, temperature and salinity were used to determine CO_2 partial pressure (pCO_2), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions concentrations, and the saturation states of calcite (ΩCal) and aragonite (ΩAg) for each aquarium. These concentrations were calculated using CO_2SYS software (Robbins et al., 2010) with K1 and K2 carbonate dissociation constants

(Mehrbach et al., 1973) refit by Dickson and Millero (1987), and KSO_4 from Dickson (1990). Physicochemical water parameters and the associated variation, for each condition, are presented in Annex X.

Aquaria were daily checked for mortality, and dead organisms removed whenever identified. At the end of the exposure09uy organisms were frozen until analysis. Before analyses, organisms were individually pulverized and homogenized with liquid nitrogen and distributed into aliquots, each one containing 0.5 g of tissue.

4.2.2. Quantification of As and BCF

Quantification of As was determined in both species (3 replicates/ condition; 1 individual/replicate) from four different conditions (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).

For As quantification, 0.5 g of soft tissue of each species was digested overnight with 2 mL of concentrated HNO_3: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 concentration determined in organisms from each condition and each species by the nominal exposure concentration (4 mg/L) (McGeer et al., 2003).

4.2.3. Biochemical analyses

For each biomarker, 0.5 g of whole soft tissue of each organisms (4 individual/replicate/species; 3 replicates/condition) was used. Biomarkers were determined in both species from four different conditions (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).

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) content, lipid peroxidation (LPO) levels, activity of antioxidant enzymes (superoxide dismutase, SOD, catalase

CAT) and biotransformation enzymes (glutathione *S*-transferase, GSTs), ratio between reduced (GSH) and oxidized (GSSG) glutathione, alkaline phosphatase (ALP) and total soluble protein .

All biomarkers measurements were performed using a microplate reader (Biotek).

Glycogen (GLY) content

The content of GLY was quantified by the phenol-sulphuric acid method, as described by Yoshikawa, (1959) in the session 3.2.2.3. 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.

Lipid peroxidation (LPO) levels

The levels of LPO were measured based on Buege and Aust (1978) as described in the session 2.2.2.6. 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 x 105 M^{-1} cm^{-1} extinction coefficient). The results were expressed in nmol of MDA equivalents per mg of protein.

Superoxide dismutase (SOD) activity

The activity of SOD was measured according to Beauchamp and Fridovich (1971) as described in the session 2.2.2.6, 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.

Catalase (CAT) activity

The activity of CAT was determined following Johansson and Borg (1988) method, as described in the session 2.2.2.6. Formaldehyde standards (0–150 µM) were used as the standard curve. The absorbance was read at 540 nm. Results were expressed in U per mg of protein. One unit (U) of enzyme activity represents the formation of 1.0 nmol formaldehyde, per min.

Glutathione S-transferase (GSTs) activity

The activity of GSTs was measured according to Habig et al. (1974) as described in the session 2.2.2.6, at 340 nm (9.6 mM^{-1} cm^{-1}) during 5 min. Results were 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.

Reduced (GSH)/ oxidized (GSSG) glutathione ratio

The contents of GSH and GSSG were measured at 412 nm as described by Rahman et al. (2006). GSH and GSSG standards were used for GSH and GSSG quantifications separately (0–60 µmol·L^{-1}). Extraction was performed using 0.6% sulfosalicylic acid in potassium phosphate buffer (100 mM dipotassium phosphate; 5 mM EDTA; 0.1% (v/v) Triton X-100; pH7.5). Absorbance was measured at 412 nm, and GSH and GSSG concentrations were determined in nmol/mg protein. The GSH/GSSG ratio was calculated dividing the GSH (µmol·g) content by 2x the concentration of GSSG (µmol·g).

Alkaline phosphatase (ALP) activity

The activity of ALP was measured at room temperature using González et al. (1994) method, by measuring the defradation of *p*-nitrophenol, at 420 nm. The extraction was performed with Tris pH 9.0 with 0.01% of TritonX-100. 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 µmol of nitrophenol, per min.

Protein (PROT) content

The concentration of protein was assayed using the Biuret method Robinson and Hogden (1940), at 540 nm. Bovine serum albumin (BSA) was used as the standard. PROT quantification was used to express the above biomarkers per mg of protein.

4.2.4. Statistical 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: i) for each species and pH level: no significant differences exist between As exposed and non-exposed clams; ii) for each species and As exposure concentration: no significant differences exist between species under different pH levels; iii) 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 lowercase letters represented differences for *R. decussatus* between As concentrations at each pH level (7.8 and 7.3), while uppercase letters represented differences for *R. philippinarum* between As concentrations at each pH (7.8 and 7.3). In figures, significant differences between pH conditions, for each species at each As concentration, were represented with asterisks. At each pH and As

condition significance levels (Monte Carlo *ρ*-values) related to the each biomarker between species are presented in Annex XI. The same table also presents significance levels between As concentrations (for each species and each pH level) and the significance levels between pH levels (for each species and each As concentration).

4.3. Results

4.3.1. Mortality

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).

4.3.2. Arsenic accumulation and BCF values

Results obtained for both species at the same pH (7.8 and 7.3) showed that organisms accumulated As when exposed to this metalloid. However, a significant accumulation was only observed in both species exposed to As at pH 7.3, when compared with non-exposed clams at the same pH (Table 4.1).

Regarding each species at 4 mg/L of As, results showed that at low pH both species presented higher As concentration and ability to accumulate As (BCF ≥ 0.73± 0.02) than under control pH (7.8) (BCF ≤ 0.65±0.20) (Table 4.1), but significant differences were only showed for *R. philippinarum*.

When comparing both species at each exposure concentration and pH level (7.8 and 7.3), results showed no significant differences in As concentrations of both clams at pH 7.3 and 7.8 combined and 0 mg/L of As, as well as, at pH 7.8 and 4 mg/L of As (Annex XI). On the other hand, at pH 7.3 and 4 mg/L of As the introduced species presented significant higher As concentration than the native species (Table 4.1). Similar results were observed for BCF values at pH 7.3 and 4 mg/L of As.

Table 4.1 - Total As concentration (mg/Kg fresh weight) and Bioconcentration Factor (BCF) in *Ruditapes decussatus*, and in *R. philippinarum* (mean±SD). For each species, significant differences ($p \leq 0.05$) among pH levels As concentrations are represented with different letters (a-b for Rd and for Rp). Significant differences ($p \leq 0.05$) between species, at each condition, are represented with an asterisk.

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

4.3.3. 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*.

When comparing the effects of pH present results 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.

4.3.4. Biomarkers

Glycogen (GLY) content

The results of GLY, for both species and the same pH level, showed no significant differences between exposed and non-exposed organisms (Fig. 4.2). Similar results were observed for each species, exposed to different pH (7.8 and 7.3) levels but at the same As concentration (0 and 4 mg/L of As) (Fig. 4.2).

When comparing both species at the same As concentration and pH level results showed significantly lower GLY content in *R. philippinarum* than in *R. decussatus*, independently on the As concentration and pH level.

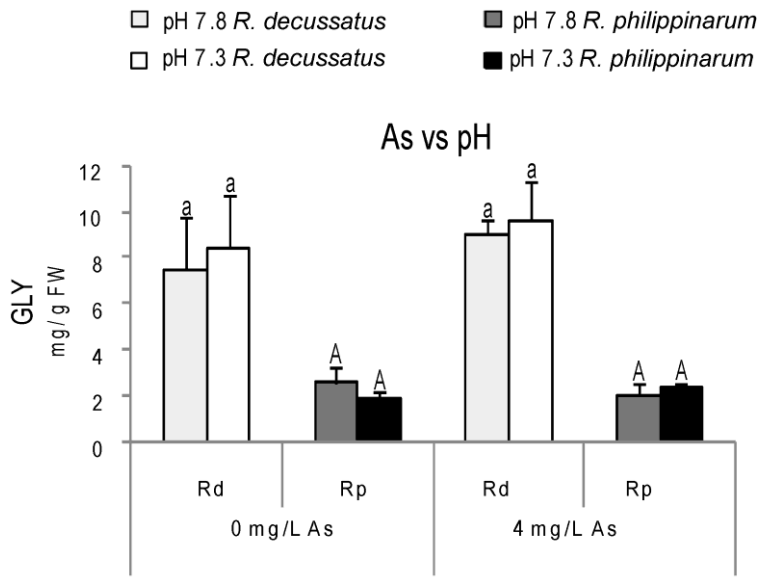

Figure 4.2 - Glycogen content (GLY, mg/g FW) in *Ruditapes decussatus* (Rd), and in *R. philippinarum* (Rp), exposed to different As conditions (0 and 4 mg/L of As) and pH levels (7.8, 7.3) (mean±SD). For each species, significant differences ($p \leq 0.05$) among salinities and pH levels are represented with different letters (a-a for Rd and A-A for Rp).

Lipid peroxidation (LPO) levels

The results of LPO, for each species under the same pH level, showed no significant differences between exposed and non-exposed organisms (Fig. 4.3, Annex XI). However, *R. philippinarum* showed an increase on LPO levels when exposed to As and pH 7.8, but no significant differences between exposed and non-exposed clams (Fig. 4.3).

When analyzing the LPO levels in species exposed to different pH levels but under the same As concentration, the results showed that *R. decussatus and R. philippinarum* only presented significantly high values at low pH and 4 mg/L of As and at low pH and 0 mg/L of As, respectively (Fig. 4.3).

Comparing both species, results demonstrated that the introduced species presented significantly higher LPO levels than native one, independently of the pH level and As concentration (Fig. 4.3, Annex XI).

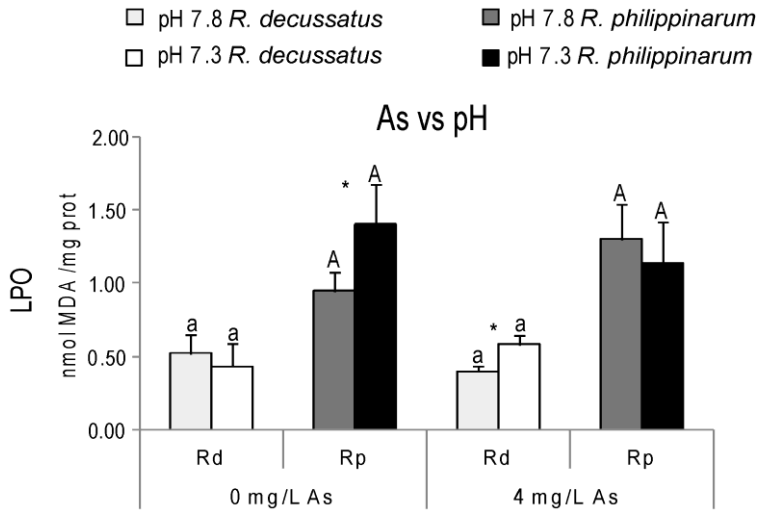

Figure 4.3 - Lipid peroxidation levels (LPO, nmol MDA/mg prot) in *Ruditapes decussatus* (Rd), and in *R. philippinarum* (Rp), exposed to different As conditions (0 and 4 mg/L of As) and pH levels (7.8, 7.3) (mean±SD). For each species, significant differences ($p \leq 0.05$) among salinities and pH levels are represented with different letters (a-a for Rd and A-A for Rp). Significant differences ($p \leq 0.05$) between pH conditions, for each species at each As condition are represented with an asterisk.

Superoxide dismutase (SOD) activity

Regarding SOD, results showed no significant differences for the native species exposed and non-exposed to As at the same pH level (Fig. 4.4). A similar pattern was observed in *R. philippinarum* exposed and non-exposed to As at low pH. Nevertheless, *R. philippinarum* at control pH significantly increased the SOD activity at As exposure (Fig. 4.4).

For each species, exposed to different pH levels but under the same As concentration similar SOD activity values were found independently of As concentration, except for *R. philippinarum* exposed to 0 mg/L where SOD activity was significantly higher in clams under low pH (Fig. 4.4 and Annex XI).

When comparing both species at the same As concentration and pH level, results demonstrated a significantly higher SOD activity in the introduced clam than in the native clam at all tested conditions (Annex XI).

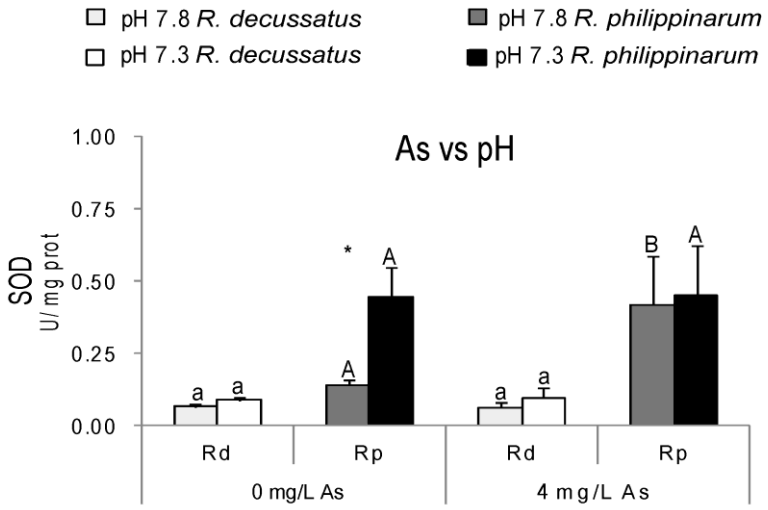

Figure 4.4 - Superoxide dismutase activity (SOD, U/mg prot) in *Ruditapes decussatus* (Rd), and in *R. philippinarum* (Rp), exposed to different As conditions (0 and 4 mg/L of As) and pH levels (7.8, 7.3) (mean±SD). For each species, significant differences among salinities and pH levels are represented with different letters (a-a for Rd and A-B for Rp). Significant differences ($p \leq 0.05$) between pH conditions, for each species at each As condition are represented with an asterisk.

Catalase (CAT) activity

The activity of CAT for *R. decussatus* significantly decreased in organisms exposed to As and pH 7.8 but at low pH the activity of this enzymes was similar between exposed and non-exposed clams (Fig. 4.5). For *R. philippinarum* clams exposed to As increased the CAT activity under pH 7.8 but the activity of this enzyme decreased in exposed clams under pH 7.3 (Fig. 4.5).

When analyzing CAT activity in each species exposed to different pH levels but the same As concentration results showed significantly higher values in *R. decussatus* under pH 7.8 at 0 mg/L *R. decussatus* and in *R. philippinarum* at the same pH but exposed to As (4 mg/L) (Fig. 4.5 and Annex XI).

In addition, comparing both clams under the same As concentration and pH level, results showed a significantly higher activity of CAT in the introduced species than in the native species independently on the tested conditions (Annex XI).

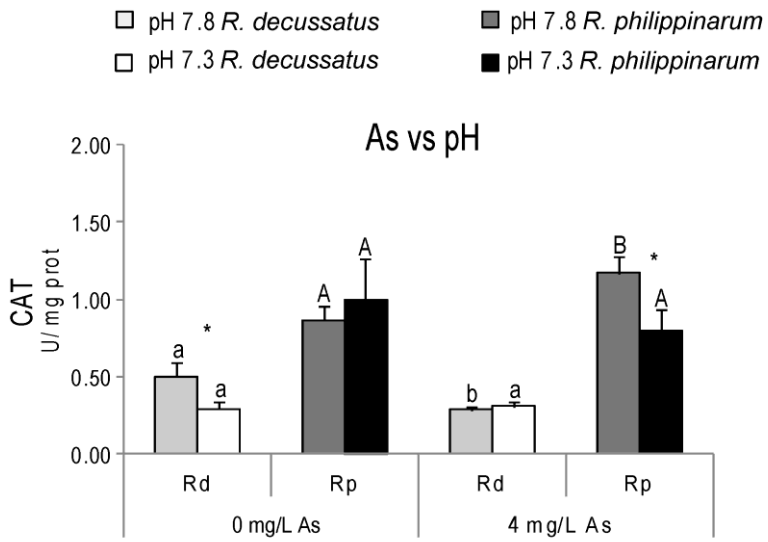

Figure 4.5 - Catalase activity (CAT, U/mg prot) in *Ruditapes decussatus* (Rd), and in *R. philippinarum* (Rp), exposed to different As conditions (0 and 4 mg/L of As) and pH levels (7.8, 7.3) (mean±SD). For each species, significant differences among salinities and pH levels are represented with different letters (a-b for Rd and A-B for Rp). Significant differences ($p \leq 0.05$) between pH conditions, for each species at each As condition are represented with an asterisk.

Glutathione S-transferase (GSTs) activity

Relatively to GSTs activity, the native species, *R. decussatus*, under the same pH level did not show significant differences between organisms exposed and non-exposed to As (Fig. 4.6). Similar results were only observed in *R. philippinarum* exposed and non-exposed to As at low pH (Fig. 4.6), while at pH 7.8 this species significantly increased the GSTs activity when exposed to As (4 mg/L).

For each species, at the same As concentration, higher GSTs activity was found at low pH, but significant differences were only found in both species exposed to 0 mg/L of As (Fig. 4.6).

When comparing both species under the same pH level and As concentration, results showed significantly higher GSTs activity in the introduced than in the native species, except at low pH and 4 mg/L of As (Annex XI).

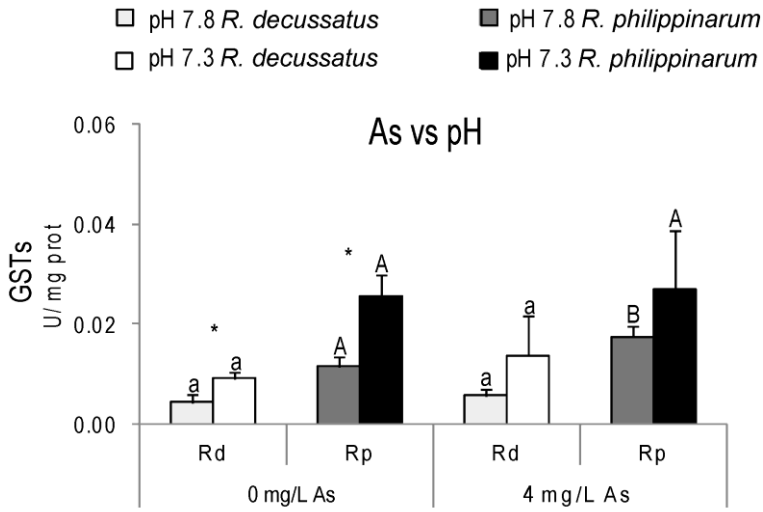

Figure 4.6 - Glutathione *S*-transferases activity (GSTs, U/mg prot) in *Ruditapes decussatus* (Rd), and *R. philippinarum* (Rp), exposed to different As conditions (0 and 4 mg/L of As) and pH levels (7.8, 7.3) (mean±SD). For each species, significant differences among salinities and pH levels are represented with different letters (a-a for Rd and A-B for Rd). Significant differences ($p \leq 0.05$) between pH conditions, for each species at each As condition are represented with an asterisk.

Ratio between reduced (GSH) and oxidized (GSSG) glutathione

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. 4.7).

Analyzing GSH/GSSG ratio in each species exposed to different pH levels (7.8 and 7.3) but at the same As conditions (0 mg/L As and 4 mg/L As), results showed no significant differences between pH conditions (Fig. 4.7), except for *R. philippinarum* exposed to As.

The results obtained also showed that when comparing GSH/GSSG ratio between species at the same pH level and As concentration, no significant differences between species were observed, except at pH 7.8 and 4 mg/L of As with significantly higher values in *R. philippinarum* (Annex XI).

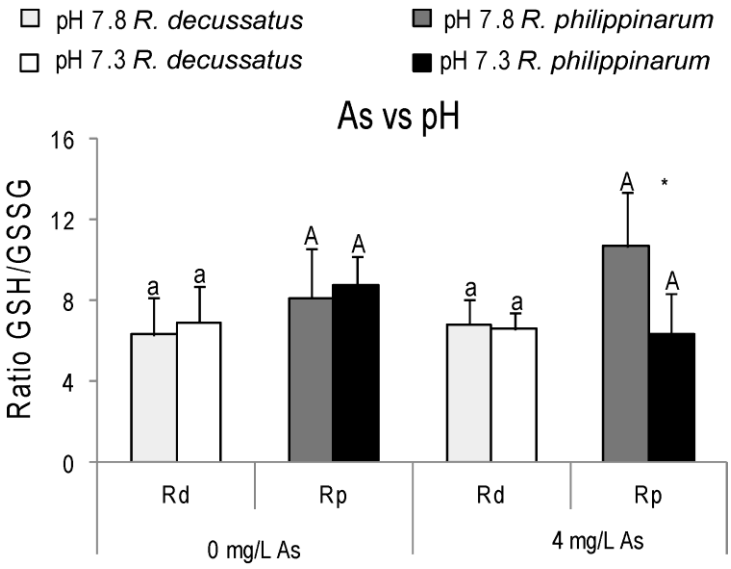

Figure 4.7 - Reduced and oxidized glutathione ratio (GSH and GSSG) in *Ruditapes decussatus* (Rd), and in *R. philippinarum* (Rp), exposed to different As conditions (0 and 4 mg/L of As) and pH levels (7.8, 7.3) (mean±SD). For each species, significant differences among salinities and pH levels are represented with different letters (a-a for Rd and A-A for Rp). Significant differences ($p \leq 0.05$) between pH conditions, for each species at each As condition are signalled with an asterisk.

Alkaline phosphatase (ALP) activity

The activity of ALP in the native species at each pH did not show significant differences among exposed and non-exposed clams (Fig. 4.8). Similar results were observed at low pH between *R. philippinarum* exposed and non-exposed clams to As (Fig. 4.8). 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 (Fig. 4.8).

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 (Annex XI).

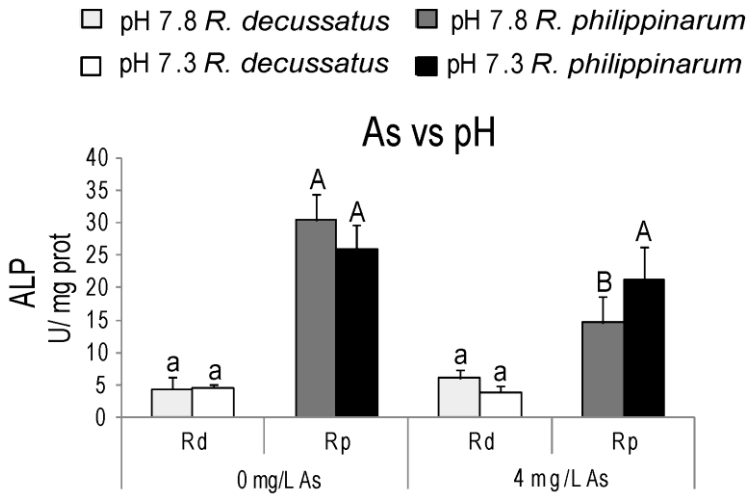

Figure 4.8 - Alkaline phosphatase activity (ALP, U/mg prot) in *Ruditapes decussatus* (Rd), and in *R. philippinarum* (Rp), exposed to different As conditions (0 and 4 mg/L of As) and pH levels (7.8, 7.3) (mean±SD). For each species, significant differences among salinities and pH levels are represented with different letters (a for Rd and A-B for Rp).

4.4. Discussion

Changes in environmental conditions, namely seawater acidification, 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; Vernberg, 2012; 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* clam species was compared when under the combination of As and different pH levels.

4.4.1. Arsenic accumulation and BCF values

The present findings revealed that, in general, the combined exposure of As and pH did not affect the survival of both species. The results obtained further showed that *R. decussatus* and *R. philippinarum* under both pH levels increased As concentrations when exposed to this metalloid. 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 (Gailer et al., 1995; Moschino et al., 2012; Moreira et al., 2016a, 2016b; Velez et al., 2016a; Zhang et al., 2015) (Table XII).

In addition, the present study also demonstrated that, both *R. decussatus* and *R.*

philippinarum species exposed to As presented higher concentrations and higher BCF values under low pH (7.3) than under control pH (7.8). These results support the hypothesis 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) (Annex XII).

The present findings also demonstrated that both species accumulated similar As concentrations when chronically exposed to 4 mg/L of As at pH 7.8. In accordance with the obtained results, similar As accumulation patterns at pH 7.8 between two bivalve species were previously demonstrated for other bivalve species (Moreira et al., 2016a). When exposed to low pH and As (4 mg/L), the introduced species presented significantly higher As concentration and BCF values than the native species. 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) (Annex XII).

4.4.2. Dietary risk assessment

Our findings revealed that the consumption in one week of just 0.16 to 0.48 Kg of both bivalves species (exposed to As, at both pH) will be enough for an adult (70 Kg) to exceed the PTWI (PTWI 0.015mg/Kg/ week of As). Similar results were found for *R. decussatus* (0.15-0.95 kg) and *R. philippinarum* (0.08–0.81 kg), collected from the Ria de Aveiro and the Óbidos lagoon (Velez et al., 2015a, b) (chapter 2) and from the coast of China (Yang et al., 2013), to exceed the PTWI for As.

The results obtained also showed that low pH affected the As accumulation since both species at low pH accumulated more As than both species at pH 7.8. Consequently, the amount of clam soft tissue necessary to exceed the PTWI by an adult of 70 kg is lower in both species exposed to As under low pH. Thus, from the present findings, it was possible to 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 amount of *R. philippinarum* is needed to exceed the PTWI under an acidified scenario, special attention should be taken in the future when selecting this clam species as food source.

4.4.3. Biomarkers

Several aquatic organisms use GLY reserves 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) which may be related to the capacity of clams to regulate acid-base balance and detoxification mechanisms without GLY depletion. The bivalves *C. gigas* and *Macoma balthica* 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. (2016b) 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, 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 (22–38 °C).

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 defence mechanisms are not able to eliminate the excess of ROS, the peroxidation of lipids is induced, resulting in physicochemical 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, has been used as a biomarker of oxidative stress in many organisms (Flora, 2009; Jozefczak et al., 2012; Marí et al., 2009).

In the present study, *R. decussatus* mantained the LPO levels, antioxidant and biotransformation enzymes, and GSH/GSSG ratio when exposed to As, independently of pH levels. These observations 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., 2016b; Zhang et al., 2015). 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 present 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 (Moreira et al., 2016a, 2016b; Velez et al., 2016a; Ventura-Lima et al., 2009) (Annex XII). On the contrary, in the present study As affected *R. philippinarum* responses 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 defence 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 (Moreira et al., 2016a; Zhang et al., 2015).

Regarding both species exposed to the same As conditions, in general, the results obtained showed high 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. 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. 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). In contrast, 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). Comparing both species, in the present study, at the same As concentration and pH level results demonstrated higher LPO levels, biotransformation and antioxidant enzymes in the introduced species than native one. 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 defence mechanisms (Moreira et al., 2016a).

Alkaline phosphatase is a plasma membrane enzyme, envolved in osmorregulation mechanisms, described by several authors in marine bivalves, its activity is known to be influenced by metals (Mazorra et al., 2002; Seitkalieva et al., 2015). Present 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. According to Lovett et al. (1994) low levels of ALP in clams suggest that membranes are an ineffective barrier to most molecular substances in organisms. 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) (Annex XII). 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) (Annex XII). Moreover, the present results indicated that the introduced clam *R. philippinarum* presented higher ALP activity than native species among all tested conditions. The introduced oyster *C. gigas* also presented higher ALP activity than the close related native species *C. angulata* under pH 7.8, 7.3 and the combined effect of low pH and As (Moreira et al., 2016a).

4.5. 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 organisms exposed to pH 7.3 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 pH 7.8 and 7.3 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 pH 7.3 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 and both species were able to avoid oxidative stress when under combined effect of pH and As.

In conclusion, the performance of both species under seawater acidification and As contamination scenario was not affected, although the introduced species had accumulated twice the amount of As than the native clams.

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Chapter 5

General conclusion and final considerations

5.1. General conclusion and final considerations

Organisms in marine systems are exposed to a complex multitude of stressors, that create a wide range of associated environmental and ecotoxicological risks. Some examples include climate change related stressors such as alterations in the range and variability of physical and chemical conditions (e.g. extremes in temperature, alterations in pH and salinity) and the magnitude and duration of exposure to metals and metalloids arising from human activities (Roberts et al., 2013). These environmental stressors have been identified as key and/or emerging drivers that could significantly influence the marine near-shore ecosystems and their inhabiting organisms, namely bivalves.

Bivalves, which are mostly filter-feeders contacting directly with water and sediment, are greatly affected by metal(loid)s contamination (Al-Subiai et al., 2011; Jebali et al., 2014; Moschino et al., 2012; Pellerin and Amiard, 2009). On the other hand, climate change may also affect marine organisms limiting their growth, reproduction and spatial distribution (Parker et al., 2013; Sorte et al., 2013). Recently published studies have demonstrated that climate change may contribute to the release of metal(loid)s contaminants from sediment to the seawater column, increasing their bioavailability and consequently their bioaccumulation on biota (Roberts et al., 2013; Rodríguez-Romero, 2014). Thus, metal(loid) contamination and climate change may represent a threat to native and introduced species, resulting into biodiversity loss and the provision of valuable ecosystem resources and services.

The study developed and included in this thesis aimed to understand if the native species, *Ruditapes decussatus*, and the introduced species, *Ruditapes philippinarum*, co-exist in the same areas of the Ria de Aveiro and the Óbidos lagoon, to assess their accumulation, tolerance and the performance of both species when co-existing. In addition, it was also assessed the response of sympatric clam species from the Ria de Aveiro when exposed to single (salinity, temperature, pH, As concentrations) or combined stressors (pH and As concentrations).

The co-existence of *R. decussatus* and *R. philippinarum* in the Ria de Aveiro and the Óbidos lagoon, as well as, the accumulation of metal(loid)s and biochemical performance of both species were evaluated in Chapter 2 using multiple endpoints: species density, metal(loid)s concentrations in the sediments and in organisms of both species (bioaccumulation pattern), the human health risks associated with the consumption of these clams and their biochemical performance under environmental conditions. Results indicated that both native and introduced species co-exist in the Ria de Aveiro and the Óbidos lagoon, being the native species not yet supplanted by the introduced one, presenting the native clams in the Ria de Aveiro higher density than the introduced species in 3 areas (co-exist in 7) while in the Óbidos lagoon the native species had the highest density among all study areas. The results further revealed that the density and spatial distribution of *R. philippinarum* from both ecosystems may be related with the sediment

contamination. Also, the contamination levels in sediments were related with sediments physicochemical parameters, since areas with higher contamination levels presented high total organic matter content and high fine particles, being an opposite tendency observed in areas with low contamination. Results also indicated that both species tended to present high metal(loid)s concentrations in areas with the highest contamination levels, suggesting that both clams can be considered potentially good sentinel species, especially in the Óbidos lagoon. The most abundant elements accumulated in both species from the Ria de Aveiro were As, Ni and Cu, while in the Óbidos lagoon, Cr was also found in high quantities, in addition to those previously cited. However, bivalves consumption can constitute a health risk for humans due to As concentrations even when collected from the lowest contaminated areas (PTWI <0.8Kg). It was also possible to reveal that for both species from both ecosystems, the majority of As was accumulated in the soluble fraction, suggesting that As may be more toxic to organisms being accumulated by lower trophic levels and biomagnified along the food chains. In general, both species from the Ria de Aveiro and the Óbidos lagoon did not increase the different oxidative stress levels with the increase of sediment contamination, suggesting that possibly these organisms are not responding to sediment contamination, but rather to a combination of stresses. The metal(loid)s partitioning may be responsible for the similar biochemical performance in both species from the Ria de Aveiro and the Óbidos lagoon among the sampling areas. In brief, the results revealed that both species co-habit in both ecosystems and presented similar accumulation, metal partitioning, and biochemical performance when present in the same area.

It must be noted that, although both environmental studies provided useful information about the accumulation of contaminants, density, and risk to human consumption, they were performed only during one seasonal period. Due to that, further research is needed to approach longer sampling periods, which could bring new light on the population dynamics and/or on the sustainability of harvesting levels in the Ria de Aveiro and the Óbidos lagoon. In addition, it would be of interest to create a monitoring program in both marine ecosystems in order to prevent the replacement of native species by the introduced ones, not only due to the ecological value of this species but also due to their socio-economic importance. This program should include the assessment of contaminants concentrations in environmental matrices, assessing biological effects of contaminants at different levels of biological organization, as well as the distribution of native and introduced species. On the other hand, the analysis of the geochemical speciation of sediment and metal(loid)s bioavailability may also elucidate why most elements accumulated in both organisms were not the most abundant in sediments. Additionally, the influence of pH, temperature, and salinity shifts on the bioavailability of metal(loid)s present in contaminated sediments should be assessed. It is also important to clarify how confounding factors, such as seasonality, age, and abiotic factors, may influence the performance of both species, as well as the effects of climate change in both species.

It is likely that the current state of the Ria de Aveiro and the Óbidos lagoon, may not present

the ideal conditions for *R. philippinarum* population expansion, which could justify why this species has not yet supplanted the native ones, as occurred in Venice lagoon. However, predicted climate change may alter this scenario. For example, in the United Kingdom estuaries, *R. philippinarum* is considered a naturalized species where its density is possibly regulated by the cold weather, since high mortality of this species was associated with temperature decrease in Poole Harbour (south coast of England) (Humphreys et al., 2007, 2015).

Several environmental characteristics may act as confounding factors and may induce biochemical alterations in *R. decussatus* and *R. philippinarum* from specific areas masking the effects caused by metals and As contamination. Therefore laboratory controlled experiments were conducted to better understand the performance of each species under controlled conditions. Native and introduced individuals were exposed in the laboratory to different stressors to determine the effects of single (As contamination, thermal stress, salinity shifts and ocean acidification) or combined stressor(s) (As contamination and ocean acidification), as well as the cause-effect relationships between biomarkers and these single/combined stressor(s) (Chapter 3 and 4). Additionally, it was important to understand if introduced species are more tolerant to these stressors when compared with their native counterparts.

Regarding single exposures, the consequences of As exposure in the performance of native and introduced species were assessed by selecting sublethal (4.0, 8.2 and 17.0 mg/L) and lethal (34 and 70 mg/L) As concentrations (Chapter 3.2). The sublethal and lethal exposures resulted in an increase of As concentration with the increase of As exposure and in similar As partitioning between species in all tested conditions. Both species were able to regulate detoxification mechanisms up to 8.2 mg/L of As, avoiding cellular damages, while at the highest exposure concentrations (17, 34 and 70 mg/L), these mechanisms were not sufficient, resulting in high damage and mortality of both species. The oxidative stress biomarkers tested proved to be quick and sensitive tools to reveal the sublethal and lethal effects in both species. Since there is scarce information about As effects on native and introduced species, this study may contribute to acknowledge about the As tolerance for both species, showing that under an As contamination scenario (concentrations up to 8.2 mg/L) both species will be affected, since they were not able to maintain tolerance mechanisms against As exposure leading to their dead. Nevertheless, deeper investigation is still needed regarding the partition of As in each subcellular fraction (cellular debris, metal-rich granules, organelles, heat-sensitive proteins and heat-stable proteins) and the As speciation in sediments. Lastly, since there is a gap between sub-individual and higher level of biological/ecological responses in aquatic ecosystems exposed to contaminants, it would be interesting to go further at higher organization levels through the performance of multitrophic studies to better understand the complexity of patterns and processes in nature.

Beyond the As concentrations effects tested, according to particular temperature assays (chapter 3.3), it was possible to understand that the native species was the most tolerant to future chronic warming conditions since temperature rise induced less subcellular and molecular changes

in *R. decussatus* than in *R. philippinarum*. The biochemical and molecular changes in the introduced species may be related to the increase of oxidative stress and apoptosis in this species, while the native species appear to inhibit biochemical and molecular responses. These results suggested that the native species close their valves in order to reduce chronic exposure to high temperatures (21 and 25 ºC). Although, in long term, this strategy may, however, influence their health status and, ultimately, their survival and replacement. In this way, the subcellular and molecular biomarkers (oxidative stress and gene expression) contributed to the increase of knowledge about the performance of both species under a global warming scenario.

Salinity and pH are among the abiotic variables that can affect the clams *R. decussatus* and *R. philippinarum* (chapter 3.4). The results obtained showed that oxidative stress was induced in *R. decussatus* exposed to salinity shifts, while this species did not show alterations on the measured biochemical biomarkers when exposed to seawater acidification scenario predicted until the end of the century (ΔpH=0.5). On the other hand, the introduced species showed to be especially vulnerable to ocean acidification, as well as to the highest tested salinity (35), since these factors induced oxidative stress, while at low salinity (14) detoxification mechanisms avoided damages in the membrane. In conclusion, the native species was affected either by low and high salinities while the introduced species was particularly affected by high salinity. Relatively to pH changes, only the introduced species was affected by seawater acidification.

In brief, due to present studies under laboratory conditions, it was possible to understand that temperature, salinity, and pH shifts may affect more one species (*R. philippinarum*) than the other (*R. decussatus*). In addition, it is possible that temperature rise, salinity changes, and seawater acidification will influence the density and spatial distribution of these species, contributing for the replacement or not of the native species. Therefore, it is important to understand how these factors will affect their behavior and reproduction when cohabiting at the same area.

After defining the tolerance and the biochemical response of both species to As contamination and pH levels, acting alone, a multiple biomarker approach was employed to assess the combined effects of both stressors on the performance of *R. decussatus* and *R. philippinarum* (Chapter 4). The results obtained demonstrated that low pH may influence the As accumulation in bivalves since both species accumulated higher amounts of As when exposed to low pH (7.4) levels compared to organisms under control pH (7.8). These findings indicate that predicted seawater acidification potentiates health risks associated with the consumption of native and introduced species since less amount of both species exposed to As and under low pH is needed for an adult to exceed the PTWI. Relatively to biochemical performance, the present study further revealed that the combination of seawater acidification and As contamination did not negatively affect both clams species. Although the introduced species had accumulated twice the amount of As than the native clams.

Since the studies performed focused mainly on cellular biomarkers and gene expression, it seems important to integrate traditional chemical and biomarker measures with omics technologies (such as proteomics and metabolomics), behavioral biomarkers and histopathological analyses to redefine the surveillance of coastal water pollution and assess the climate change effects in non-model organisms, such as bivalves, to understand the complete picture of stressful events.

In a context of climate change, despite the relevant information disclosed in the present thesis about the performance of both species, future research is needed to understand: i) will climate change affect the reproduction of *R. decussatus* and *R. philippinarum* and consequently their stocks in different ecosystems?; ii) which will be the most affected species during larval stages?; iii) when combined, can environmental stressors alter the susceptibility of *R. decussatus* and *R. philippinarum*, as well as other economical relevant bivalve species, to contaminants and diseases?; iv) what will be the impact of climate change on global shellfish stocks and its consequences on fisheries resources?; v) what management measures should be applied?.

Finally, the findings reported here contributed to answer the main question "Is it possible for native and introduced Ruditapes species to live in sympatry?", demonstrating that different strategies used by both species under thermal stress, salinity changes, seawater acidification and As contamination may contribute to the co-existence of *R. deccussatus* and *R. philippinarum* in the Ria de Aveiro.

5.2. References

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Annexes

Annex I - Physicochemical parameters from the Ria de Aveiro, along the 15 sampling areas: temperature (ºC), pH; salinity, gravel, sand and fines content (percentage of total sediment dry weight) and the total organic matter (TOM, %) (mean±SD).

Areas	Temperature	pH	Salinity	Main sediment type	Median	Gravel	Sand	Fines	TOM
I	19.5	7.04	39	Clean medium sand	1.40±0.05	1.10±1.08	98.84±1.00	0.18±0.04[a]	0.42±0.06[a]
D	19.3	7.57	36	Clean medium sand	1.71±0.46	0.05±0.07	96.68±4.37	0.42±0.60[a]	0.47±0.26[a,b]
O	20.9	7.40	28	Verry silty medium sand	1.9±0.00	2.73±2.25	69.73±7.57	27.58±9.66[b]	0.32±0.10[a]
B	21.1	7.57	36	Clean medium sand	0.00±00	2.00±0.59	96.07±2.14	3.86±1.68[c]	2.12±1,51[a-c]
N	19.4	7.16	28	Clean medium sand	1.25±0.01	0.44±0.03	99.23±5.74	0.43±0.12[a]	0.71±0.22[b,e]
A	20.1	7.59	34	Clean corse sand	1.40±0.04	2.13±1.89	85.20±16.65	0.69±0.18[a]	1.22±0.36[b,e]
J	18.0	6.82	40	Clean medium sand	2.00±0.14	00±00	98.84±1.31	0.32±0.37[a]	0.49±0.04[a,e]
H	20.1	7.71	40	Silty medium sand	2.00±0.64	0.43±0.40	86.14±13.58	19.77±10.38[b]	0.96±0.78[a,e]
C	22.6	7.80	35	Silty medium sand	2.00±0.54	0.00±0.00	89.90±8.46	9.71±8.52[a,b]	0.76±0.10[b,e]
E	21.7	8.53	32	Silty medium sand	1.10±0.84	5.01±4.26	88.11±6.61	15.42±14.77[a,b]	5.29±4.67[a,c-e]
G	21.6	7.04	35	Very silty medium sand	2.30±0.53	0.00±0.00	73.81±23.63	32.72±13.44[c]	0.58±0.21[a,e]
F	21.6	7.35	32	Silty fine sand	2.80±0.58	0.03±00	79.01±15.81	20.86±15.94[c]	4.89±3.01[a,c-e]
K	22.5	6.86	32	Very silty very fine sand	3.49±1.03	0.53±0.27	42.69±22.78	52.15±24.72[d]	4.62±2.97[c,d]
M	19.9	7.21	33	Mud	>4	0.74±0.64	26.18±18.90	73.35±17.12[d]	6.15±1.10[d]
L	21.6	7.35	33	Silty fine sand	2.10±0.66	6.01±4.32	80.00±4.91	15.40±4.17[b]	3.03±1.26[c]

Annex II - Element concentration and total element concentration (mg/Kg DW) in sediments from the Ria de Aveiro, along the sampling areas (mean±SD). Areas are presented according to an increasing contamination gradient. Underlined numbers represent concentrations higher than TEL values. Highlighted values represent the two most abundant elements in each of the sampling areas. For each element concentration and total element concentration, significant differences ($p \leq 0.05$) among areas are signalled with different letters (a-e).

Areas	As	Cr	Ni	Pb	Cu	Cd	Hg	Total
I	1.06 ± 0.13^{a}	1.02 ± 0.28^{a}	0.57 ± 0.10^{a}	1.07 ± 0.06^{a}	0.55 ± 0.01^{a}	0.02 ± 0.00^{a}	0.01 ± 0.01^{a}	4.31 ± 0.09^{a}
D	0.82 ± 0.16^{a}	1.22 ± 0.07^{a}	0.68 ± 0.13^{a}	0.93 ± 0.02^{b}	0.68 ± 0.39^{a}	0.02 ± 0.00^{a}	0.01 ± 0.01^{a}	4.37 ± 0.11^{a}
O	1.23 ± 0.30^{b}	1.18 ± 0.18^{a}	0.88 ± 0.18 ^{a,b}	1.40± 0.12^{c}	0.63 ± 0.11^{a}	n.d.	n.d.	5.32 ± 0.13^{a}
B	1.45 ± 0.07^{b}	1.27 ± 0.11^{a}	0.91 ± 0.14^{b}	1.41 ± 0.13^{c}	0.55 ± 0.01^{a}	0.03 ± 0.01^{a}	0.01 ± 0.01^{a}	5.63 ± 0.07^{a}
N	1.74 ± 0.22^{b}	1.57 ± 0.27^{a}	1.15 ± 0.20^{b}	2.25 ± 0.30^{d}	0.91 ± 0.16^{b}	0.03 ± 0.00^{a}	0.02 ± 0.01^{a}	7.64 ± 0.16^{b}
A	1.57 ± 0.77^{b,c}	2.61 ± 0.23^{b}	1.22 ± 0.57^{b}	1.57 ±0.77^{a-d}	1.31 ± 0.12^{c}	0.04 ± 0.01^{a}	0.01 ± 0.01^{a}	8.32 ±0.36^{b}
J	1.51 ± 0.25^{b}	2.51 ± 0.54^{b}	1.42 ± 0.39^{b}	2.01 ± 0.30^{d}	0.98 ± 0.20^{b}	0.03 ± 0.00^{a}	n.d	8.45 ± 0.24^{b}
H	1.44 ± 1.43^{a,b,c}	3.47 ± 2.77^{a,b}	1.28 ± 1.63^{b}	2.18 ± 2.24^{a-e}	1.89 ± 1.61^{b,c}	0.05 ± 0.02^{a,b}	0.01 ± 0.01^{a}	10.33 ± 1.39^{b,c}
C	2.53 ± 0.94^{b,c}	3.55 ± 1.26^{b}	2.46 ± 0.90^{b}	3.41 ± 1.20^{d,e}	2.03 ± 0.88^{b,c}	0.09 ± 0.03^{b,c}	0.06 ± 0.02^{b}	14.13 ± 0.75^{c}
E	2.43 ± 0.75^{b}	2.76 ± 0.18^{b}	4.22 ± 3.62^{b,c}	3.17 ± 0.46^{d}	1.45 ± 0.06^{b,c}	0.05 ± 0.02^{a,b}	0.09 ± 0.03^{b}	14.16 ± 0.74^{c}
G	2.27 ± 0.91^{b,c}	5.59 ± 2.20^{b,c}	2.19 ± 0.82^{b}	2.83 ± 0.82^{d}	1.43 ± 0.65^{b,c}	0.07 ± 0.02^{b}	0.03 ± 0.01^{a}	14.42 ± 0.74^{c}
F	6.14 ± 2.75^{c}	5.46 ± 0.89^{c}	5.75 ± 3.02^{c}	10.64 ± 5.36^{e}	3.63 ± 1.17^{d}	0.16 ± 0.05^{c,d}	0.15 ± 0.11^{b}	31.93 ± 1.91^{d}
K	3.97 ± 0.39^{c}	6.85 ± 1.74^{c}	7.68 ± 5.04^{c}	15.25 ± 4.25^{e}	4.49 ± 1.45^{d}	0.10 ± 0.02^{c}	0.03 ±0.02^{a}	38.38 ± 2.45^{d}
M	4.21 ± 1.98^{c}	16.95 ± 3.21^{d}	8.61 ± 2.72^{c}	5.33 ± 1.93^{e}	4.46 ± 1.61^{d}	0.20 ± 0.05^{d}	n.d.	39.78 ± 1.64^{d}
L	4.78 ± 1.89^{c}	8.57 ± 3.71^{c}	4.76 ± 2.32^{b,c}	5.19 ± 0.00^{e}	26.22 ± 8.99^{e}	0.17 ± 0.05^{c,d}	0.06 ± 0.02^{b}	49.73 ± 2.83^{e}
TEL	7.24	52.30	15.90	30.24	18.70	0.68	0.13	
ERL	8.20	81.00	20.90	46.70	34.00	1.20	0.15	
PEL	41.60	160.00	42.80	112.00	108.00	4.21	0.70	
ERM	70.00	370.00	51.60	218.00	270.00	9.60	0.71	

TEL- Threshold Effect Level (MacDonald et al., 1996); ERL - Effects Range Low (Long and Morgan, 1990; Long et al., 1995); PEL - Probable Effects Levels (MacDonald et al., 1996); ERM - Effects Range Median (Long and Morgan, 1990; Long et al., 1995).

Annex III - Metal(loid)s concentrations (mg/kg DW) and Biota-Sediment Accumulation factor (BSAF) of *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp) from the Ria de Aveiro, collected in each of the sampling areas (mean±SD). Areas are presented according to an increasing contamination gradient.Values in bold represent the most abundant elements in clams. Highlighted values represent BSAF > 1. For *R. decussatus* and *R. philippinarum* significant differences ($p \leq 0.05$) among areas are signalled with different letters (a-d for Rd and A-E for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with asterisk.

		As		Cr		Ni		Pb	
Area	species	Clams	BSAF	Clams	BSAF	Clams	BSAF	Clams	BSAF
O	Rd	**15.63±4.03^{a}**	12.75±3.29^{a}	3.68±2.14^{a,b,c}	3.13±1.81^{a}	**8.12±5.01^{a,b}**	9.19±5.67^{a,b,c}	1.41±0.31^{a}	1.00±0.22^{a}
	Rp	**13.08±3.78^{A}**	10.67±3.08^{A}	4.33±0.35^{A,B}	3.68±0.30^{a}	**4.77±1.44^{A}**	5.40±1.63^{A}	1.59±0.19^{A}	1.13±0.14^{A}
B	Rp	**13.60±3.18^{A}**	9.39±2.20^{A}	**4.18±1.17^{B}**	3.28±0.92^{A}	**5.82±3.09^{A,B}**	6.39±3.39^{A,C}	1.33±0.72^{A,B}	0.94±0.51^{B}
N	Rd	**9.94±0.53^{b}**	5.73±0.31^{b}	**2.54±1.02^{a,b,c}**	1.62±0.65^{a}	**2.57±0.77^{a}**	2.24±0.67^{a}	1.10±0.44^{a,b}	0.49±0.19^{b}
	Rp	**8.06±2.55^{A}**	4.64±1.47^{B}	1.76±0.13^{C}	1.12±0.08^{B,D,E}	2.00±0.45^{B}	1.74±0.39^{B}	1.05±0.19^{B}	0.47±0.08^{C}
C	Rp	**7.38±3.41^{A}**	2.92±1.35^{B}	**1.93±1.17^{B,C}**	0.54±0.33^{B}	**3.38±2.02^{A,B}**	1.38±0.82^{B,C,D}	0.65±0.48^{B}	0.19±0.14^{C}
E	Rd	**10.52±1.34^{b}**	4.34±0.55^{c}	1.58±0.56^{a}	0.46±0.16^{b,c}	**3.00±0.78^{a}**	0.71±0.18^{b,c}	0.78±0.10^{b,c}	0.25±0.03^{b}
	Rp	**11.77±0.99^{A}**	4.85±0.41^{B}	1.31±0.62^{C,D}	0.38±0.18^{B,D,E}	**4.10±2.09^{A,B}**	0.97±.49^{B,C,D}	0.80±0.13^{B}	0.25±0.04^{C}
G	Rd	**11.45±0.10^{a,b}**	5.04±1.32^{b,c}	2.40±0.01^{b}	0.43±0.19^{b}	**3.70±0.00^{b}**	1.69±0.32^{b}	2.05±0.00^{d}	0.72±0.07^{d}
	Rp	**11.22±3.32^{A}**	3.96±0.06^{B}	3.13±1.26^{A,B,E}	0.52±0.14^{B}	**4.41±0.87^{A}**	1.49±0.96^{B}	1.42±0.49^{B}	0.55±0.38^{C}
F	Rd	**11.98±5.76^{a,b}**	1.95±0.54^{d}	2.22±0.65^{a,b,c}	0.41±0.12^{b}	**4.62±0.83^{b}**	0.80±0.14^{c}	1.24±0.43^{a,c}	0.12±.0.04^{c,e}
	Rp	**10.88±5.76^{A}**	1.77±0.94^{D}	1.72±1.09^{C,D,E}	0.31±0.20^{B,D}	**3.83±1.31^{A,B}**	0.67±0.23^{D}	1.17±0.29^{A,B}	0.11±0.03^{D}
K	Rd	**30.76±4.04^{b}**	7.75±1.02^{b,d}	2.12±0.47^{a,b}	0.31±0.07^{b,c}	**2.54±1.28^{a}**	0.33±0.17^{d}	0.80±0.10^{b,c}	0.05±0.01^{c}
	Rp	**26.78±6.95^{A}**	6.75±1.75^{A,B}	2.24±0.13^{D}	0.33±0.02^{D}	**4.01±0.69^{A}**	0.52±0.09^{D}	1.09±0.18^{B,C}	0.07±0.01^{E}
M	Rd	**36.00±3.77^{c*}**	8.54±0.90^{a,d*}	3.35±0.57^{c}	0.20±0.03^{b,c*}	**3.88±0.83^{a}**	0.45±0.10^{d}	1.15±0.40^{a,c}	0.22±0.08^{b,e}
	Rp	**69.73±8.43^{B}**	16.55±2.00^{A}	2.96±1.2^{B,C,D}	2.95±1.29^{E}	**4.73±1.19^{A}**	0.55±0.14^{D}	1.40±0.30^{A,C}	0.26±0.06^{B}

Annex IV - Metals concentrations (mg/kg DW) and Biota-Sediment Accumulation factor (BSAF) of *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp), collected in each of the sampling areas from the Ria de Aveiro (mean±SD). Areas are presented according to an increasing contamination gradient. Values in bold represent the most abundant elements in clams. Highlighted values represent BSAF > 1. For *R. decussatus* and *R. philippinarum* significant differences ($p \leq 0.05$) among areas are signalled with different letters (a-C for Rd and A-D for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with asterisk.

		Cu		Cd		Hg		MeHg
Areas	species	Clams	BSAF	Clams	BSAF	Clams	BSAF	Clams
O	Rd	**8.33±1.83^{a}**	13.16±2.90^{a}	0.43±0.12^{a}				
	Rp	**8.90±1.33^{A}**	14.06±2.0^{A}	0.36±0.05^{A,C}				
B	Rp	2.88±0.04^{B}	5.26±0.06^{B}	0.36±0.09^{A,B,C}	3.94±0.96^{A,C}			
N	Rd	2.44±1.10^{b}	2.67±1.21^{b,c}	0.24±0.07^{a}	8.77±2.66^{a}	0.05 ±0.01^{a}	0.02±0.00^{a}	0.04±0.01^{a}
	Rp	**6.67±1.23^{A}**	7.31±1.35^{C}	0.23±0.05^{B}	8.14±1.83^{B}	0.07±0.01^{a}	0.03±0.00^{A}	0.04±0.01^{A}
C	Rp	**3.18±1.66^{B}**	1.56±0.82^{C,D}	0.26±0.12^{A,B,C}	2.81±1.30^{A,C,D}			
E	Rd	3.96±0.72^{b}	2.73±0.50^{c}	0.26±0.03^{a}	5.09±0.52^{b}	0.24±0.06^{b}	2.63±0.70^{b}	0.09±0.02^{b}
	Rp	4.42±0.41^{C}	3.04±0.28^{B}	0.29±0.07^{A,B,C}	5.72±1.28^{B,C}	0.13±0.05^{A}	1.42±0.55^{B}	0.09±0.01^{B}
G	Rd	**5.20±0.01^{c}**	3.64±1.14^{a,b}	0.23±0.01^{a}	5.68±0.00^{b}			
	Rp	**6.74±1.85^{A}**	4.09±1.13^{B}	0.30±0.05^{A}	6.28±1.47^{A}			
F	Rd	**3.78±1.19^{b}**	1.04±0.33^{b}	0.36±0.07^{a}	2.15±0.41^{c}	0.11±0.02^{c}	0.74±0.15^{c}	
	Rp	**3.48±2.34^{B,C}**	0.96±0.65^{D}	0.36±0.02^{A,C}	2.18±0.09^{D}	0.13±0.04^{A}	0.85±0.23^{B}	
K	Rd	**4.62±1.05^{b}**	1.03±0.23^{b}	0.27±0.05^{a}	2.78±0.54^{c}	0.09±0.01^{c}	2.54±0.30^{b}	0.06±0.01^{c}
	Rp	**4.20±2.07^{B,C}**	0.93±0.46^{D}	0.24±0.02^{B}	2.47±0.21^{C,D}	0.09±0.01 ^{B}	2.54±0.30^{C}	0.07±0.01^{C}
M	Rd	**5.00±1.75^{a,b,c}**	1.12±0.55^{b}	0.41±0.09^{c}	2.00±0.43^{c}			
	Rp	**3.63±0.95^{B,C}**	0.81±0.21^{D}	0.41±0.06^{A}	1.99±0.29^{D}	0.10±0.02^{A}		

Annex V - Metal(loid)s concentration in soluble and insoluble fraction of *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp) along the nine sampling areas from the Ria de Aveiro (mean±SD). For each element and for each species it is highlighted the highest concentration (soluble fraction *vs* insoluble fraction).

		As		Cr		Ni		Pb		Cu		Cd		Hg	
Areas	species	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble
O	Rd	1.73±0.39	1.14±0.47	0.24±0.04	0.75±0.15	0.30±0.05	1.36±0.095	0.06±0.01	0.21±0.06	0.63±0.24	1.03±0.13	0.03±0.01	0.05±0.02	0.00±0.00	0.01±0.00
	Rp	1.2±0.33	1.42±0.47	0.21±0.04	0.65±0.11	0.42±0.24	0.53±0.08	0.07±0.01	0.24±0.04	0.77±0.27	1.01±0.08	0.03±0.01	0.05±0.01	-	0.01±0.00
B	Rp	1.36±0.31	1.44±0.50	0.13±0.02	0.71±0.25	0.24±0.08	0.92±0.64	0.03±0.03	0.24±0.012	-	0.79±0.04	0.03±0.01	0.04±0.01	-	-
N	Rd	1.27±0.20	0.72±0.10	0.09±0.03	0.42±0.20	0.18±0.08	0.33±0.13	0.04±0.02	0.18±0.07	0.28±0.10	0.31±0.01	0.02±0.01	0.03±0.01	0.00±0.00	0.02±0.00
	Rp	0.95±0.32	0.66±0.26	0.09±0.04	0.27±0.06	0.16±0.06	0.24±0.05	0.06±0.02	0.15±0.02	0.93±0.22	0.4±0.05	0.02±0.00	0.03±0.01	0.00±0.00	0.01±0.00
C	Rp	0.93±0.28	0.82±0.17	0.19±0.13	0.30±0.07	0.40±0.22	0.28±0.25	0.05±0.07	0.11±0.02	0.17±0.29	0.39±0.02	0.03±0.02	0.04±0.02	-	-
E	Rd	1.19±0.28	0.92±0.16	0.14±0.13	0.27±0.13	0.29±0.21	0.31±0.14	0.07±0.09	0.12±0.01	0.39±0.22	0.40±0.07	0.03±0.00	0.04±0.00	0.02±0.00	0.03±0.00
	Rp	1.49±0.17	0.86±0.03	0.07±0.04	0.29±0.02	0.16±0.00	0.66±0.41	0.02±0.00	0.14±0.03	0.32±0.08	0.57±0.08	0.02±0.00	0.04±0.01	0.01±0.00	0.04±0.00
G	Rd	1.32±0.00	0.97±0.01	0.18±0.00	0.30±0.00	0.27±0.00	0.47±0.00	0.12±0.00	0.29±0.00	0.41±0.01	0.63±0.00	0.04±0.01	0.04±0.00	-	-
	Rp	1.15±0.69	1.27±0.15	0.12±0.01	0.68±0.14	0.28±0.08	0.71±0.04	0.07±0.01	0.27±0.06	0.67±0.43	0.88±0.23	0.03±0.00	0.05±0.01	-	-
F	Rd	1.42±0.53	0.98±0.14	0.04±0.07	0.40±0.07	0.34±0.06	0.58±0.11	0.05±0.01	0.20±0.08		0.76±0.24	0.03±0.00	0.04±0.01	0.00±0.00	0.02±0.00
	Rp	1.28±0.63	0.89±0.53	0.12±0.10	0.34±0.11	0.21±0.06	0.56±0.20	0.05±0.01	0.19±0.06	0.11±0.19	0.59±0.28	0.03±0.00	0.04±0.00	0.00±0.00	0.02±0.00
K	Rd	4.35±1.32	1.80±0.73	0.09±0.01	0.33±0.10	0.24±0.11	0.35±0.10	0.03±0.01	0.13±0.01	0.39±0.05	0.54±0.17	0.02±0.00	0.03±0.01	0.02±0.00	0.00±0.00
	Rp	4.01±1.38	1.35±0.50	0.13±0.03	0.31±0.02	0.40±0.10	0.41±0.13	0.06±0.02	0.15±0.04	0.39±0.05	0.36±0.02	0.02±0.00	0.03±0.01	0.02±0.00	0.00±0.00
M	Rd	4.48±0.37	2.72±0.63	0.2±0.08	0.47±0.04	0.27±0.10	0.51±0.12	0.05±0.01	0.18±0.08	0.15±0.26	0.78±0.18	0.04±0.01	0.04±0.00	-	-
	Rp	6.63±0.69	7.31±0.78	0.03±0.00	0.56±0.21	0.20±0.02	0.74±0.22	0.06±0.02	0.22±0.05	-	0.73±0.19	0.03±0.00	0.05±0.01	0.00±0.00	0.02±0.00

Annex VI - Sediment physicochemical parameters along the 5 sampling areas from the Óbidos lagoon: temperature (ºC), pH, salinity, type of sediment, median, sand and fines content (%), and the total organic matter (TOM, %) (mean±SD). For each parameter, significant differences ($p \leq 0.05$) among areas are signalled with distinct letters (a-d).

Areas	Temperature	pH	Salinity	Main sediment type	Median	Sand	Fines	TOM
A	22.00 ± 1.20^{a}	7.30 ± 0.31^{a}	21.41 ± 2.01^{a}	Clean fine sand	2.24 ± 1.12^{a}	96.59 ± 1.16^{a}	4.41 ± 1.16^{a}	$1.58 \pm 0.25^{a.b}$
B	26.30 ± 1.42^{b}	7.62 ± 0.42^{a}	38.30 ± 1.12^{b}	Clean medium sand	1.66 ± 0.96^{a}	97.90 ± 1.09^{a}	$2.09 \pm 0.91^{a.b}$	1.55 ± 0.12^{a}
C	25.30 ± 1.21^{b}	7.87 ± 0.25^{a}	32.90 ± 0.99^{c}	Clean medium sand	1.76 ± 0.87^{a}	98.17 ± 0.98^{a}	1.83 ± 0.62^{b}	1.13 ± 0.17^{b}
D	25.20 ± 1.34^{b}	7.57 ± 0.32^{a}	32.06 ± 1.14^{c}	Mud	>4	33.42 ± 6.23^{b}	66.58 ± 6.25^{c}	9.65 ± 1.65^{c}
E	25.10 ± 1.10^{b}	7.38 ± 0.43^{a}	32.09 ± 0.87^{c}	Mud	>4	10.60 ± 0.90^{c}	89.40 ± 0.90^{d}	12.31 ± 2.55^{c}

Annex VII - Element concentration and total element concentration (mg/Kg dry weight) in sediments from the Óbidos lagoon, along the sampling areas (mean±SD). Underlined numbers represent concentrations higher than TEL and ERL values. Highlighted values represent the two most abundant elements in each of the sampling areas. For each element concentration and total element concentration, significant differences ($p \leq 0.05$) among areas are signalled with different letters (a-d).

	As	Cr	Ni	Pb	Cu	Total
A	1.18±0.13^{a}	2.98± 0.63^{a}	1.30±0.25^{a}	1.90±0.10^{a}	2.33±0.54^{a}	9.66±1.63^{a}
B	0.74±0.10^{b}	2.03±0.24^{a}	1.13±0.45^{a}	1.90±0.20^{a}	1.36±0.25^{b}	7.10±1.06^{a}
C	1.48±0.16^{a}	4.48 ± 0.15^{b}	1.58±0.17^{a}	4.70±1.30^{b}	2.53±0.11^{a}	14.72±1.58^{b}
D	7.36±1.99^{c}	22.69±7.22^{c}	11.26±3.96^{b}	13.07±4.09^{c}	19.71±7.91^{c}	74.15±25.10^{c}
E	9.36±2.99^{c}	29.46±10.48^{c}	15.53±5.69^{b}	15.86±5.03^{c}	27.74±11.63^{d}	98.01±35.77^{c}
TEL	7.24	52.30	15.90	30.24	18.70	
ERL	8.20	81.00	20.90	46.70	34.00	
PEL	41.60	160.00	42.80	112.00	108.00	
ERM	70.00	370.00	51.60	218.00	270.00	

TEL- Threshold Effect Level (MacDonald et al., 1996); ERL - Effects Range Low (Long and Morgan, 1990; Long et al., 1995); PEL - Probable Effects Levels (MacDonald et al., 1996); ERM - Effects Range Median (Long and Morgan, 1990; Long et al., 1995).

Annex VIII - Concentrations (mg/kg DW) and Biota-Sediment Accumulation factor (BSAF) of each metal(loid)s in *Ruditapes decussatus* (Rd) and *R. philippinarum* (Rp) from the Óbidos lagoon, collected in each of the sampling areas (mean±SD). Values in bold represent the most abundant elements in both species. Highlighted values represent BSAF > 1. For *R. decussatus* and *R. philippinarum* significant differences ($p \leq 0.05$) among areas are signalled with different letters (a-d for Rd and A-B for Rp). For each area, significant differences ($p \leq 0.05$) between species are represented with asterisk.

		As		Cr		Ni		Pb		Cu	
Areas	Species	Clams	BSAF	Clams	BSAF	Clams	BSAF	Clams	BAF	Clams	BSAF
A	Rd	**12.36±1.92^{a}**	10.61±1.93^{a}	**17.92±0.80^{a}**	6.17±0.08^{a}	**7.83±0.80^{a}**	6.50±4.98^{a}	3.36±0.34^{a}	1.76±0.22^{a}	5.92±0.44^{a}	2.63±0.65^{a}
B	Rd	**11.00±1.75^{a}**	15.27±4.69^{a}	**9.53±2.19^{b}**	4.82±1.51^{b}	**5.53±1.44^{b}**	5.64±2.90^{a}	2.03±0.35^{b}	1.10±0.14^{b}	4.53±0.86^{a}	3.49±1.30^{a}
	Rp	**11.69±4.56^{A}**	15.87±5.77^{A}	**8.75±1.08^{A}**	4.38±0.84^{A}	4.44±0.77^{A}	4.49±2.01^{A}	1.72±0.16^{A}	0.93±0.104^{A}	**4.64±1.98^{A}**	3.39±1.08^{A}
C	Rd	**12.92±3.34^{a}**	8.62±0.85^{b}	**8.33±2.10^{b}**	2.12±0.43^{b}	4.33±0.22^{b}	2.34±0.43^{a}	2.34±0.62^{b}	0.52±0.15^{c}	**6.53±1.54^{a}**	2.00±0.49^{a}
	Rp	**12.75±3.34**^{A}	8.62±1.87^{B}	**9.53±1.63**^{A}	2.12±0.35^{B}	3.78±1.76^{A}	2.34±0.94$^{A.B}$	2.34±0.14^{B}	0.52±0.12^{B}	**5.03±1.11^{A}**	0.35±0.18^{A}
D	Rd	9.19±1.42^{a}	1.43±0.37^{c}	**11.00±3.13^{c}**	0.54±0.28^{c}	**12.78±1.55^{c}**	1.09±0.43^{b}	3.42±1.74^{a,b}	0.18±0.08^{d}	**14.14±11.63^{a}**	0.37±0.13^{b}
	Rp	**12.03±3.69^{A}**	1.51±0.25^{C}	10.47±0.35^{A}	0.49±0.15^{C}	**12.78±2.14^{B}**	1.18±0.53^{B}	2.42±0.75^{B}	0.17±0.06^{C}	**15.22±4.73**^{B}	0.32±0.15^{B}
E	Rd	**10.14±1.59^{a}**	1.07±0.43^{c}	**17.11±11.22^{a}**	0.63±0.48^{c}	**11.19±1.88^{c}**	0.88±0.22^{b}	2.29±0.74a^{,b}	0.23±0.14^{d}	5.92±1.38^{a}	0.78±0.34^{c}
	Rp	**10.83±0.83^{A}**	1.29±0.12^{C}	**10.08±1.95^{A}**	0.35±0.06^{C}	**12.00±2.28^{B}**	0.86±0.18^{B}	2.09±0.29^{B}	0.15±0.01^{B}	7.28±1.28^{A}	2.63±0.65^{A}

Annex IX - Elements concentrations in the soluble and insoluble fractions (mg/kg) in *Ruditapes decussatus* and *R. philippinarum* along the five sampling areas (A to E, Óbidos lagoon) (mean±SD). For each element and for each species it is highlighted the highest concentration (soluble fraction *vs* insoluble fraction). For each element, for each fraction and for each species, significant differences ($p \leq 0.05$) among areas are signalled with distinct letters (a-c for Rd and A-C for Rp).

		As		Cr		Ni		Pb		Cu	
Areas	Species	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble
A	Rd	0.93 ± 0.18^{a}	0.55 ± 0.06^{a}	1.07 ± 0.31^{a}	1.08 ± 0.29^{a}	0.49 ± 0.01^{a}	0.45 ± 0.09^{a}	0.11 ± 0.04^{a}	0.30 ± 0.01^{a}	0.30 ± 0.00^{a}	0.41 ± 0.05^{a}
B	Rd	0.99 ± 0.13^{a}	0.33 ± 0.10^{a}	$0.26\pm0.04^{b.c}$	0.89 ± 0.30^{a}	0.20 ± 0.03^{a}	$0.46\pm0.19^{a.b}$	0.06 ± 0.01^{a}	0.18 ± 0.04^{b}	0.30 ± 0.06^{a}	$0.25\pm0.05^{b.d}$
	Rp	0.99 ± 0.45^{A}	0.42 ± 0.13^{A}	0.63 ± 0.14^{A}	0.42 ± 0.11^{A}	0.25 ± 0.16^{A}	0.28 ± 0.13^{A}	0.10 ± 0.04^{A}	0.11 ± 0.03^{A}	0.34 ± 0.25^{A}	0.21 ± 0.02^{A}
C	Rd	1.07 ± 0.19^{a}	0.48 ± 0.10^{a}	$0.34\pm0.13^{b.c}$	0.66 ± 0.21^{a}	0.19 ± 0.06^{a}	0.33 ± 0.04^{a}	0.08 ± 0.02^{a}	0.20 ± 0.06^{b}	0.31 ± 0.17^{a}	$0.47\pm0.14^{a.b}$
	Rp	0.94 ± 0.19^{A}	0.59 ± 0.24^{A}	0.31 ± 0.11^{A}	$0.83\pm0.23^{A.B}$	0.13 ± 0.12^{A}	0.32 ± 0.10^{A}	0.08 ± 0.02^{A}	0.20 ± 0.03^{B}	0.22 ± 0.03^{A}	$0.39\pm0.16^{A,B}$
D	Rd	0.64 ± 0.16^{a}	0.58 ± 0.03^{a}	0.47 ± 0.09^{c}	0.85 ± 0.03^{a}	0.55 ± 0.25^{b}	0.79 ± 0.07^{b}	$0.11\pm0.04^{a.b}$	0.16 ± 0.07^{b}	0.32 ± 0.21^{a}	1.40 ± 0.26^{c}
	Rp	0.69 ± 0.26^{A}	0.61 ± 0.20^{A}	$0.43\pm0.11^{A.B}$	0.83 ± 0.20^{B}	0.57 ± 0.03^{B}	0.87 ± 0.24^{B}	0.09 ± 0.05^{A}	0.16 ± 0.02^{B}	0.22 ± 0.03^{A}	1.61 ± 0.59^{C}
E	Rd	0.66 ± 0.32^{a}	0.45 ± 0.19^{a}	1.46 ± 0.10^{a}	0.60 ± 0.19^{a}	0.82 ± 0.10^{b}	0.71 ± 0.18^{b}	0.28 ± 0.10^{b}	0.13 ± 0.08^{b}	0.48 ± 0.17^{a}	0.23 ± 0.03^{d}
	Rp	0.89 ± 0.21^{A}	0.55 ± 0.24^{A}	$0.43\pm0.03^{A.B}$	0.78 ± 0.24^{A}	0.62 ± 0.15^{B}	0.91 ± 0.12^{B}	0.09 ± 0.03^{A}	0.20 ± 0.09^{B}	0.43 ± 0.24^{A}	0.44 ± 0.14^{B}

Annex X - Carbonate physicochemical parameters at each pH condition tested. Mean values (± SD) of measured pH and determined total alkalinity (TA) in water samples collected from each aquarium (temperature 19±1 °C and salinity 28 ± 1 g/L). Partial CO_2 pressure (pCO_2), bicarbonate (HCO_3^-), carbonate ion concentrations (CO_3^{2-}), saturation states of calcite (ΩCal) and aragonite (ΩAra) were calculated with CO_2SYS software (Robbins et al., 2010).

	Condition	pH	AT (µmol/Kg)	pCO_2 (µatm)	HCO_3^- (µmol/Kg)	CO_3^{2-} (µmol/Kg)	Ω_{Ara}	Ω_{Cal}
R. decussatus	7.8	7.8±0.08	2391.7±131.80	1192.0±162.35	2233.8±105.74	78.2±13.68	1.2±0.22	1.9±0.35
	7.3	7.3±0.05	2897.3±82.85	4062.6±742.39	2861.0±101.11	43.0±17.85	0.7±0.28	1.1±0.43
	7.8 4 mg/L As	7.8±0.14	2526.6±211.97	1275.3±431.61	2348.8±198.86	88.2±30.74	1.4±0.48	2.2±0.74
	7.3 4 mg/L As	7.3±0.10	2829±95.53	3912.6±96.55	2758.3±90.92	35.1±1.54	0.6±0.03	0.9±0.04
R. philippinarum	7.8	7.8±0.08	2350.7±47.34	1361.2±242.06	2217.0±35.01	66.2±12.44	1.1±0.20	1.7±0.32
	7.3	7.3±0.10	2801.7±139.54	4074.4±573.92	2733.6±131.24	33.9±6.82	0.7±0.32	0.7±0.09
	7.8 4 mg/L As	7.8±0.11	2419±183.93	1321.4±373.60	2265.4±177.03	76.1±22.72	1.2±0.36	1.9±0.56
	7.3 4 mg/L As	7.3±0.15	2637.7±71.99	3984.6±700.96	2574.9±73.48	31.1±3.29	0.5±0.05	0.8±0.08

Annex XI - Monte Carlo *ρ*-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 (*Ruditapes 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 ratio; and alkaline phosphatase (ALP) activity. Bold numbers represent significant increases for each speciesat each condition ($p \leq 0.05$).

		0 vs 4 mg/L of As		pH 7.8 vs pH 7.3		Rd vs Rp			
	Species	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 (=)				

Annex XII - Examples of studies that assessed the effects of chemicals and abiotic factors on marine invertebrates.

Species	Chemicals	Abiotic factors	REF.
Mytilus edulis (bivalve)	As (arsenobetaine and arsenocholine) (100 µg/ dm3)	-	Gailer et. 1995
Chamelea gallina and Mytilus galloprovincialis (bivalve)	-	Salinity (28; 34; 40) & pH (7.4; 7.8; 8.1)	Matozzo et al. (2013)
Saccostrea cucullata (bivalve)	As(III) (1; 5; 20 mg/L) and As(V) (1; 5; 20 mg/L)	-	Zhang et al. (2015)
Mytilus edulis,Tegillarca granosa, and Meretrix meretrix (bivalve)	Cd (0.05 mg/L)	pH (8.1, 7.8; 7.4)	Shi et al. (2016)
Crassostrea virginica and Mercenaria mercenaria (bivalve)	Cu (50 µg/L) and Cd (50 µg/L)	pH (7.60-8.18)	Götze et al., 2014
Crassostrea gigas (bivalve)	-	pH (8.0)	Timmins-Schiffman et al. (2014)
Crassostrea angulata and Crassostrea gigas (bivalve)	As (0; 4 mg/L)	pH (7.8;7.3)	Moreira et al. (2016a)
Crassostrea angulata (bivalve)	As (0; 4 mg/L)	Salinity (10, 20, 30; 40)	Moreira et al. (2016b)
Macoma balthica (bivalve)	Cd (0,01; 0,03; 0,10; 0,30 mg/L)	-	Duquesne et al. (2004)
Venerupis corrugata, Ruditapes decussatus, Ruditapes philippinarum (bivalves)	-	Salinity (0 to 42)	Carregosa et al. (2014)
Ruditapes decussatus and R. philippinarum (bivalve)	-	Temperature (22 to 42) & pH (8.2)	Anacleto et al. (2014)
Ruditapes decussatus and R. philippinarum (bivalves)	As (0 to 70 mg/L) and Hg (0 to 8 mg/L)	-	Velez et al. 2016b
Ruditapes decussatus and R. philippinarum (bivalves)	As (0; 4 mg/L)	pH (7.3;7.8)	present study
Ruditapes philippinarum (bivalve)	As (0; 4; 17 mg/L	Salinity (10; 21; 28; 35; 42)	Freitas et al. (2016)
Ruditapes philippinarum (bivalve)	As (0; 2 mg/L)	-	Velez et al. (2016a)
Ruditapes philippinarum (bivalve)	-	Salinity (14; 28; 35) & pH (7.8)	Velez et al. (2016c)
Ruditapes philippinarum (bivalve)	Carbamazepine (0.00; 0.03, 0.30; 3.00; 9.00 µg/L)	pH (7.8)	Almeida et al. (2015)
Corbicula fluminea (bivalve)	As (0.1; 0.3; 0.5; 1 mg/L)	pH (7.0)	Diniz et al. (2008)
Venerupis corrugata (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)
Scrobicularia plana (bivalve)	Hg; Cd; Zn; Cu (0.05 to 1 mM)	-	Mazorra et al. (2002)
Scrobicularia plana (bivalve)	Carbamazepine (3.00 mg/L)	pH (7.1; 7.8)	Freitas et al. (2015)
Lamellidens marginalis (bivalve)	As (0 to 5 mg/L)	-	Chakrabortya et al. (2010)
Corbicula fluminea (bivalve)	As (0.1; 0.3; 0.5;1.0 mg/L)	pH (7.0)	Diniz et al. (2008)
Arenicola marina (polychaeta)	As (2, 5, 10; 20 µg/L)	-	Casado-Martinez et al. (2012)
Cyprinus carpio (fish)	As (0; 0.1; 1.0 mg/L)	pH (8.0)	Ventura-Lima et al. (2009)