Universidade de Aveiro Departamento de Biologia
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Vanessa Silva Carregosa

Tolerance and response of clams in Ria de Aveiro to salinity changes

Tolerância e resposta de amêijoas na Ria de Aveiro a alterações de salinidades

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica da Doutora Rosa de Fátima Lopes de Freitas (Investigadora Auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro) e co-orientação científica da Professora Doutora Etelvina Maria Paula de Almeida Figueira (Professora Auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro).

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Onde sou a mim mesma devolvida
Em sal, espuma e concha regressada
À praia inicial da minha vida

Sophia de Mello Breyner Andresen

o júri

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palavras-chave

Venerupis decussata, *Venerupis corrugata*, *Venerupis philippinarum*, bivalves, biomarcadores, osmorregulação, Ressonância Magnética Nuclear, stress oxidativo, metabolómica

Resumo

As respostas dos organismos aquáticos a alterações naturais, nomeadamente, alterações de salinidade, têm recebido pouca atenção, inversamente à preocupação que tem vindo a crescer em relação aos impactos da contaminação em populações marinhas bentónicas. De facto, a salinidade é um dos factores ambientais dominantes que mais afetam os bivalves marinhos, o que limita a sua distribuição espacial no ecossistema. As marés combinadas com entradas de água doce, de rios ou períodos de chuva longos e estações secas extremas, podem alterar drasticamente a salinidade da água, provocando alterações nas populações de bivalves bentónicos, nomeadamente intertidais. Além disso, a salinidade de um determinado ambiente irá restringir a distribuição espacial das espécies, o que é especialmente importante quando se avalia a propagação de uma espécie invasora num ambiente novo. A fim de entender como espécies nativas (*Venerupis decussata* e *Venerupis corrugata*) e invasoras (*Venerupis phiippinarum*) de molluscos lidam com as mudanças de salinidade, foram investigados parâmetros fisiológicos, bioquímicos e metablómicos. Os resultados obtidos mostraram que *V. decussata* e *V. philippinarum* apresentaram elevada mortalidade em salinidades baixas (0 e 7), mas toleram as salinidades mais altas (35 e 42). Por outro lado, *V. corrugata* apresentou elevadas taxas de mortalidade tanto em salinidades baixas (0 e 7) como em salinidades altas (35 e 42). A quantificação do teor de Na e K, revelou que ao longo do gradiente de salinidade, a *V. decussata* foi a espécie com maior capacidade de manter a homeostasia iónica. Os parâmetros bioquímicos também mostraram que *V. decussata* foi a espécie que melhor lidou com as mudanças de salinidade enquanto a *V. corrugata* foi a mais sensível. Além disso, os resultados obtidos mostraram que as ameijoas, sob condições adversas de salinidade, podem alterar os seus mecanismos bioquímicos, nomeadamente aumentando as suas defesas antioxidantes, para lidar com um maior stress oxidativo resultante das condições de hipo e hipersalinidade. Entre os parâmetros fisiológicos e bioquímicos analisados (glicogénio, glucose, proteinas, níveis de peroxidação lípidica (LPO), atividade de enzimas antioxidantes; glutationa total, reduzida e oxidada), LPO, superoxide dismutase (SOD) e glutathiona S-transferase (GST) mostraram ser biomarcadores úteis para avaliar os impactos de salinidade em bivalves. Os efeitos das alterações de salinidade no perfil metabólico das três espécies foram também estudados através de Ressonância Magnética Nuclear de ^{1}H (RMN). A análise multivariada dos espectros de RMN permitiu a observação de alterações em relação à exposição de ameijoas a diferentes concentrações de salinidade. Quando expostos a baixas salinidades, as reservas energéticas destes organismos podem ser esgotadas, aumentando o desequilíbrio osmótico, afetando o desempenho metabólico e aumentando o stress oxidativo. *V. corrugata* mostrou ser a amêijoa mais sensível a mudanças de salinidade. O intervalo de salinidades entre 21 e 28 foi o ideal para *V. decussata* e *V. philippinarum* e a salinidade 21 foi a ideal para *V. corrugata*. Este estudo mostrou que as mudanças de salinidade têm impactos diferentes em espécies nativas e invasoras.

keywords

Venerupis decussata, *Venerupis corrugata*, *Venerupis philippinarum*, bivalves, biomarkers, osmoregulation, Nuclear Magnetic Resonance, oxidative stress, metabolomics.

abstract

Unlike the concern that has been growing in relation to the impacts of contamination in marine benthic populations, the responses of aquatic organisms to natural alterations, namely changes in salinity, have received little attention. In fact, salinity is one of the dominant environmental factors that most affect marine bivalves, limiting their spatial distribution in the environment. Tide combined with fresh water inputs, from rivers or heavy rainy periods, and extreme dry seasons can dramatically alter the salinity of water, causing alterations in the benthic populations, namely intertidal bivalves. Furthermore, salinity of a given environment will restrict the spatial distribution of the species, which is especially important when assessing the spread of an invasive species into a new environment. In order to understand how native (*Venerupis decussata* and *Venerupis corrugata*) and invasive (*Venerupis philippinarum*) clam species cope with salinity changes, physiological, biochemical and metabolomic patterns were investigated. The results obtained showed that *V. decussata* and *V. philippinarum* presented high mortality at low (0 and 7) but tolerate high (35 and 42) salinities. On the other hand, *V. corrugata* presented high mortality rates both at low (0 and 7) and high salinities (35 and 42). The quantification of Na and K content revealed that, along the salinity gradient, *V. decussata* was the species with higher ability to maintain the ionic homeostasis. The biochemical parameters also showed that *V. decussata* was the clam that best cope with salinity changes and *V. corrugata* was the most sensitive. Furthermore, the results obtained showed that clams under salinity stressful conditions can alter their biochemical mechanisms, such as increasing their antioxidant defences, to cope with the higher oxidative stress resulting from hypo and hypersaline conditions. Among the physiological and biochemical parameters analysed (glycogen, glucose and protein content; lipid peroxidation (LPO) levels, antioxidant enzymes activity; total, reduced and oxidized glutathione), superoxide dismutase (SOD), LPO and glutathione S-transferase (GST) showed to be useful biomarkers to assess salinity impacts in clams. The effects of salinity changes in the metabolic profile of the three species were also studied using ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy of clam extracts. Multivariate analysis of the NMR spectra enabled metabolite changes to be observed in relation to clams exposure to different salinity concentrations. When exposed to low salinities, energy reserves of clams may be exhausted, increasing the osmotic imbalance, affecting the metabolic performance and increasing the oxidative stress. *V. corrugata* showed to be the most sensitive clam to salinity changes. The optimal salinity for *V. decussata* and *V. philippinarum* was between 21 and 28 and for *V. corrugata* was salinity 21. This study showed that changes in salinity have different impacts in native and invasive species

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List of Abbreviations

BSA – Bovine Serum Albumin

CAT – Catalase

CDNB – 1-chloro-2,4-dinitrobenzene

DTPA – Diethylene Triamine Pentaacetic Acid

DTT – Dithiothreitol

EDTA – Ethylenediamine Tetraacetic Acid

GSH – Reduced Glutathione

GSH/GSSG – Reduced Glutathione/Oxidized Glutathione ratio

GSSG – Oxidized Glutathione

GST – Glutathione S-Transferase

KIO_4 – Potassium Periodate

KOH – Potassium Hydroxide

LPO – Lipid Peroxidation

MDA – Malondialdehyde

MVA – Multivariate analysis

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

NBT – Nitro Blue Tetrazolium

NMR – Nuclear Magnetic Resonance

OCR - Oxygen Consumption Rate

PCA – Principal Components Analysis

PCO – Principal Coordinates analysis

PLS-DA – Partial Least Squares - Discriminant Analysis

PVP – Polyvinylpyrrolidone

ROS – Reactive Oxigen Species

SOD – Superoxide Dismutase

TBA – Thiobarbituric Acid

TBARS – ThioBarbituric Acid Reactive Substances

TCA – Trichloroacetic Acid

1. Introduction

1.1. Aquatic Systems under global changes

1.1.1. Climate changes: salinity alterations

The alterations on climate are a global problem and have been studied in the last few decades (Hull and Tortoriello, 1979; Beare and Heaney, 2002; Milly et al., 2002; Booij, 2005; Kay et al., 2006; Solomon et al., 2007). Behind these alterations is the increase of greenhouse gas concentrations provoked by anthropogenic activities (Houghton et al., 1996; Beare and Heaney, 2002; Karl and Trenberth, 2003; Booij, 2005; Solomon et al., 2007). The consequences of these events may occur at several levels, as described by the International Panel on Climate Change (IPCC, 2007). The increase of sea level, evapotranspiration, runoff and river discharge, changes in ocean circulation, extreme precipitation, changes in sea surface temperature, wind speed and even changes in nutrient supply and distribution of plankton are some of the changes predicted by IPCC (2002; 2008). IPCC also predicted that global climate changes will occur in the next hundred years and the main alterations in marine environment include water acidification and changes in water salinity (Booij, 2005; Kay et al., 2006). It is expected that the increase of mean sea level will intensify flooding and provoke flood of low-lying coastal areas, erosion of lagoon sand barriers and invasion of saltwater in estuaries and aquifers, which will cause a freshwater lack and loss of natural ecosystems in these environments (Nicholls et al., 2007; FitzGerald et al., 2008; Nicholls, 2010). Thus, due to erosion of barriers in lagoon systems and intensification of flooding, it is expected the increase of salinity in estuaries (Hull and Tortoriello, 1979; Beare and Heaney, 2002). In fact, estuaries are particularly affected by climate changes, especially by salinity fluctuations, due to tidal inputs and mainly due to long periods of extreme precipitation, decreasing the salinity of the water, and dry and hot seasons increasing the salt concentration. Thus the organisms that live in these ecosystems, periodically experience hypo and hypersaline stresses.

In a warmer world dominated by human influences, longer periods of precipitation and more intense variations of salinity are predicted to become seriously frequent. These events will certainly affect the organisms living in ecosystems where the salinity fluctuations are common. Due to these changes, it is very important and extremely urgent to study the effects of salinity fluctuations in aquatic organisms, especially those living in estuaries and lagoon systems, like bivalves.

The impact of climate change on salinity shifts is studied since 1979 (Hull and Tortoriello, 1979) and since then, other studies have been performed in this field of work and it is known that salinity profoundly affects chemical, physical and biological dynamics of estuaries (Gibson and Najjar, 2000). Johnson et al. (1991) affirmed that the effect on density, circulation and stratification is the most important physical consequence of salinity. Furthermore, biological dynamics are affected, since salt concentrations in a transitional water system affect the density and occurrence of populations in ecological niches according to their salinity tolerance (Attrill and Rundle, 2002). Nicholls et al. (2007) affirmed that salt concentration alterations, induced by climate change, affect the ecological equilibrium of transitional water systems, forcing organisms living in these very productive environments, to readapt in relation of their distribution. Velasco et al. (2006) investigated the biomass of primary producers in a hypersaline stream and concluded that the main factor determining the composition and structure of macroinvertebrate communities in a protected area in Spain (Rambla Salada) was salinity. Furthermore, it has been shown that salinity alterations disrupts the organisms affecting their distribution, survival, growth and reproduction (Hall and Burns, 2002; Gonçalves et al., 2007; Brucet et al., 2010). The exposure of larvae states to salinity changes have influence in the survival capacity, growth and development of organisms (Giménez and Anger, 2001; Giménez and Torres, 2002; Giménez, 2003). Giménez and Anger (2001) discovered that higher losses of carbon and nitrogen at lower (15 and 20) than at higher (32) salinities leads to a loss of biomass during embryogenesis of the estuarine crab *Chasmagnathus granulata* and concluded that salinity changes may have effects in the survival of early larvae in the field. Giménez and Torres (2002) also studied the influence of salinity in *C. granulata* during embryonic development and found that a group of physiological and development processes and variability in biomass are affected in embryos when exposed to a salinity stress, which may influence the survival and growth in advanced stages of its life cycle.

When assessing the spread of an invasive species into a new environment salinity is one of the major factors limiting the spatial distribution of marine species (Widdows and Shick, 1985; Berger and Kharazova, 1997). Thus, the study of interactions between the alien and native marine species under stressful conditions, namely salinity shifts, has become a focus of interest, especially when it comes to economically relevant species.

1.1.2. Alien species

One of the major threats to biological diversity is now acknowledged to be biological invasions caused by alien species, which has been recognized as an important element of global change (Pravoni et al., 2006). Elliot (2003) describes that there are many aspects in which introduced marine organisms can be regarded as being no different from chemical pollutants and encourages the use of the term biological pollution.

Exotic, alien, or allochthonous species are defined as species that are introduced out of their native habitat by the man (intentionally or accidentally) or naturally (Ruiz et al., 1997; Occhipinti-Ambrogi and Savini, 2003). When this introduction become a threat to biodiversity, economy or/and public health, the species are identified as invasive.

Marine species are probably the easiest group of animals to transport to a new environment. Since the beginning of ship traveling organisms have been accidentally transported on ballast of the ships, making the marine invasions historical (Bax et al., 2003). Navigation, aquaculture, channels building, some recreation activities, discharges of ballast water, tourism and sportive fishery represent some of the vectors that contribute to the introduction of exotic marine organisms (Leppäkoski, 1991; Bax et al., 2003; Ruiz et al., 1997). Thousands of freshwater, estuarine and marine species have been established far away from their native regions (Elton, 1958; Carlton and Geller, 1993).

Some of the exotic species adapt to the new habitat becoming part of the ecosystem, coexisting with the native species. However, some non-native species just compete with the native becoming invasive if they have more favourable characteristics (Bax et al., 2003). Like Charles Darwin proposed, natural selection will command the most adapted species to live in certain habitat and under certain conditions. For example, the native species can lose their priori advantage in an environment where they were well-adapted if anthropogenic alterations rapidly alter the environmental conditions and they had to compete with exotic species (Pravoni et al., 2006). This means that if the environmental conditions suddenly changed, the exotic species will compete with the native because the former are as well or better adapted to the new conditions (Byers, 2002). Invasions can be considered threats for native species. The alteration on ecosystem properties and consequent influence in other species include reduction of food availability, changes on concentrations of phytoplankton and zooplankton; change in flow of nutrients, influencing the biogeochemical cycle; quality of physical resources, including free space,

temperature and light (Gutiérrez et al., 2003; Crooks, 2002). The invasions also have impacts on economic and social issues, affecting the activities involved on marine environments – fisheries, aquaculture, tourism activities and recreational activities (Bax et al., 2003). Invasive species are not only directly involved in social impacts, but they have also an indirect responsibility on the decrease of local people's well-being, degrading and reducing the quality of their natural environment.

On the other hand, in some cases, alien species could also be positive. It can be one reason to create new economic activities and consequently, increase workstations related to this activities and others like project management of exotic marine species (Bax et al., 2003). Positive effects of invasion of exotic species also include the opportunity of native species to escape to predation, taking advantage of shells of living molluscs that provide a structural barrier. It can also create other micro-habitats on the empty spaces between shells and protect other organisms from waves, currents, temperature and others stresses (Gutiérrez et al., 2003).

The most part of marine exotic species are found in the tidal and subtidal zones (Bax et al., 2003). Streftaris et al. (2005), showed that zoobenthos represents about 57 % of the non-indigenous species in European seas, being the dominant group of organisms invading new habitats. The same study demonstrated that the Mollusca phylum represents 23 % of all alien species in seas of Europe.

Bivalves are one of the examples of invasion in oceans, colonising several aquatic ecosystems with particularly ecological and economic impacts (Sousa et al., 2009). Some invasions of bivalves are positive for invertebrate density and species richness, but on the other hand, there are cases of bivalves invasions associated with decreases, or even extinction, of some species (Solidoro et al., 2000; Pravoni et al., 2006; Sousa et al., 2009).

1.2. Bivalves as bioindicators

Bivalves are characterised by the presence of two shells or valves, articulated in its dorsal portion by a corneal ligament. Their shells are constituted by one layer with protein composition and two layers composed by calcium carbonate ($CaCO_3$) (Gosling, 2003; González, 2012). They filter the water catching organic matter and phytoplankton as food. In species that live buried in sediment, feeding and breathing are performed through two siphons (one inhalant and other exhalant). Bivalves can be found in fresh or seawater, and their survival capacity and life quality

depends on environmental (abiotic) and biological (biotic) factors. The three species under analysis in this study are gonochoric (with separated sexes in different organisms), although hermaphroditism is rarely detected (González, 2012). In bivalves, fertilization occurs externally after females and males discharge the gametes in water and especially during de summer (González, 2012). When gametes are fertilized, larval development initiates with cleavage of the embryo yielding a trochophore pyriform, an invertebrate free-swimming larva. The straight-hinged larva or Dlarva stage (D-stage, the second larval form) presents already two valves protecting a complete digestive system, and the velum, a locomotor and feeding organ. During this stage while the larvae swim, feeding and growing, a protuberance in shell near the hinge, called umbo, develops until larvae approach maturity. A foot and gills are formed in the maturity stage. Metamorphosis occurs when the branchia is developed and the velum is lost. In this phase of their life cycle, clams change to a sedentary benthic life style. Thanks to the foot, postlarvae organisms (with similar appearance to adults) bury in sediment and rapidly become adults (Gosling, 2003; González, 2012).

The sessile condition and feeding habits (filtration), put bivalves constantly subjected to environmental stressful conditions, such as fluctuations in water temperature, oxygen concentrations and salinity, predators, alterations on food availability and the quality of the surrounding environment (Almeida et al., 2007). Salinity, temperature, dissolved oxygen, light and pH are some of the abiotic factors that influence the biological processes of bivalves and their activity and the presence of parasites, competitors and quantity of food available represent the potential biotic threats (Berger and Kharazova, 1997).

1.2.1. Responses of bivalves to salinity alterations

Bivalves, such as many other organisms, have the ability to adapt themselves to different alterations in the surrounding environment, based on regulating processes, which maintain physiological homeostasis of individuals (Manduzio et al., 2005). These organisms are examples of benthic species and have been considered good aquatic bioindicators for having a wide geographical distribution, tolerance to several adverse conditions, great capacity of bioaccumulation, sedentary behaviour and single sampling (Pruell et al., 1986; Usero et al., 1997; Gómez-Ariza et al., 2000; Reid et al., 2003; Luedeking and Koehler, 2004; Albentosa et al., 2007; Liu et al., 2011; Kamel et al., 2012; Antunes et al., 2013; McEneff et al., 2014).

Because molluscs bivalves are filterers and due to their habitat characteristics and functional morphology, these organisms become a “mirror” of the environment. The analysis of several organic parts of bivalves, allows to obtain indicators of the condition of sediment and water column where they inhabit. Their bioaccumulation action, as the capacity of concentrating many elements existing in the environment (e.g. metals or organic compounds), make these organisms very important indicators of pollution levels in their ecosystem.

Typical responses of aquatic organisms to salinity changes include, besides mortality, biochemical, physiological and metabolic responses and the reduction of feeding activity and growth rates (Shumway, 1977a, 1977b; Navarro, 1988; Guerin and Stickle, 1992; Matozzo et al., 2007; Carregosa et al., 2014a). In particular, marine organisms living in estuaries are subjected to tidal and rain periods, causing short-term and long-term changes in salt concentrations. These events force these organisms to appeal to physiological mechanisms to be able to survive under these stress conditions (Navarro and Gonzalez, 1998).

The abiotic factors have consequences in the bivalves accumulation capacity, since it may limit the filtration rate. Thus, the monitoring of the bivalves’ health is an important indicator of microbiological and chemical quality of their production areas. Since bivalves are among organisms that are highly influenced by salinity fluctuations, because they are mostly estuarine or near shore nature, it is important to understand the implications of such alterations on these organisms.

Bivalves may immediately close their valves when the surrounding salinity concentration changes as a mechanism of defence against osmotic stress (Kim et al., 2001; Gosling, 2003). Akberali (1978) and Elston et al. (2003) demonstrated, respectively, that *Scrobicularia plana* and *Venerupis philippinarum* are able to close their valves when exposed to low salinities so that they can resist to this osmotic stress. Kim et al. (2001) suggested that valves closure in *V. philippinarum* when it is exposed to low salinities (5, 10, 15 and 20), resulting into a reduction of Oxygen Consumption Rate (OCR), and respiration rate.

Navarro (1988) showed that when the mussel *Choromytilus chorus* was exposed to a range of salinities (15, 18, 24 and 30), low feeding activity and high metabolic rate at lower salinities, promoted a decrease growth efficiency. Similar results were found by Navarro and Gonzalez (1998). These authors exposed the scallop *Argopecten purpuratus* to different salinity concentrations (18, 21, 24, 27 and 30) and concluded that its scope for growth was negatively affected by lower salinities (18 to 24) due to low feeding activity, loss of energy in excretion and

respiration activity, ingestion and absorption rates. Long periods of exposure to low salinity have important effects on the performance and osmoregulatory mechanisms, inducing a significant decrease of the ingestion and scope for growth rates of *Chlamys opercularis* and *Patinopecten caurinus* (Shumway, 1977b; Bernard, 1983). As described by Sarà et al. (2008), the scope for growth of the *Brachidontes pharaonis* is negatively affected by low salinity (15) in a range of salinities from 37 to 15.

Since water is considered the most important molecule of life, its internal loss on cells is certainly a threat to organisms (Yancey, 2005) and cope with this changes is extremely important for survival of organisms exposed to such alterations. Osmoregulation is one of the protection mechanisms, and perhaps the most effective one, working for survival of marine organisms under salinity alterations (Shumway, 1977a; Berger and Kharazova, 1997). Normally, osmoregulation in a new environment, namely with different salt concentration, is performed by inorganic cations, such as Na, Cl, and K (Evans et al., 2005; Bianchini et al., 2008). However, osmoregulation performed by inorganic cations represents a less energy costly mechanism than the one regulated by organic compounds (Carregosa et al., 2014a). Additionally, major osmotic components in cells of multicellular organisms are organic osmolytes, although the extracellular fluids are mostly composed by inorganic compounds, such as NaCl (Yancey, 2005). Osmoregulation achieved by organic molecules, include the functioning of osmolytes, is used by organisms to maintain cell volume when they are under osmolarity stress (Yancey, 2005). Organic osmolytes have unique properties such as protecting metabolic reactions and counteracting the destabilizing forces on macromolecules, which confers them an important role on the prevention of cellular damage (Carregosa et al., 2014a).

Osmoconformers are organisms that adapt their fluids osmolarity according to the external environment. This type of organisms are most commonly found in the oceans and include vertebrates and some arthropods (Yancey, 2005). Whereas some osmoregulators in oceans (sharks, hagfishes, skates, fishes…) have regulator organs, namely gills and kidneys, which maintain the osmolarity of their internal body fluids, avoiding in general, the use of organic compounds, osmoconformers need these molecules to regulate their metabolism and match their body osmolarity to the surrounding environment. Organic osmolytes include, among others, small carbohydrates like sugars and amino acids (for example, glycine, proline, taurine) (Yancey et al., 1982; 2001). These compounds have a very diffuse occurrence. While some organic osmolytes, such as glycine and betaine, are found in every kingdoms of life, others like taurine is more common in marine organisms and in some mammalian organs (Yancey, 2005).

Aquatic organisms under osmotic pressure can also respond to these adverse conditions with oxidative stress. The study of different stages related to this mechanism can give relevant information about their physiological status. Oxygen plays an important role in the species diversification and in their distribution on the ecosystems (Manduzio et al., 2005). Many biological reactions and processes have oxygen as base, making this molecule essential to aerobic organisms, but it can also be dangerous due to its great oxidizing capacities (Abele, 2000; Manduzio et al., 2005).

Reactive Oxygen Species (ROS), atoms or molecules that are extremely unstable and potentially reactive (Manduzio et al., 2005; Almeida et al., 2007), are generated by all the reactions involving oxygen consumption (Abele, 2000). Organisms rely on a respiratory chain and enzymatic systems to use oxygen, but they also need some mechanisms to deal or eliminate the toxic effects of oxygen (Ďuračková, 2008). When this mechanisms are not balanced meaning that preference is given to the formation of oxidants, allowing the generation of reactive metabolites of oxygen and nitrogen (ROS and RNS, Reactive Nitrogen Species), oxidative stress can be established, leading to oxidation of key cell components like proteins, fatty acids and DNA (Sies, 1997; Hayes et al., 2004; Manduzio et al., 2005; Wakamatsu et al., 2008; Niki, 2012; Antunes et al., 2013). Oxidants are also produced as a result of aerobic metabolism, being a common outcome during the development of natural physiological processes in cell, but in adverse conditions, it can be produced at higher levels (Sies, 1997), forcing the cell to fight against this uncontrolled production of oxidants to avoid cell damage (Storey, 1996). Superoxide anion radical ($O2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}HO$) formation are intermediate steps for oxygen reduction (Sies, 1997; Griendling and FitzGerald, 2003). Free radicals become toxic to the cell when the protective mechanisms fail, leading to a damage on molecules, cells, organs and even to death of the organisms. Damages in mitochondria caused by superoxide, can lead to apoptosis – cellular suicide (Abele, 2002). One way of interception of toxic free radicals is performed by enzymatic antioxidants. Superoxide dismutases, catalases and glutathione peroxidases are the main classes of antioxidant enzymes. Specialized antioxidant defences pass through catalase (CAT), that detoxificate H_2O_2, superoxide dismutase (SOD), for decomposition of $O2^{\bullet -}$, oxidized glutathione (GSSG), glutathione S-transferase (GST) (Sies, 1997; Griendling and FitzGerald, 2003).

1.2.2. Tested species

The clams *Venerupis decussata*, *Venerupis corrugata* and *Venerupis philippinarum* were used in the present study. These species belong to Animalia kingdom, Mollusca phylum, Bivalvia class,

Figure 1. Clam species: A - *Venerupis corrugata*; B - *Venerupis philippinarum*; C - *Venerupis decussata*.

Veneroida order, Veneridae family, Venerupis genus (ITIS report). Several studies demonstrated that these species are found worl-wide (Flassch and Leborgne, 1992; Usero et al., 1997; Allam et al., 2000; Elston et al., 2003; Pravoni et al., 2006; Delgado and Pérez-Camacho, 2007; Bebianno and Barreira, 2009; Dang et al., 2010; Figueira and Freitas, 2013).

Venerupis decussata (Linnaeus, 1758) (Figure 1C), formerly known as *Ruditapes decussatus*, also known as grooved carpet shell or European clam (Usero et al., 1997) is characterized by its yellowish colour with brown stains, radial and concentric ridges. It is an euryhaline species that lives in sheltered areas of the coast, bays, estuaries and river mouths. This bivalve lives buried in sediment up to 12 cm. Feeding and breathing are performed by two siphons separated along its whole length. The fertilization of this species occurs in water, where females lay their oocytes and males deposit the sperm, since they have separated sexes.

V. decussata is native from Europe and it is distributed along Atlantic coast from Norway to Congo, English Channel, Mediterranean Sea and in Red Sea (Parache, 1982; Gosling, 2002). This species is mainly produced in France, Spain, Portugal and in the Mediterranean basin (Schuller, 1998; FAO 2011). The European clam has a great economic value and a consequent high commercial value, representing an important resource (Matias et al., 2009; 2013). In Portugal, this species is hardly produced and harvested, representing a large portion of the aquaculture production (27 % in 2009; DGPA, 2011), being the Ria de Aveiro one of the main production areas (Matias et al., 2009; 2013).

Venerupis philippinarum (Adams & Reeve, 1850) (Figure 1B), formerly known as *Ruditapes philippinarum*, is characterised by a solid, equivalve and inequilateral shell, with many variations in colour and pattern, generally brownish. This species, also known as Japanese carpet shell or Manila clam, lives buried in sediment approximately at 4 cm to surface in intertidal and subtidal zones.

The manila clam is native from Indo-Pacific regions (Gosling 2003), being the wild populations found in Asiatic coast (Philippines, South and East China Seas, Yellow Sea, Sea of Japan, Sea of Okhotsk and around Southern Kuril Islands) (FAO).

Manila clam was accidentally introduced in east part of Pacific coast, North America, in the beginning of 1930s, imported together with Pacific oysters, *Crassostera gigas* (Flassch and Leborgne, 1992). Late, due to the unstable yields and overfishing of European *V. decussata*, force the intencional import of *V. philippinarum* with aquaculture proposes (Breber, 1985; Pellizzato et al., 1989; Gosling, 2003). At the beginning of 1970s this species was introduced in France (Bodoy et al., 1981; Flassch and Leborgne, 1992; Gosling 2002) and rapidly spread along European coastal systems, becoming in some places the main contributor to the local fisheries. Because this species showed to have a faster growing that *V. decussata*, other countries, like Ireland, Italy, England, Spain (Flassch and Leborgne, 1992; ICES, 2011) also imported it into European waters following the large aquaculture hatchery. Thus, presently, *V. philippinarum* is one of the mollusc species that have been able to settle far away from its natural habitat (Melià and Gatto, 2005; Melià et al., 2004) being one of the most exploited bivalves species (Usero et al., 1997; Allam et al., 2000; Pravoni et al., 2006; Dang et al., 2010; Figueira et al., 2012; Moschino et al., 2012; Figueira and Freitas, 2013; FAO, 2014a).

The great capacity of *V. philippinarum* to introduce itself into a new environment, coupled with its fast growth give to this species a high commercial value (Usero et al., 1997), which have been changed sharply the exploitation of living resources in aquatic ecosystems, with Manila clam representing 2.36 million tonnes of produced organisms in 2002 (FAO, 2010).

It is unknown when and how Manila clam was introduced in Portugal, but it was registered for the first time in the Tagus estuary in 2000, in extensive intertidal and shallow areas (ICES, 2011). At the same time that abundance of *V. philippinarum* increased, it was noticed a massive decrease in abundance of the native *V. decussata* (Pravoni et al., 2006; ICES, 2011), living in sympatric in same places. This species is the most commonly cultured clam species (Clam fisheries and Aquaculture), being the fourth species more produced in world in 2011 with 3.68 million tonnes among fishes, crustaceans, molluscs and others (FAO, 2013). Some authors have been described

V. philippinarum as being more capable to survive to physical, environmental and anthropogenic stressors than other species, which make this species able to take the ecological niche of native species in the locals where *V. philippinarum* was introduced (Solidoro et al., 2000; Pravoni et al., 2006).

Venerupis corrugata (Gmelin, 1791) (Figure 1A), formerly known as *Venerupis pullastra*, is also known as pullet carpet shell (and), present an equivalve and not equilateral shell and its coloration can vary from cream to light brown, grey or yellowish white, with darker bands representing the growth stages. Unlike *V. decussata* and *V. philippinarum*, the siphons of this species are joined along their entire length, except in the end zone. The outside of the shell, periostracum, is fine and flat with concentric and irregular ridges usually more pronounced in the posterior area and radial ridges very fine. It leaves buried in sand and silty mud, up to 5 cm and it can be found from the low tide mark to nearly 40 m of depth of water column.

This species is distributed from the North of Norway to Atlantic coast of Morocco undergoing by Iberian Peninsula and the majority of the harvesting of this species occurs in Portugal, Spain, France and Italy (FAO, 2010). The intensive capture of this species started in 1926 (Anacleto et al., 2013; FAO, 2014c).

According to FAO, in 2009 the production of fishes and molluscs in Portugal represented almost 100 % of total aquaculture production. According the last update information (INE, 2013), 42 % of the total shellfish production represents the national annual production of clams in Portugal, being extremely important to the national socioeconomic framework, since it implies, directly or indirectly, thousands of employees.

The organisms included in Bivalvia phylum are economically relevant in Portugal, representing a significant part of national fishery (IPIMAR, 2008). These organisms are part of Portuguese cuisine, being much appreciated by their consumers especially in summer (Nunes and Campos, 2008).

1.3. Objectives

Unexpected and irreversible consequences are expected for the native communities when different stressors act together, namely biological invasions and salinity alterations (Occhipinti-Ambrogi and Savani, 2003; Whitfield et al., 2007). Indeed, salinity is one of the most relevant environmental factors that have impact in marine organisms, restricting their spatial distribution (Widdows and Shick, 1985; Berger and Kharazova, 1997). Thus, salinity changes in aquatic systems are especially important when assessing the spread of an invasive species in a new environment. For this reason, the present work was conducted with the aim to investigate the influence of salinity alterations in native (*V. decussata* and *Venerupis corrugata*) and invasive (*Venerupis philippinarum*) clam species. The three clam species, collected at the Ria de Aveiro (where they live in sympatry), were exposed to a range of salinities under controlled laboratory conditions. To assess the salinity effects on these species, ionic content, physiological, biochemical and metabolic alterations were investigated. Powerful tools, such as RMN, were used to assess the biological impacts of salinity changes on these three Veneridae clams aiming to identify the mechanisms activated as response to this osmotic stress.

2. Materials and Methods

2.1. Study area

In the present study, clams (*Venerupis philippinarum*, *Venerupis decussata* and *Venerupis corrugata*) were collected at the Mira Channel in the Ria de Aveiro (Figure 2), which is considered the less impacted channel in this system (Castro et al., 2006; Freitas et al., 2014). Ria de Aveiro is a shallow coastal lagoon in Northwest of Portugal, representing one of the most notable geographical accidents of the Portuguese coast. This lagoon system, comprises a complex system of a longitudinal channel and several ramifications (Lopes et al., 2007) and is about 45 km long (NNE-SSW) and 8.5 km wide. The area covered with water at high tide is approximately 47 km^2 and at low tide is about 43 km^2 (Barroso et al., 2000).

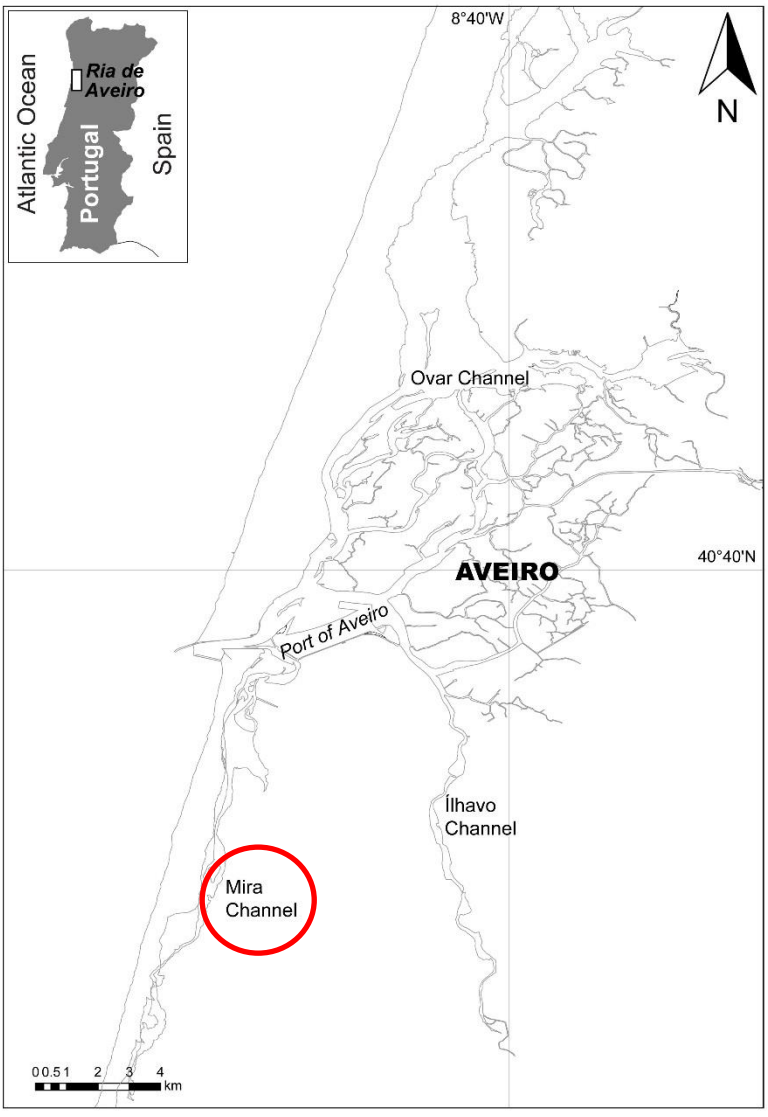

Figure 2. Study area: Ria de Aveiro

The Ria de Aveiro presents significant intertidal zones (mud flats and salt marshes) and it is connected to the Atlantic Ocean only through a narrow channel with 1.3 km of length, 350 m width and 20 m of depth (Dias et al., 2000). The water exchange is performed through the navigation channel, by the tidal inputs (Dias et al., 1999) and there are many rivers and streams that flow into Ria de Aveiro, being Rio Vouga, Antuã and Fontão (on North) and Rio Boco (on South) the principal fluxes (Rebelo and Pombo, 2001).

It is notorious the seasonal and spatial salinity variation in the Ria de Aveiro (Dias et al., 2011). The adjacent rivers, periods of rain, hot and dry seasons and sea water inputs are the agents responsible for the wide range of salinities (0-36) in this ecosystem. However, the water circulation is dominated by the sea water penetrating the Ria de Aveiro (70 x 10^6 m^3 in spring tides) comparatively with the input of freshwater (1.8 x 10^6 m^3 per tidal circle) (Moreira et al., 1993). In terms of seasonal variation, during the winter and at the beginning of Spring the lowest salinities are found, while the highest values of salinity are registered during late Spring and Summer (Dias et al., 2011). As a consequence of the spatial gradient of salinity (from about 0 at the freshwater discharges from the tributaries, and about 36 at the connection with sea), this lagoon system represents a habitat for many different species. Here, like in all marine habitats, the benthic community distribution (including the species used in this research) is strongly influenced by the hydrodynamics and salinity gradient (Rodrigues et al., 2011), which is one of the most important factor for spatial distribution of the species.

2.2. Sampling procedure

In the present study, clams were collected from a subtidal area. Although the three species live in sympatry in this lagoon, they may not co-existe in the same site. Considering this, the sampling area was selected taking into account the co-existence of the 3 species to ensure that they were under the same conditions. A total of approximately 200 organisms were collected in the sampling site and at same time (October of 2012). In order to minimize the effect of body size on biochemical and physiological responses to salinity changes, organisms of similar size were collected. The harvest was carried out by professional divers (Figure 3). The species were confirmed and brushed carefully on board to remove fine sediments and transported to the laboratory in ice-cold plastic containers.

A sample of sediment from the sampling site was collected using a corer with 20 cm diameter. The sediment was transported in containers with ice (0 °C) and in the laboratory it was preserved at -20 °C until further analysis. These sediments were used for grain size analysis and organic matter content determination (total volatile solids). At the sampling site, redox potential (Eh), pH, salinity and temperature were measured at sediment surface with specific probes.

Figure 3. Harvesting the organisms.

2.3. Laboratory procedures

After clams collection, 63 organisms of each species were weighted and measured (width and length) in laboratory (Figure 4).

To reduce the content in potential pathogenic microorganisms, organic and inorganic contaminants, and to provide an adaptation period to the laboratory conditions, clams were acclimated for 48 h, under continuous aeration (Freitas et al., 2012b), by placing organisms in plastic tanks with artificial seawater (salinity 28)). According to previous studies (Freitas et al., 2012b), the salinity of 28 was selected as representing control conditions, resembling the natural conditions of clams in their natural habitat.

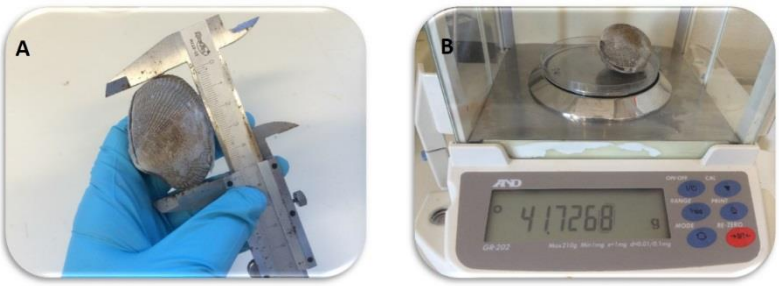

Figure 4. Measurement (A) and Weight (B) of organisms.

After acclimation, the organisms were exposed during 144 hours to salinity assays (Elston et al., 2003), consisting of the exposure of 9 organisms/salinity level (3 replicates per level, 3 individuals/replicate). The salinities used were: 0, 7, 14, 21, 28, 35 and 42 (Figure 5). It is important to note that salinity is considered to be dimensionless, being defined by UNESCO Practical Salinity Scale of 1978 (PSS78) as a conductivity ratio (NASA, 2010).

A plastic container with 1 L of water was used for each replicate. Water was prepared with commercial salt (Tropical Marin – sea salt, the pharmaceutical grade sea salt especially for modern reef aquaria). A temperature of 18 ± 1 °C was maintained during acclimation and experimental periods, each container was maintained under continuous aeration and the photoperiod was fixed to 12 h light and 12 h dark. During the experiment, the water of each container was renewed every other day and dead organisms were removed from the containers whenever the water was changed. Organisms were considered dead when their shells gaped and failed to shut again after external stimulus. At the end of the experiment, surviving organisms were frozen at -80 °C for further analysis.

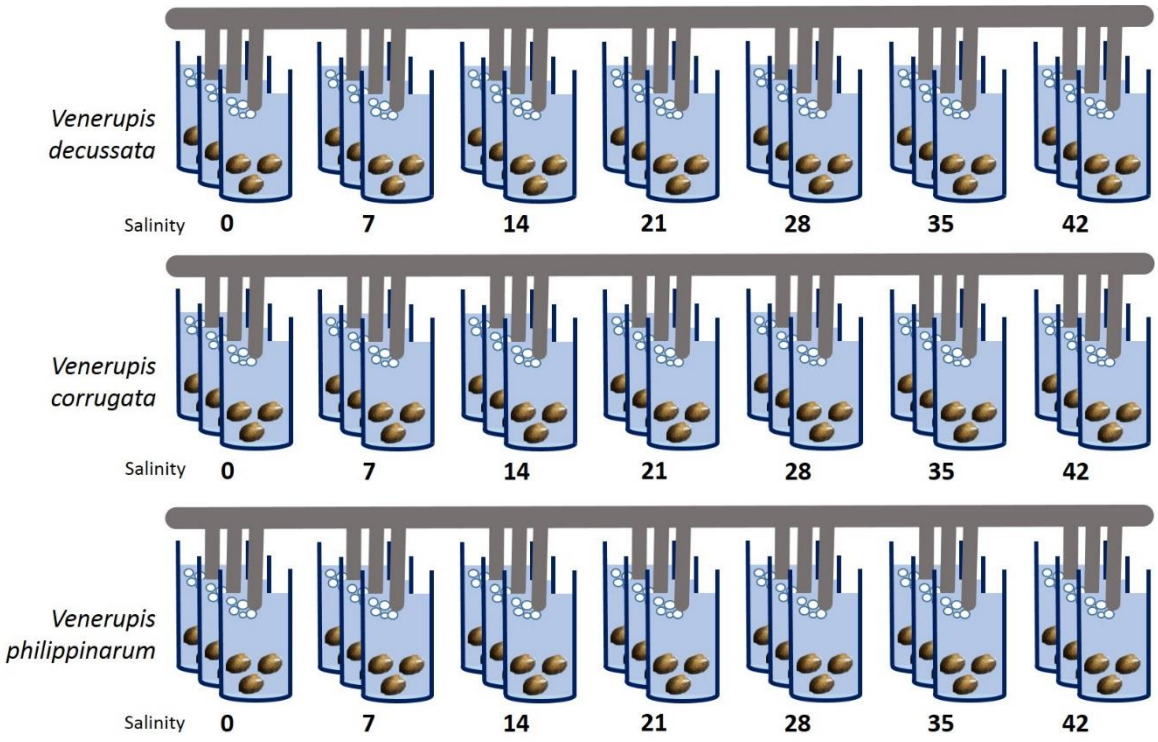

Figure 5. Experimental design for clams exposure to different salinities (0, 7, 14, 21, 28, 35 and 42).

2.3.1. Sediments grain size and Organic matter content

To determine sediment grain size of the sampling site, the procedure described by Quintino et al. (1989) was followed. The sediment was weighed (approximately 120 g), washed with destilated water and the chemical destruction of organic matter was performed with successive increasing concentrations of hydrogen peroxide (H_2O_2): 30, 60 and 120 volumes (Figure 6A). After H_2O_2 addition, the samples were dried in an oven at 60 °C until obtaining a constant weight (from 24 to 48 h) and the total weight was determined (*P1*). The chemical dispersion of sediments was carried out for 24 h with decahydrate pyrophosphate tetra-sodium (30 g/L) - agent which allows disaggregation of particles. A wet sieving was performed, by wet sieving through a 63 µm mesh and the material retained at this mesh was dried again in an oven at 60 °C until obtain a constant weight (*P2*). The weight of fraction lower than 63 µm was determined by the difference between *P1* and *P2*. Sediments with diameter higher than 63 µm (*P2*) were mechanically dry sieved using sieves with mesh sizes of 63 pm (4 ϕ) and 4 mm (-2 ϕ), with an interval of 1 ϕ (ϕ = $-\log_2$ particle size expressed in mm) (Figure 6B). The fractions retained on each sieve were weighed and the percentage was determined in relation to the total dry weight. The median (*P50*) was measured from the percentages obtained, value where 50 % of the cumulative percentage of the sample is located. The sediments were classified according to the Wentworth scale, based on the median value and taking into account the level of fines (Table 1).

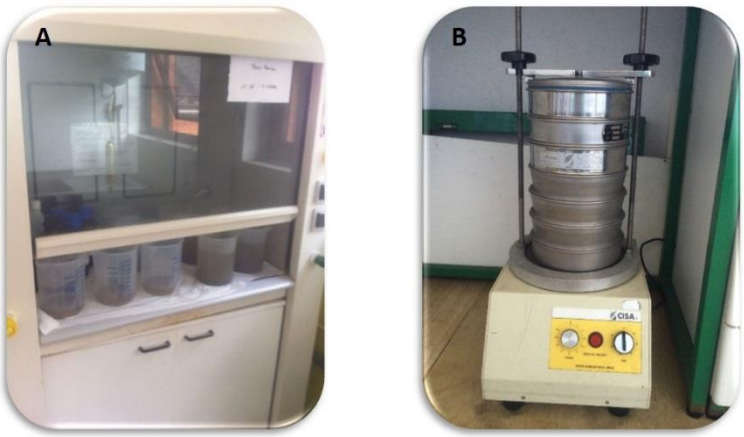

Figure 6. A- Destruction of organic matter with H_2O_2; B- Dry separation of sediments on a battery of sieves

Table 1. Classification of sediments, adapted from Wentworth (Doeglas, 1968).

<table>
<tr><th rowspan="2">Median (φ)</th><th colspan="2" rowspan="2">Sediment Classification</th><th colspan="3">Fines content (%)</th></tr>
<tr><th>< 5</th><th>5 - 25</th><th>25 - 50</th></tr>
<tr><td>(-1) - 0</td><td rowspan="5">Sand</td><td>Very Coarse</td><td rowspan="5">Clean</td><td rowspan="5">Silty</td><td rowspan="5">Very silty</td></tr>
<tr><td>0 - 1</td><td>Coarse</td></tr>
<tr><td>1 - 2</td><td>Medium</td></tr>
<tr><td>2 - 3</td><td>Fine</td></tr>
<tr><td>3 - 4</td><td>Very Fine</td></tr>
<tr><td>> 4</td><td colspan="2">Mud</td><td colspan="3">Above 50 %</td></tr>
</table>

Total organic matter (TOM) content was determined following the procedure described by Byers et al. (1978). Sediment samples were firstly dried in an oven at 60 °C after which 1 g of each sample was weighted. Loss by ignition was performed during 5 h at 450 °C – for a minimal risk of volatizing inorganic carbon (Kristensen and Andersen, 1987) - in a muffle furnace. After 30 min in a dessicator, the ashes were weighted once again. TOM was expressed as a percentage of total sediment dry weight.

2.3.2. Quantification of elements

Total concentrations of 4 elements (Na; K; Ca; and Mg) were measured in clams' soft tissues. For this procedure, organisms (excluding shells) were mechanically homogenised, under liquid nitrogen and then transferred to Teflon bombs and the biological samples digested overnight (for *ca.* 18h) at 115 °C with 2 mL of 65 % HNO_3 (Suprapur, Merk). The cooled digest was made up to 5 mL using 1 M HNO_3, and the concentrations of elements were determined by ICP-MS. All element quantifications were carried out by a certified laboratory at the University of Aveiro. Regarding quality controls, the calibration of the apparatus was made with IV standards, and they were verified with standard reference materials (National Institute of Standards and Technology, NIST SRM 1643e). The accuracy of these measurements ranged between 90 and 110 % (information given by the laboratory). All samples below this accuracy level were rejected and the analysis repeated. Determinations were performed using 3 replicates.

2.3.3. Metabolomic, physiological and biochemical analysis

Bivalves have been proposed as good sentinel organisms in pollution monitoring studies through the analysis of biochemical biomarkers. Thus, physiologycal analysis (protein, glycogen and glucose content), biochemical measurements (lipid peroxidation, LPO; superoxide dismutase, SOD; catalase, CAT; glutathione S-transferase, GST; total glutathione, GSHt and reduced glutathione, GSH) and quantification of elements (sodium, Na; potassium, K; calcium, Ca; and magnesium, Mg) were preformed to analyze the responses of these organisms under salinity stressful conditions.

To understand the variations in metabolomic patterns of these species, two different high sensitive technologies were used: two-dimensional gas phase chromatography coupled to spectrophotometer detector flight time (GC x GC – ToFMS), and Nuclear Magnetic Resonance (NMR). Volatile organic compounds (VOCs) were analyzed by GC x GC – ToFMS, which represents a very high resolving power for metabolomic studies employing two orthogonal mechanisms to detect and separate the compounds in samples (Rocha et al., 2013). NMR is a high resolution technique which is capable to discriminate the intensity of metabolites like aliphatic, polar and aromatic compounds and provide information about the molecular structure of organic molecules and biomolecules in solution.

In the present study the three approaches were used in order to evaluate the effects of salinity changes in three species of clams living in simpatry in Ria de Aveiro – *V. decussata*, *V. philippinarum* and *V. corrugata*.

2.3.3.1. Physiological and biochemical analysis

The responses of organisms to biomarkers are essential to assess their physiological status at molecular, cellular and individual levels (Hamer et al., 2008). Physiological and biochemical analysis have been used to study mostly the effects of anthropogenic pollution and stresses in bivalves. Kamel et al. (2012) studied the biochemical responses and antioxidant defence (glutathione S-transferase, GST) in *V. decussata* when exposed to treated municipal effluents. Figueira et al. (2012) investigated the impact of cadmium contamination in two clam species, *V. philippinarum* and *V. decussata*. The effect of metals was also studied in *V. decussata* by Roméo and Gnassia-Barelli (1997), Hamza-Chaffai et al. (1999), Moraga et al. (2002), Smaoui-Damak et al.

(2009) and Figueira et al. (2012). Recent studies by Antunes et al. (2013) used *V. decussata* and *V. philippinarum* to assess the impacts of pharmaceutical drugs on clams.

Reactive oxygen species (ROS) are formed by oxygen through several transfers of electrons and bio-molecules in cell, such as nucleic acids, lipids, proteins and polysaccharides, represent different substrates of ROS (Manduzio et al., 2005). Formation of ROS is inevitable in aerobic cells (Haeys et al., 2004) and is necessary mechanisms to eliminate these compounds to avoid the cell damage. Oxidative stress occurs when exist an imbalance between the formation of ROS and the cellular antioxidant defence system.

The formation of ROS, responsible for oxidative stress, leads to some cellular and metabolic alterations, such as protein degradation and lipid peroxidation of membranes (Viarengo et al., 1990). The response to oxidative stress include the increase of activity of antioxidant enzymes, oxidative modification of lipids, saccharides, proteins and nucleic acids or substitution or reparation of damaged molecules in cell (Ďuračková, 2008).

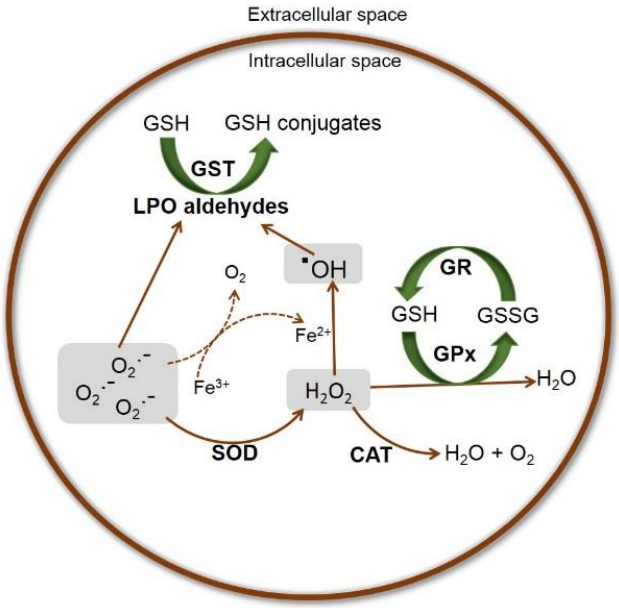

Figure 7. Oxidative stress. Legend: Superoxide ($O_2\bullet^-$); hydrogen peroxide (H_2O_2); superoxide dismutase (SOD); catalase (CAT); water (H_2O); oxygen (O_2); Glutathione peroxidase (GPx); glutathione (GSH); hydroxyl radical (•HO); oxidized glutathione (GSSG); glutathione reductase (GR); Glutathione-S-transferase (GST); lipid peroxidation (LPO). Highlighted in grey, are some of the most important reactive oxygen species (ROS) in cells.

To prevent cell from protein oxidation, lipid peroxidation and DNA damage, provoked by oxidative stress (Figure 7), antioxidant enzymes, like CAT and SOD work as primary defence against oxidative damage (Livingstone, 2001), functioning as a strategy to reduce the ROS.

In biological systems, complex reactions involving free radicals, especially oxygen free radicals (unstable atoms or molecules, with one or more lone electrons), normally results in different kinds of radicals through several chain mechanisms (Di Giulio et al., 1989; Manduzio et al., 2005). One of the reactive oxygen species, superoxide radical anion ($O_2\bullet^-$), results from one-electron reduction [Equation 1]. Iron is involved in the production of •HO the Haber-Weiss reaction (Storey, 1996; Di Giulio et al., 1989; Manduzio et al., 2005). Together with superoxide radical anion, Fe^{3+} react, yielding Fe^{2+} and O_2 [Equation 2], which will be used to form •HO. Hydrogen peroxide is converted to hydroxyl radicals by Fe^{2+} [Equation 3].

SOD decompose $O_2\bullet^-$ to H_2O_2 [Equation 4], which is converted to H_2O and molecular O_2 by CAT [Equation 5] (Storey, 1996; Di Giulio et al., 1989; Geret et al., 2003; Manduzio et al., 2005; Almeida et al., 2007). H_2O_2 is also reduced to water by GPx in association with GSH oxidation [Equation 6] (Di Giulio et al., 1989; Geret et al, 2003; Almeida et al., 2007). GSSG is reduced to GSH by the enzyme GR, helping to maintain the redox status (Di Giulio et al., 1989). Conjugation of foreign compounds with GSH normally leads to formation of less reactive products that are excreted. Here, GST have an antioxidant function and conjugate GSH among the end-products of lipid peroxidation (LPO aldehydes) transforming them into glutathione conjugates, nonpolar compounds. (Storey, 1996; Griendling and FitzGerald, 2003; Hayes et al., 2004; Almeida et al., 2007; Wakamatsu et al., 2008). Despite the antioxidant defences, ROS can indirectly affected the cell, due to reactive secondary metabolites resulting from the oxidation of these macromolecules (Marnett et al., 2003). GST and GPx are examples of enzyme defences against the degradation products of oxidative stress (Hayes et al., 2004). Chain reactions that amplify the damages on lipids, result from the peroxidation of polyunsaturated fatty acids in membranes and become a problem for the cell (Hayes et al., 2004).

$$O_2 + e^- \rightarrow O_2^{\bullet -} \qquad \text{[Equation 1]}$$

$$O_2^{\bullet -} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \qquad \text{[Equation 2]}$$

$$Fe^{2+} + H_2O_2 \rightarrow \bullet OH + OH^- + Fe^{3+} \qquad \text{[Equation 3]}$$

$$2O_2^{\bullet-} \xrightarrow[2H^+]{} H_2O_2 + O_2 \qquad \text{[Equation 4]}$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad \text{[Equation 5]}$$

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \qquad \text{[Equation 6]}$$

Evidences of oxidative stress in organisms under analysis in the present study can be studied observing alterations in antioxidant enzyme activities; antioxidant levels and oxidative damage in cell.

In the present study, biochemical and physiological analysis were individually performed in three organisms per condition (one of each replicate). For biochemical measurements, frozen organisms (soft tissues) were mechanically pulverized under liquid nitrogen and frozen (-80 °C) until further analysis. For protein, glycogen and glucose quantification, extractions were performed in proportion of 1:2 (w/v), with sodium phosphate buffer 50 mM, pH 7.0 (disodium hydrogen phosphate dihydrate 50 mM; sodium dihydrogen phosphate monohydrate 50 mM, Ethylenediamine tetraacetic acid (EDTA) 1mM, Triron X-100 1% (w/v)). For superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and total glutathione (GSHt), homogenates were resuspended in potassium phosphate buffer 50 mM (1:2, w/v), pH 7.0 (dipotassium phosphate 50 mM; potassium dihydrogen phosphate 50 mM; EDTA 1 mM; Triton X-100 1% (v/v); polyvinylpyrrolidone (PVP) 1% (v/v); Dithiothreitol (DTT) 1 mM). For lipid peroxidation (LPO) and reduced glutathione (GSH), the soft tissue was diluted in trichloroacetic acid (TCA) 20% v/v (1:2). All samples were homogenised in an ultrasonic probe (2 cicles of 15 s each) and centrifuged for 10 min at 10 000 *g* and 4 °C. Supernatants were divided into aliquots and either stored at -80 °C or used immediately. Whenever necessary, samples were diluted with same potassium phosphate buffer or TCA as extraction was performed.

Total protein content

Total protein contents were determined by the spectrophotometric Biuret method (Robinson and Hogden, 1940), using bovine serum albumin (BSA) as standards (0-40 mg/mL). This method is used to find peptide bonds or to find out the protein content, since each amino acid in the peptide has the same frequency of peptide bonds.

For each sample 50 µL of extract and 600 µL of Biuret reagent was used. The mixture was shacked, making up-and-down. The colorimetric reaction was carried out at 30 °C for 10 min and

absorbance was measured at 540 nm. The final results were expressed in mg per g of fresh weight.

Polypeptide separation by SDS-PAGE

Proteins were separated by SDS-PAGE, carried out in 4-20 % of polyacrylamide (Mini-PROTEAN TGX – Bio-Rad) following 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 Colour Protein Marker II – nzy tech genes & enzymes) and calculated using Quantity One program software (Bio-Rad) (Figueira et al., 2005).

Total carbohydrate content (Glycogen)

Glycogen was quantified according to the phenol-sulphuric acid method, as described by Yoshikawa (1959). This method detects almost all carbohydrates (mono-, di-, oligo- and polisaccharides), but absorbance of each is different. Sulphuric acid breaks the bonds of polysaccharides, oligosaccharides and disaccharides, turning them into monosaccharides; dehydrates pentoses into furfural and hexoses to hydroxymethyl furfural. These compounds react with phenol and produce a yellow-gold colour (Nielsen, 2010).

Glycogen concentrations were determined with comparison against glucose standards (0-5 mg/mL). All the samples were diluted 25 times and 50 µL of *V. philippinarum*, 10 µL of *V. decussata* and *V. corrugata* (adding 40 µL of phosphate buffer (the same used for extraction) to make up 50 µL) were used. To every sample, 100 µL of phenol (5 %) and 600 µL of H_2SO_4 (96 %) were added and then incubated at room temperature for 30 min. Absorbance was measured spectrophotometrically at 492 nm and results were expressed as mg per g of fresh weight.

Glucose content

Glucose was quantified using a RTU-glucose kit (bioMérieux SA). Glucose oxidase catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide (H_2O_2). Through an oxidative coupling reaction catalyzed by peroxidase, H_2O_2 reacts with 4-aminoantipyrine and phenol (included in RTU-glucose kit). The intensification of colour quinoneimine is proportional to the amount of glucose present in the sample.

To every sample 10 µL of extract was used and 600 µL RTU-glucose solution was added. Samples were incubated at room temperature for 20 min and glucose concentrations were

compared with a glucose standard (0-5 mg/mL). Absorbance was measured spectrophotometrically at 505 nm and the results were expressed as mg per g of fresh weight.

Lipid peroxidation

LPO is a well-known mechanism of cellular injury and is used as indicator of oxidative damage in cells and tissues. Malondialdehyde (MDA) maybe is the most abundant aldehyde product, so therefore, the measure of MDA has been used as an indicator of oxidative stress in invertebrates (Wheatley, 2000). Lipid peroxidation implies the reorganization of the double bonds of unsaturated lipids, formation of lipid radicals, the capture of oxygen and possibly, the degradation of lipid membranes. Following the procedure described by Ohkawa et al. (1979), lipid peroxidation (LPO) was measured by the quantification of ThioBarbituric Acid Reactive Substances (TBARS), being addressed as a measure of membrane damage. This method is based on the reaction of MDA, with 2-thiobarbituric acid (TBA) 0.5 %, derived from LPO, forming TBARS, which can be read spectrophotometrically because of its characteristic color. To 100 µL of sample (diluted in TCA 20 %) 400 µL of TBA (0.5 %) and 300 µL of TCA (20 %) was added. The reaction was performed during 25 min at 96 °C. Samples were immediately transferred to ice, to stop the reaction. The absorbance was measured at a wavelength of 535 nm, with an extinction coefficient of 1.56 mM^{-1} cm^{-1} and final results were expressed as nmol of MDA per g of fresh weight.

Catalase activity

Catalase is an enzyme that protect the cell from reactive oxygen species (ROS) avoiding oxidative damages. It promotes the decomposition of hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2). The method used to measure the activity of catalase is based on the reaction of this enzyme with methanol in the presence of hydrogen peroxide (H_2O_2) (Lars et al., 1988).

To 25 µL of extract sample (previously diluted 2 times) and standards of formaldehyde (0-150 µM) was 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. After incubate the samples and standards at room temperature for 20 min in a stirrer, 37.5 µL of potassium hydroxide (KOH) (10 M) was 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 (KIO_4), to oxidize the reaction between formaldehyde and purpald and lead to a coloured compound formation. The standard curve was performed with formaldehyde standards

and the absorbance was measured in a microplate reader at 540 nm after more 5 minutes of incubation. The final results were expressed in units (U) of CAT per g of fresh weight, being one unit defined as the quantity of enzyme responsible for the formation of 1.0 nmol of formaldeyde, per minute.

Superoxide dismutase activity

Superoxide dismutase (SOD) is an enzyme with high importance in antioxidant defence. It catalyses the superoxide ($O_2\bullet^-$) into oxygen and hydrogen peroxide (H_2O_2). Superoxide reduces NBT^{2+} in formazan (a chromogenic product which displays a blue colour). SOD, in turn, intercepts the $O_2\bullet^-$ blocking the formation of formazan. Thus, the less intense blue colour (meaning less amount of formazan), the higher content of SOD.

The activity of this enzyme was determined following the method of Beauchamp and Fridovich (1971) with some modifications and adapted to microplate. This method is based on the reduction of superoxide anion levels by SOD. To 25 µL of each sample (previously diluted 4 times) were added 250 µL of reaction buffer (Tris-HCl 50 mM, pH 8.0; diethylene triamine pentaacetic acid (DTPA) 0.1 mM; hypoxanthine 0.1 mM and nitro blue tetrazolium (NBT) 68.4 µM) and 25 µL of xanthine oxidase (56.1 mU/mL) to start the reaction, converting the xanthine and oxygen into uric acid and H_2O_2 yielding superoxide anions. To 25 µL of standards of SOD (0.25–60 U/mL) was added 25 µL of extraction buffer, 225 µL of reaction buffer and 25 µL of xanthine oxidase. The samples and the standards were incubated for 10 min at room temperature in a stirrer. The standard curve was performed with SOD standards. SOD activity was measured in a microplate reader at 560 nm and the results were expressed as U per g of fresh tissue. One unit of SOD activity represents a reduction of 50 % of NBT.

Glutathione S-transferase activity

Glutathione-S-transferase (GST) is an enzyme that is part of a defence strategy and the efficiency depends on glutathione synthase to provide GSH and also depends on transporters actions to remove glutathione conjugates from the intracellular space (Hayes and McLellan, 1999). GST converts the tripeptide glutathione (GSH) into xenobiotic compounds, conjugating GSH with 1-chloro-2,4-dinitrobenzene (CDNB), an electrophilic substrate, forming one thioether (with an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$), that can be measured by increasing absorbance at 340 nm.

In the present work, the activity of this enzyme was measured following the procedure described by Habig et al. (1974) with some modifications to microplate method (96 flat bottom wells). To 50 µL of extracted sample (previously diluted 4 times) were added 200 µL of a reaction solution containing 1-Chloro-2,4-dinitrobenzene (CDNB) 60 mM (14.2 % of total volume), reduced glutathione (GSH) 10 mM (85.3 % of total volume) and potassium phosphate buffer 0.1 M, pH 6.5 (dipotassium phosphate 0.1 M, potassium dihydrogen phosphate 0.1 M) - 0.47 % of total volume.

Absorbance values were obtained in a microplate reader at 340 nm (ε =9.6 mM^{-1} cm^{-1}), at intervals of 10 s for 5 min. The GST activity was expressed in U per g of fresh weight, where U corresponds to the amount of enzyme that catalyzes the conversion of 1 µmol of substrate per min.

Total glutathione

Glutathione is an important antioxidant preventing cell damage caused by reactive oxygen species such as free radicals and peroxides. It exists in reduced (GSH) and oxidized (GSSG) forms. This enzyme interferes in the synthesis and degradation of proteins, regulation of enzymes and protection of the cell from ROS (Manduzio et al., 2005).

Total glutathione (GSHt) content (the sum of the two forms) was quantified according to the 5,5'-dithiobis-2-nitrobenzoic acid (DTNB)-glutathione reductase (GR) method described by Anderson (1985) and adapted to microplate method. Glutathione standards (0-500 µmol L^{-1}) were prepared to compare against GSHt concentrations. To 23 µL of standards and samples (previously diluted 2 times) it was added 240 µL of potassium phosphate buffer 50 mM, pH 7.0 (dipotassium phosphate 50 mM; potassium dihydrogen phosphate 50 mM), 9.23 µL of NADPH (nicotinamide adenine dinucleotide phosphate) 30 mM, 23 µL of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) 10 mM and 4.62 µL of GR 10 U mL^{-1} (together with NADPH, GR transform the GSSG to GSH) and then incubated for 5 min at room temperature. Absorbance was measured in a microplate reader at 412 nm and the content of GSHt was expressed in µmol per g of fresh weight.

Reduced and oxidized glutathione content

Reduced glutathione content (GSH) was determined adapting the procedure described by Moron et al. (1979). Glutathione standards (0-500 µmol/L) were prepared in TCA 20 % (v/v) to compare against the GSH values. 50 µL of supernatant and standards were neutralized with 20 µL of sodium hydroxide (NaOH) 2M. To this mixture 500 µL of potassium phosphate buffer 50 mM (pH 7.0) and 50 µL of DTNB 10 mM (with 620 µL as final volume) were added. During 5 min, the samples and standards were incubated at room temperature. Finally, the absorbance was measured spectrophotometrically at 412 nm. Values of GSH were expressed as µmol per g of fresh weight.

Oxidized glutathione (GSSG) was obtained calculating the difference between GSHt and GSH. GSSG content was expressed as µmol per g of fresh weight.

2.3.3.2. Nuclear Magnetic Resonance (NMR) Spectroscopy

High resolution Nuclear Magnetic Resonance (NMR) spectroscopy is an important technique for rapid and non-invasive analysis of complex systems providing information on a large number of different compounds, with different concentrations (Graça et al., 2008). This technique is based on the magnetic properties of the atomic nuclei when placed in a strong magnetic field providing important information about the molecular structure of organic molecules and biomolecules. NMR has been the dominant method for analysing organic compounds, because in most situations it is possible to determine the entire structure using a reduce number of analytical tests. It has also been increasingly used in the area of inorganic chemistry allowing valuable information to be obtained about molecular structures. Besides the wide use of NMR spectroscopy in structural chemistry, the technique has also, more recently, been used in metabolomics applied in several contexts (e.g. drug development and assessment, food analysis disease research). Specific reports comprise studies related with tumor metavolic profiling (Rocha et al., 2010) for metabolic profiling and also detailed characterization of food (Duarte et al., 2002; 2006). Although there are already some studies related to the metabolic effects of climate changes on marine organisms (e.g. Liu et al., 2011a; 2011b studied toxicological effects induced by mercury exposure of *V. philippinarum*), metabolic differences have not been studied on marine species when subjected to a stress caused by salinity fluctuations.

Principals of NMR Spectroscopy

The nuclei of some atoms have the ability to rotate around its axis, when subjected to a magnetic field, and this property is named as spin. The hydrogen nucleus, the proton (^{1}H) is one of these nuclei. The nuclear spin is associated to an angular moment, P, and generates a magnetic moment (μ) characterizing each nucleus. Magnetic (μ) and angular (P) moments are related by $\mu=\gamma P$, where ɣ is the gyromagnetic ratio of the nucleus, a characteristic of the nucleus (Günther, 1998). The angular moment of spin depends on the spin quantum number, I, which takes up values different from zero for the nuclei with spin. For proton, I = 1/2 (Graça et al., 2008).

By placing a proton in an external magnetic field, its magnetic moment can be oriented parallel (up) and anti-parallel (down) in relation to the external field. NMR spectroscopy is based on the occurrence of transitions between these states, by absorbing radiation in the frequency range of radio waves (60 to 750MHz). The exact value of energy absorbed is strongly dependent on the chemical environment in which the proton is found and this dependency is translated by a

quantity called chemical shift. The sample (containing the magnetic nuclei) is excited by selective absorption of radiation, then returns to the fundamental state, with the emission of radiant energy in the field of radio frequencies; this gives rise to the absorption peak registered in the NMR spectrum.

The detailed information that can be obtained - on the molecular structure of the sample, or on the global internal dynamics of the molecules - is related to the exact determination of the chemical shifts (in ppm) corresponding to specific frequencies emitted. The ^{1}H NMR spectra (graph of absorbance according to the chemical shift) is based on the different location of the peaks, because its position depends on electronic environment around the proton.

In the present study, for NMR analysis 15 samples were selected (9 samples of *V. philippinarum*, including 1 at salinity 0, 2 at salinity 7, 3 at salinity 28 and 3 at salinity 42; 3 samples of *V. decussata* at salinity 28; 3 samples of *V. corrugata* at salinity 28) in order to study the range of salinities that the three species tolerate.

The final analysis included the study of *V. philippinarum* metabolic alterations when subjected to four different salinities: 0, 7, 28 and 42, to understand the metabolic effect of the salinity; data of the three species when subjected at salinity 28 were analyzed, aiming to study the differences between clam species under the same salinity conditions.

Extraction and preparation for NMR analysis

Metabolite extraction was performed using a water/methanol/chloroform method described by Hines et al. (2007). After grinding the clams' soft tissue (0.5 g per sample) in liquid nitrogen, 2 mL of methanol, 0.425 mL of distillated water and 1 mL of chloroform were added. The mixture was sonicated in an ultrasonic probe (2 cycles of 15 s each) and then centrifuged (2 500 *g*, 4 °C, 10 min). The aqueous layer was removed and transferred into a new tube, and the lower phase was discarded. To the aqueous phase, 1 mL of chloroform and 1 mL of water were added and the mixture was vortexed and centrifuged (2 500 *g*, 4 °C, 10 min), giving rise to two layers. The aqueous phase and the lipidic phase were separated to different tubes, dried in a centrifugal concentrator (UNIVAP 100 H) and stored at -80 °C until NMR analysis. Before spectral acquisition, the dry polar extracts (aqueous phase) were resuspended in 600 µL of sodium phosphate buffer (0.1 M in D_2O, pH 7.4, containing 0.5 mM sodium 3-trimethylsilyl-2,2,3,3-*d*4-propionate (TSP) as chemical shift standard); the dry nonpolar extracts (lipid phase) were resuspended in 650 µL deuterated chloroform ($CDCl_3$), both followed by vortexing and centrifugation (12 000 rpm, 10

min). For analysis, 550 µL of supernatant of polar extracts and 600 µL of supernatant of nonpolar extracts were transferred into 5 mm NMR tubes.

To compare different salinities, *V. philippinarum* was analyzed at salinities 0, 7, 28 and 42. The differences among the three species were evaluated at salinity 28.

^{1}H NMR spectroscopy

All ^{1}H NMR spectra were acquired on a Bruker Avance DRX-500 spectrometer using a BBI probe, perating at a frequency of 500.13 MHz for proton. The one-dimensional (1D) ^{1}H NMR spectra were acquired at 298 K, with a NOESYPR1D pulse sequence (Bruker pulse program library) and referencing chemical shifts internally to the TSP signal at δ 0.00 for aqueous extracts and chloroform signal at δ 7.26 for lipids extracts. Water suppression was achieved by irradiation of the water peak during recycle (RD = 4 s) and mixing time (t_m = 100 ms). A 90° pulse lengh of 12 µs was used and 256 transients were collected into 32 K data points with 14 ppm spectral width. All 1D spectra were processed with a line broadening of 0.3 Hz, manually phased and baseline corrected. 2D homonuclear (total correlation spectroscopy, TOCSY) and heteronuclear (^{1}H-^{13}C) correlation spectra were acquired for selected samples in order to aid spectral assignment. Assignment was based on consultation of the Bruker Biorefcode spectral database and several other non-comercial databases.

2.4. Data analysis

The GSH/GSSG ratio, considered to be an index of cellular redox status (e.g. Ault and Lawrence, 2003), was determined based on the data described above.

Data from biochemical and physiological parameters and the element content were submitted for hypothesis testing using permutation 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 was followed, with the salinity as the main fixed factor. When the main test revealed statistical significant differences ($p \leq 0.05$), pairwise comparisons were performed. The t-statistic in the pair-wise comparisons was evaluated in terms of significance among different salinities. The null hypothesis tested for each parameter were “no significant differences exist among salinities”, when comparing the different salinities for each species and “no significant differences exist among species”, when comparing the three

species in each salinity. Significance levels ($p \leq 0.05$) between salinities or species are presented with letters. The matrix gathering the biochemical and physiological descriptors, for each species, per salinity 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 the species condition, which was then submitted to ordination analysis, performed by Principal Coordinates (PCO). Pearson correlation vectors of physiological and biochemical descriptors (correlation > 0.5) were provided as supplementary variables being superimposed on the top of the PCO graph.

For NMR data analysis, each set of spectra was used to set up the data matrices for the multivariate analysis (MVA). This method has the advantages of taking all the variables into account in one single analysis and, more important, allows the construction of predictive models. Therefore, MVA provides the appropriate tools for metabolomics data analysis.

Thus, for aqueous extracts, all signals in δ 0.5-9.5 region except water spectral region (δ 4.60 – 4.80) were included for analysis; for lipid extracts the region used for analysis was δ 0.5 – 10.0, except chloroform spectral region (δ 7.03 – 7.48).

Probabilistic quotient normalization (PQN) of the spectra using the median spectrum to estimate the most probable quotient was carried out and the spectra were aligned by the recursive segment-wise peak alignment (RSPA) method (Veselkov et al., 2009)to reduce variability in the peak positions using MATLAB R2012a. The region of δ 4.60- 4.80 and δ 7.03 – 7.48 was removed to eliminate the effects of imperfect water suppression and chloroform signal, respectively, prior to normalization and alignment. The resulting datasets were then imported into SIMCA-P 11.5 (Umetrics, Umeå, Sweden) software for multivariate statistical analysis.

Principal Components Analysis (PCA) is used to obtain an overview of the similarities and differences between the samples analysed, and Partial Least Squares - Discriminant Analysis (PLS-DA) is used to explore the differences between classes and exclude confounding factors derived from differences of each individual differences. The aim of scaling is to avoid the dominance of the higher intensity signals over lower ones, emphasizing the differences between the spectra in the next steps of MVA (Veselkov et al., 2011). Unit variance (UV) scaling divides each point of the data matrix by the standard deviation of the respective column (peaks). All imported data were autoscaled (i.e unit variance) and Principal Component Analysis (PCA) and Partial-Least Squares-Discriminant Analysis (PLS-DA) were performed on the datasets. To evaluate the differences between the samples groups, the separation obtained in PLS-DA scores is not enough, being

necessary an appropriate validation model. For that propose, R-statistical software (version 2.15.2) was used along with the Plotrix package (Lemon, 2006) to produce PLS-DA loadings plots color-coded as a function of variable importance in the projection (VIP). The loading plots affords information about the contribution of each peak to the separation in the scores plots. The resulting plots provide the information in a same shape as that of a spectrum, together with a colour code representing the variable importance for the discrimination between the classes.

Scores plots were analysed to see the distribution of each sample under analysis. At this point, all the results were analysed in two-dimensional scores plots, representing the distribution of samples in the model. The trends registered between the different classes were evaluated, validating the model. This validation was performed taking into account the following parameters: R^2X, R^2Y and Q^2. In PLS-DA model, R^2X is the explained variance of X explained and R^2Y is the explained variance of Y. Q^2 value represent the validation of R^2 and can be used to test the validity of the model, whereas higher Q^2 values are usually associated with best discrimination between classes.

Loading plots of spectra give to each point a corresponding colour representing their importance in the separation of the samples. The relevant peaks, those with stronger contribution to the trend between classes, were integrated and normalized to total spectral area, usingAMIX 3.9.5 (BrukerBioSpin, Rheinstetten, Germany).

Integral variations were subjected to the Shapiro (normal distribution for $p>0.05$), t-student and Wilcoxon test (statistical relevance for $p\leq0.05$).

Shapiro test determined if the data followed ($p>0.05$) or not ($p\leq0.05$) a normal distribution. For data which followed a normal distribution, the t-student test was applied and for those which do not followed a normal distribution was applied the Wilcoxon test. The p-value obtained in the statistical tests provides information about the significance of the differences between the classes. In the present study, the null hypothesis is “differences between the averages of the classes are equal to zero”, meaning that the integrals are not significantly different between the classes. When p-value was lower than 0.05, means that the integrals are significantly different between the groups.

3. Results

3.1. Environmental data

The physic-chemical characteristics of the sampling site, including sediment classification, percentage of fine particles, median values (ϕ), total organic matter (TOM), salinity, redox potential (Eh), temperature and pH, are presented in Table 2. The results obtained revealed that sediment from the sampling site was classified as very silty medium sand, with high percentage of fines (25.93 %) and high organic matter content (> 3 %, cf. Table 2).

Table 2. Environmental parameters of the sampling site: temperature, pH, salinity, redox potential (Eh), percentage of total organic matter (TOM), percentage of fine particles, median value in units of phi (Φ).

Environmental data						
Temperature (°C)	**pH**	**Salinity**	**Eh (mV)**	**TOM %**	**Fines %**	**Median ϕ**
19.9 ± 1	8.45 ± 1.91	28 ± 2	-173.05 ± 14.07	3.34 ± 0.21	25.93 ± 2.31	1.88 ± 0.04

3.2. Biometric data

Concerning the size and weight of the clams collected, *V. corrugata* was the lightest and the smallest species, while *V. philippinarum* was the biggest and the heaviest one. The *V. decussata* individuals presented an average weight of 27 ± 3 g, an average length of 49 ± 2 mm and 38 ± 2 mm wide. *V. corrugata* specimens had an average weight of 10 ± 2 g and measured 38 ± 3 mm in length with 25 ± 2 mm of wide. *V. philippinarum* clams presented an average length of 50 ± 2.7 mm, 39 ± 3 mm wide and weight of 37 ± 5 g.

3.3. Mortality

When exposed to different salinities (0, 7, 14, 21, 28, 35 and 42), *V. corrugata* showed significantly ($p \leq 0.05$) higher mortality than the other two clams (*V. philippinarum* and *V. decussata*) at most of the salinities tested. Differences were especially noticeable at the lowest (0) and highest salinities (35 and 42) (Figure 8), where *V. corrugata* presented 100 % of mortality. At salinity 0, the 3 species showed high mortality rates, being *V. decussata* the species that revealed the highest survival capacity, revealing approximately 33 % of mortality against 77.8 % for *V. philippinarum* and 100 % for *V. corrugata* (cf. Figure 8). When exposed to the highest salinities (35 and 42), *V. decussata* and *V. philippinarum* presented 100% of survival while *V. corrugata*

presented 100% of mortality, identifying this species as the most sensitive (cf. Figure 8). Although *V. decussata* presents 33 % of mortality at salinity 0, is the species that can tolerate a greater range of salinities.

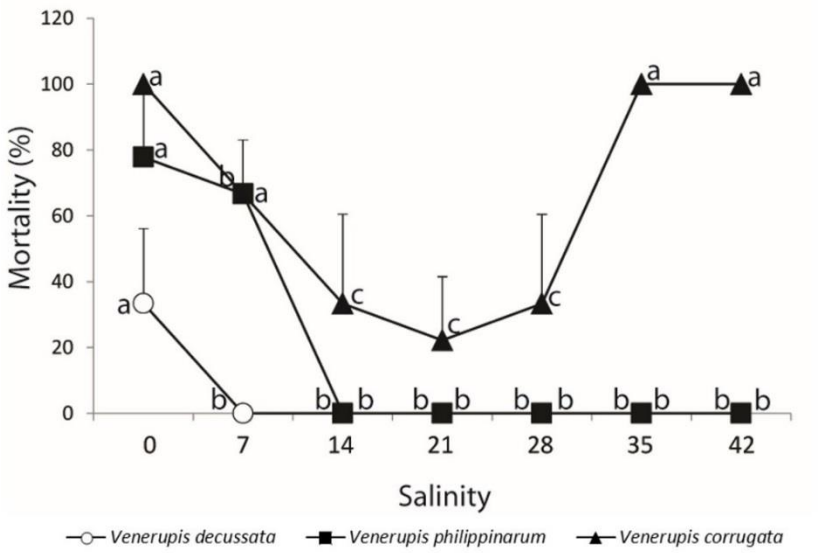

Figure 8. Mortality rate (%) in *Venerupis philippinarum*, *Venerupis decussata* and *Venerupis corrugata* when exposed to increasing salinities (0, 7, 14, 21, 28, 35, 42). Values are the mean of three replicates ± standard deviation. For each species, different letters (a-c) represent significant differences (p≤0.05) among salinities.

3.4. Elemens content

For each species, the concentration of the elements Na (A), K (B), Ca (C) and Mg (D) along the salinity gradient is present in Figure 9.

Along the salinity gradient *V. decussata* maintained fairly constant the amount of Na, except at the highest salinity (42), where this species significantly increased (p≤0.05) the content of Na (Figure 9A). Along the exposure gradient, both *V. philippinarum* and *V. corrugata* gradually increased the Na content with significant differences among salinities, especially between the lowest (0 and 7) and the highest (≥ 28) salinities for *V. philippinarum*, and between 7 and 28 for *V. corrugata* (cf. Figure 9A). *V. decussata* showed significant differences from *V. philippinarum* at salinities 0, 21, 28 and 35. Along the salinity gradient *V. corrugata* presented no significant differences from *V. philippinarum*, while significant differences were found between *V. corrugata* and *V. decussata* (Table 3).

The total K accumulated was significantly different between *V. corrugata* and the other two species (cf. Table 3) with *V. corrugata* presenting the highest and *V. philippinarum* the lowest K content. *V. decussata* and *V. philippinarum* presented a similar behaviour with few significant differences between both species (cf. Table 3). *V. decussata* and *V. philippinarum* maintained the concentration of K along the salinity gradient with no significant differences, while in *V. corrugata* the content of this element increased with the salinity, presenting significant differences along the gradient (cf. Figure 9B).

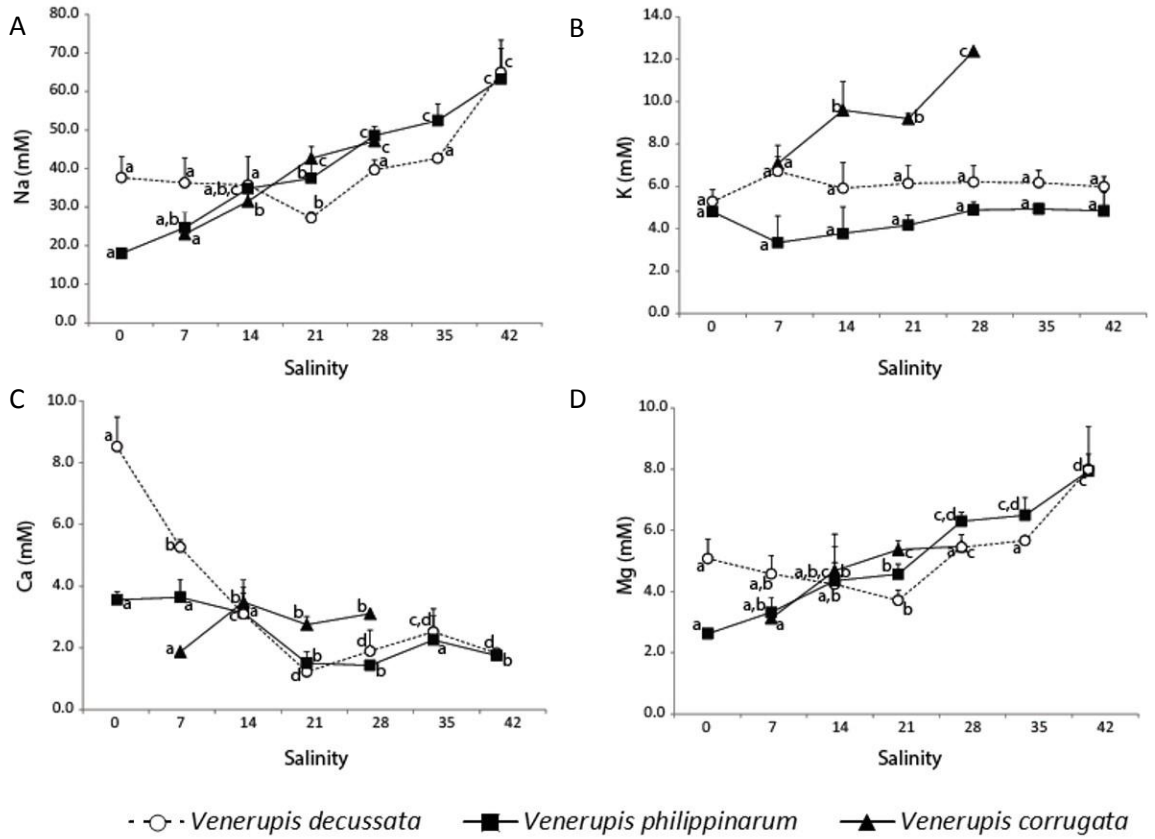

Figure 9. Concentration of Na, K, Ca and Mg (mM) in *Venerupis decussata*, *Venerupis philippinarum* and *Venerupis corrugata* when exposed to increasing salinities (0, 7, 14, 21, 28, 35, 42). Values are the mean of three replicates ± standard deviation. For each species, different letters (a-d) represent significant differences ($p \leq 0.05$) among salinities.

Regarding the Ca content, *V. decussata* presented significant differences between the lowest (0 and 7) and the highest (≥ 21) tested salinities, where the concentration of Ca was lower (Figure 9C). *V. philippinarum* showed a similar behaviour, except for salinities 0 and 7, presenting lower values with no significant differences between these two salinities. Thus, the differences registered along the salinity range, were less pronounced in *V. philippinarum* than in *V. decussata*

(cf. Figure 9C). In *V. corrugata* an opposite trend was noticed. For this species, the lowest value was registered at salinity 7 with significant differences with other salinities (14, 21 and 28). *V. decussata* and *V. philippinarum* only showed significant differences at lowest salinities (0 and 7), while *V. corrugata* showed a significantly different behaviour from the other two species, except at salinity 14 (cf. Table 3).

The observed variation of Mg content was similar to the Na pattern for all species, with *V. decussata* revealing less significant differences on the concentration of this element along the salinity gradient. *V. corrugata* and *V. philippinarum* showed a significant increase of Mg with the increase of salinity (Figure 9D). Significant differences were noticed at salinities 0, 21 and 28 between *V. decussata* and *V. philippianrum* (cf. Table 3). At salinities 7 and 14, *V corrugata* did not show any significant differences from the other two species, while at salinities 21 and 28, *V. corrugata* presented significant differences when compared to *V. decussata* (cf. Table 3).

Table 3. Concentration of Na, K, Ca and Mg (mM) in *Venerupis decussata*, *Venerupis philippinarum* and *Venerupis corrugata* along the salinity range (0, 7, 14, 21, 28, 35 and 42). Values are the mean of three replicates ± standard deviation. For each element and for each salinity, different letters (a-c) represent significant differences ($p \leq 0.05$) among species.

Element	***Salinity***	***V. decussata***	***V. philippinarum***	***V. corrugata***
Na	0	37.44 ± 5.44^{a}	17.76 ± 0.80^{b}	
	7	36.09 ± 4.56^{a}	$24.47 \pm 9.19^{a,b}$	22.82 ± 1.37^{b}
	14	35.49 ± 0.01^{a}	$34.60 \pm 12.74^{a,b}$	31.26 ± 0.70^{b}
	21	26.99 ± 1.55^{a}	37.30 ± 4.75^{b}	42.45 ± 5.33^{b}
	28	39.47 ± 2.49^{a}	48.36 ± 2.38^{b}	46.91 ± 2.16^{b}
	35	42.46 ± 0.31^{a}	52.25 ± 4.36^{b}	
	42	64.74 ± 6.13^{a}	63.07 ± 10.10^{a}	
K	0	5.32 ± 0.56^{a}	4.84 ± 0.00^{a}	
	7	6.75 ± 0.86^{a}	3.39 ± 0.89^{b}	7.11 ± 1.44^{a}

	14	5.95 ± 0.87[a]	3.82 ± 1.24[a]	9.63 ± 0.23[c]
	21	6.18 ± 1.74[a,b]	4.21 ± 0.49[a]	9.23 ± 1.34[c]
	28	6.24 ± 0.76[a]	4.92 ± 0.26[b]	12.41 ± 0.16[c]
	35	6.21 ± 0.57[a]	4.97 ± 0.23[b]	
	42	6.02 ± 0.48[a]	4.88 ± 0.61[a]	
Ca	0	8.50 ±0.96[a]	3.55 ± 0.19[b]	
	7	5.24 ± 0.17[a]	3.63 ± 1.35[b]	1.86 ± 1.66[c]
	14	3.08 ± 1.91[a]	3.12 ± 2.20[a]	3.47 ± 0.00[a]
	21	1.21 ± 0.10[a]	1.49 ± 0.36[a]	2.74 ± 0.20[b]
	28	1.88 ± 0.68[a]	1.42 ± 0.16[a]	3.10 ± 0.26[b]
	35	2.51 ± 1.30[a]	2.24 ± 1.01[a]	
	42	1.82 ± 0.05[a]	1.74 ± 0.11[a]	
Mg	0	5.03 ±0.63[a]	2.59 ± 0.11[b]	
	7	4.53 ± 0.93[a]	3.29 ± 0.11[a]	3.10 ± 0.25[a]
	14	4.20 ± 1.21[a]	4.31 ± 1.51[a]	4.64 ± 0.03[a]
	21	3.67 ± 0.39[a]	4.53 ± 0.33[b]	5.32 ± 0.26[b]
	28	5.40 ± 0.42[a]	6.25 ± 0.28[b]	5.43 ± 0.63[a,b]
	35	5.62 ± 0.13[a]	6.44 ± 0.57[a]	
	42	7.93 ± 0.50[a]	7.88 ± 1.44[a]	

3.5. Biochemical and physiological analysis

3.5.1. Total protein content and peptide alterations

For protein content, *V. philippinarum* and *V. decussata* evidenced a similar pattern along the salinity range (Figure 10A). The protein content was constant at lower salinities (0 to 21) and gradually increased from 28 to 42, with significant ($p \leq 0.05$) differences between the lowest (≤ 21) and the highest (> 21) salinities (cf. Figure 10A). In *V. corrugata* lower protein content was found at salinities 7 and 28, where no significant ($p > 0.05$) differences were found to the other species (Table 4). At salinities 14 and 21, *V. corrugata* showed a significant higher protein content, compared to the other two species (cf. Figure 10A). However, this species showed significant differences along the salinities tolerated (cf. Figure 10A). Table 4 presents the differences, in terms of protein content, among salinities for each species. *V. decussata* and *V. philippinarum* did not present significant differences between the lowest (0-21), but was registered a significant increase to higher salinities (28-35), while *V. corrugata* presented significant differences between the highest and the lowest (7 and 28) tolerated salinities and the remaining ones (14 and 21), with the highest value being observed at salinity 14. Similar protein concentrations were found between salinities 14 and 21 that were significantly higher than at 7 and 28. Significant differences were not found between the protein pattern of *V. decussata* and *V. philippinarum* along the salinity range and *V. corrugata* only presented significant differences from the other two species at salinities 14 and 21 (cf. Table 4).

Regarding polypeptides expression, the levels of the most abundant ones comparing the ones characterizing individuals under salinity 28 were represented as heatmaps (Figure 10B, C and D). In *V. philippinarum* (Figure 10B), for all salinities, ca. 30 % of the proteins did not change their levels, compared to salinity 28. For the remaining 70 %, most of the changes occurred at the lower salinities (≤ 21), with the appearance of a new band and the decrease of 64 % of the polypeptides. At higher salinities (35 and 42), a low number (30%) of polypeptides presented changes (cf. Figure 10B). For *V. decussata* (Figure 10C), was registered ca. 48 % of alterations in polypeptide expression at higher salinities (> 28) and 44 % at lower salinities (< 21). At salinities 35 and 42, 18 % of such changes represented induction and 77 % was related to repression of polypeptides expression. At lower salinities (0, 7 and 14), repression was represented by 27 % of alterations and 17 % of that alterations corresponding to induction of polypeptides. At salinity 21 only 14 % of polypeptides demonstrated alterations, with 86 % of polypeptides not showing any

changes in their expression (cf. Figure 10C). In relation to *V. corrugata* were observed 65 % of alterations in polypeptide expression, being 49 % related to repression and 16 % to induction of that polypeptide expression at salinities analyzed (7, 14 and 21) (Figure 10D).

Table 4. Protein content (mg/g FW) in *Venerupis decussata*, *Venerupis philippinarum* and *Venerupis corrugata* along the salinity range (0, 7, 14, 21, 28, 35 and 42). Values are the mean of three replicates ± standard deviation. For each salinity, different letters (a-b) represent significant differences ($p \leq 0.05$) among species.

Salinity	***V. decussata***	***V. philippinarum***	***V. corrugata***
0	19.38 ± 2.91[a]	20.38 ± 2.25[a]	
7	19.71 ± 1.66[a]	19.05 ± 0.23[a]	20.79 ± 3.10[a]
14	20.01 ± 2.76[a]	19.84 ± 0.06[a]	34.11 ± 1.81[b]
21	23.05 ± 3.58[a]	19.71 ± 1.00[a]	30.91 ± 0.40[b]
28	28.30 ± 2.51[a]	27.41 ± 3.47[a]	22.86 ± 6.86[a]
35	30.05 ± 3.37[a]	30.90 ± 3.80[a]	
42	32.44 ± 2.99[a]	31.97 ± 5.77[a]	

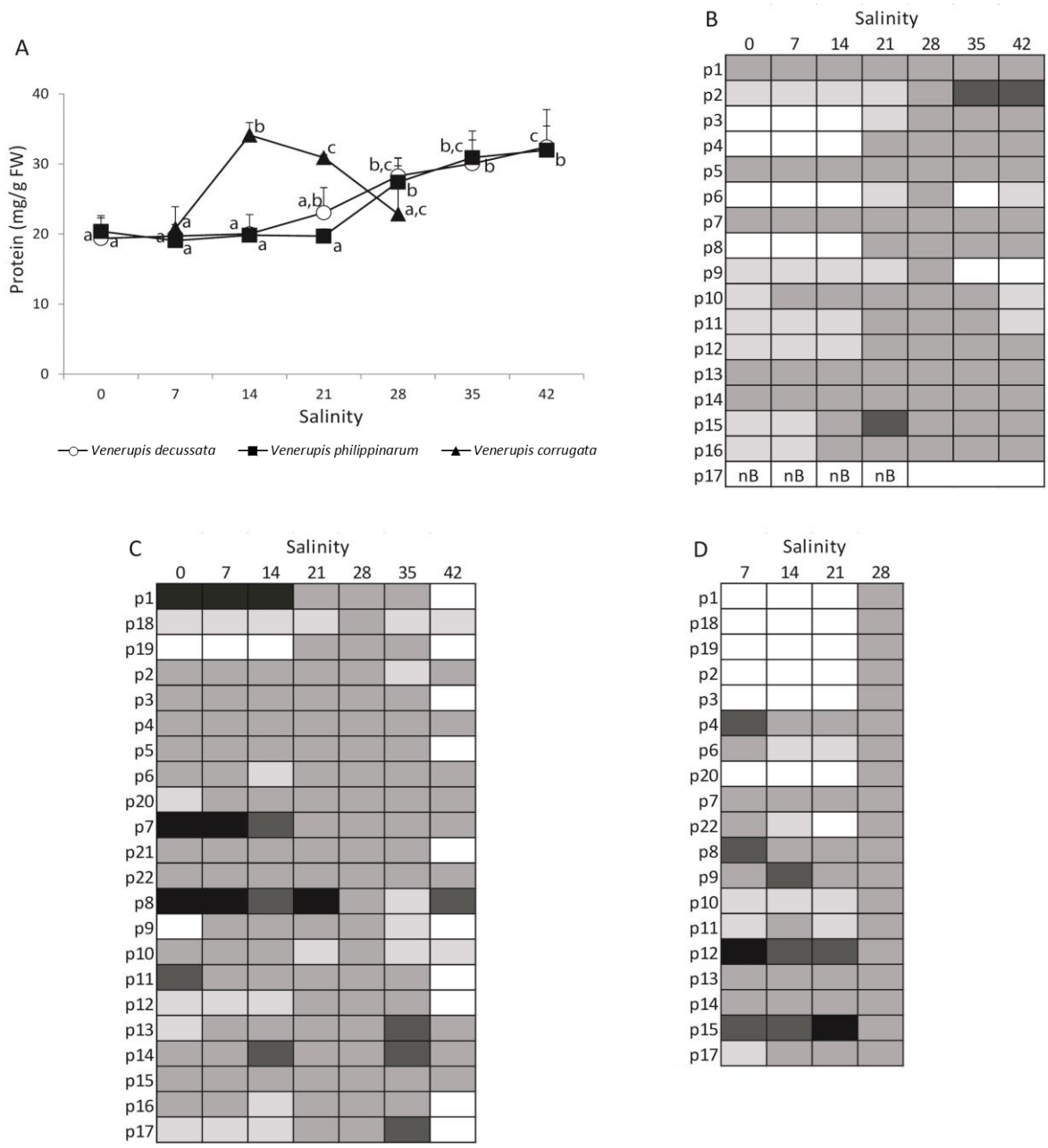

Figure 10. A - Protein content (mg/g FW) in *Venerupis decussata*, *Venerupis corrugata* and *Venerupis philippinarum* when exposed to increasing salinities (0, 7, 14, 21, 28, 35, 42). Values are the mean of three replicates ± standard deviation; for each species different letters (a-c) represent significant differences ($p \leq 0.05$) among salinities. Protein expression B – in *Venerupis philippinarum*, when exposed to increasing salinities (0, 7, 14, 21, 28, 35 and 42); C – in *Venerupis decussata*, when exposed to salinities (0, 7, 14, 21, 28, 35 and 42); D – in *Venerupis corrugata*, when exposed to increasing salinities (7, 14, 21 and 28); the different colours represent repression (white and light grey), no alteration (median grey) or induction (dark grey and black) of peptides in comparison with salinity 28; p1-p22 represent the different polypeptides identified; New bands are also marked (nb); values are the mean of n=3.

3.5.2. Total carbohydrates content (Glycogen)

In terms of glycogen content (Figure 11 and Table 5), a significant difference ($p \leq 0.05$) was observed between the three species, with *V. corrugata* presenting the highest values and *V. philippinarum* the lowest ones. When compared to the other two species, *V. corrugata* showed higher glycogen content that was maintained along the salinity gradient tolerated by this species without significant differences among the salinities (cf. Figur 11). *V. philippinarum* presented a significant increased at salinities 28 and 35 and a significant decrease at salinity 42 (cf. Figure 11), while *V. decussata* showed a significant increase of glycogen content at salinity 42.

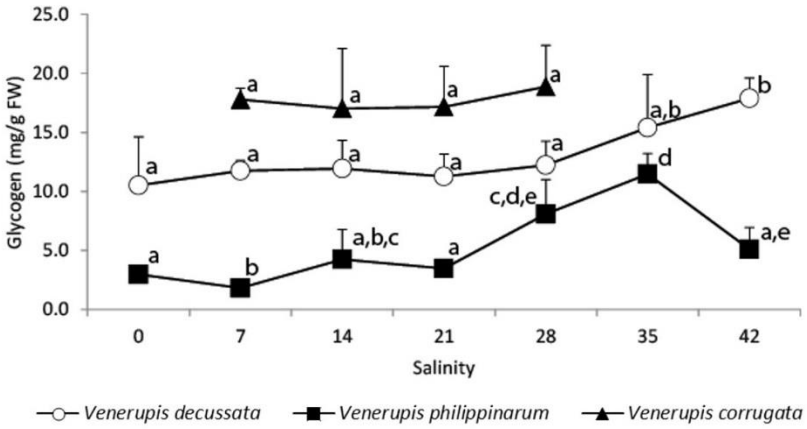

Figure 11. Glycogen content (mg/g FW) in *Venerupis decussata*, *Venerupis corrugata* and *Venerupis philippinarum* when exposed to increasing salinities (0, 7, 14, 21, 28, 35, 42). Values are the mean of three replicates ± standard deviation. For each species, different letters (a-e) represent significant differences ($p \leq 0.05$) among salinities.

Table 5. Glycogen content (mg/g FW) in Venerupis decussata, Venerupis philippinarum and Venerupis corrugata along the salinity range (0, 7, 14, 21, 28, 35 and 42). Values are the mean of three replicates ± standard deviation. For each salinity, different letters (a-c) represent significant differences ($p \leq 0.05$) among species.

Salinity	*V. decussata*	*V. philippinarum*	*V. corrugata*
0	10.51 ± 4.1^{a}	2.97 ± 0.6^{b}	
7	11.76 ± 0.8^{a}	1.81 ± 0.3^{b}	17.79 ± 0.9^{c}
14	11.95 ± 2.3^{a}	4.25 ± 2.53^{b}	17.02 ± 5.1^{a}
21	11.27 ± 1.9^{a}	3.48 ± 0.5^{b}	17.15 ± 3.4^{a}
28	12.23 ± 2.0^{a}	8.10 ± 2.9^{b}	18.80 ± 3.4^{c}
35	15.40 ± 4.51^{a}	11.49 ± 1.74^{a}	
42	17.89 ± 1.72^{a}	5.11 ± 1.82^{b}	

When comparing species, significant differences were observed between *V. decussata* and *V. philippinarum* along the salinity range except for 35 (Table 5). At lowest (7) and highest (28) salinities that *V. corrugata* could tolerate, this species showed significant differences with the other two species, but at salinities 14 and 21, this species showed no significant differences comparing with *V. philippinarum* (cf. Table 5).

3.5.3. Glucose content

Figure 12 presents the glucose content for all species, revealing significant differences between species, with *V. decussata* being the species with the highest values. Along the salinity gradient all clam species increased the glucose content (cf. Figure 12). *V. corrugata* and *V. philippinarum* showed a significant decrease in glucose content at the highest salinity tolerated by each species (28 and 42, respectively). For these two species was also noticed a significant increase at salinity 21 for *V. corrugata* and at salinity 35 for *V. philippinarum* (cf. Figure 12). Along the salinity range, *V. decussata* presented no significant differences, except at salinity 0, being the

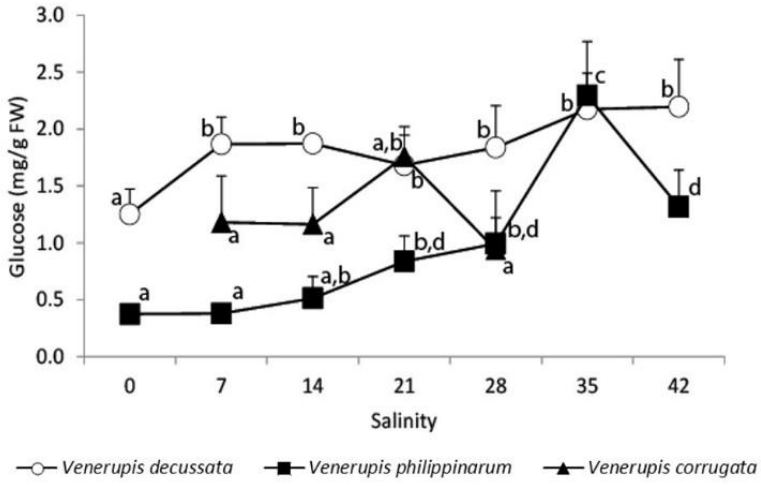

Figure 12. Glucose content (mg/g FW) in *Venerupis decussata*, *Venerupis corrugata* and *Venerupis philippinarum* when exposed to increasing salinities (0, 7, 14, 21, 28, 35, 42). Values are the mean of three replicates ± standard deviation. For each species, different letters (a-d) represent significant differences ($p \leq 0.05$) among salinities.

glucose content fairly constant along the salinity range (cf. Figure 12).

Comparing *V. decussata* and *V. philippinarum*, significant different behaviour were observed, except at salinity 35, where these two species presented the same values (Table 6). On the contrary, at salinity 14 *V. corrugata* presented significant differences comparing with *V. decussata* and *V. philippinarum* (cf. Table 6).

Table 6. Glucose content (mg/g FW) in *Venerupis decussata*, *Venerupis philippinarum* and *Venerupis corrugata* along the salinity range (0, 7, 14, 21, 28, 35 and 42). Values are the mean of three replicates ± standard deviation. For each salinity, different letters (a-c) represent significant differences (p≤0.05) among species.

Salinity	*V. decussata*	*V. philippinarum*	*V. corrugata*
0	1.25 ± 0.22[a]	0.37 ± 0.03[b]	
7	1.87 ± 0.24[a]	0.38 ± 0.07[b]	1.18 ± 0.41[a]
14	1.87 ± 0.07[a]	0.51 ± 0.19[b]	1.16 ± 0.32[c]
21	1.68 ± 0.34[a]	0.84 ± 0.22[b]	1.76 ± 0.19[a]
28	1.84 ± 0.37[a]	0.99 ± 0.23[b]	0.94 ± 0.52[a,b]
35	2.17 ± 0.32[a]	2.30 ± 0.47[a]	
42	2.19 ± 0.42[a]	1.32 ± 0.32[b]	

3.5.1. Lipid peroxidation

Concerning LPO (Figure 13), although the three species showed the same trend, with higher values at the lowest and the highest salinities, *V. corrugata* was the species with higher and *V. philippinarum* was the one with lowest LPO values. The results showed that *V. decussata* and *V. philippinarum* significantly decreased LPO levels with the increase of salinity up 28, with significant differences between salinities 0 and 28 (cf. Figure 13). After this decrease, it was observed a slight increase up to salinity 42, but with no significant differences comparing with other salinities (≤ 28). *V. decussata* and *V. philippinarum* presented a similar pattern although the former presented more pronounced differences between salinities. *V. corrugata* presented an abrupt increase at salinity 28, but the statistical analysis showed no significant differences comparing the salinity 28 with the other salinities tolerated by this species (7, 14 and 21; cf. Figure 13).

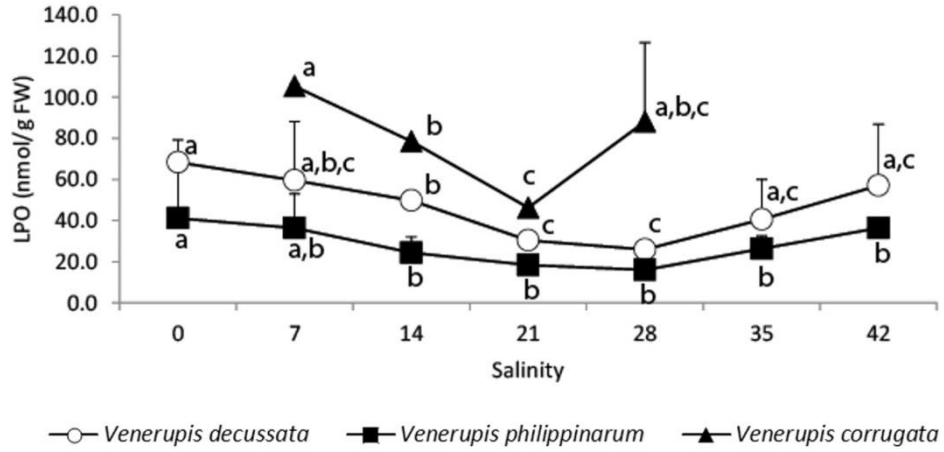

Figure 13. Lipid peroxidation (LPO, nmol/g FW) in *Venerupis decussata*, *Venerupis corrugata* and *Venerupis philippinarum* after exposure to a range of salinities (0, 7, 14, 21, 28, 35 and 42). Values are the mean of three replicates ± standard deviation. For each species, different letters (a-c) represent significant differences (p≤0.05) among salinities.

Between the three clam species, the main differences were noticed at salinities 14, 21 and 28, where were found significant differences between the three species (Table 7). On the extreme of the salinity range (0, 7 and 35, 42), *V. corrugata* and *V. philippinarum* did not show significant differences (cf. Table 7).

Table 7. Lipid peroxidation (LPO, nmol/g FW) in *Venerupis decussata*, *Venerupis philippinarum* and *Venerupis corrugata* along the salinity range (0, 7, 14, 21, 28, 35 and 42). Values are the mean of three replicates ± standard deviation. For each salinity, different letters (a-c) represent significant differences (p≤0.05) among species.

Salinity	*V. decussata*	*V. philippinarum*	*V. corrugata*
0	6.83 ± 1.09^{a}	4.12 ± 2.30^{a}	
7	5.95 ± 2.87^{a,b}	3.64 ± 1.68^{a}	10.53 ± 0.09^{c}
14	4.98 ± 0.28^{a}	2.44 ± 0.77^{b}	7.85 ± 0.00^{c}
21	3.04 ± 0.38^{a}	1.85 ± 0.23^{b}	4.63 ± 0.05^{c}
28	2.60 ± 0.24^{a}	1.60 ± 0.50^{b}	8.82 ± 3.83^{c}
35	4.05 ± 1.96^{a}	2.63 ± 1.96^{a}	
42	5.70 ± 2.97^{a}	3.65 ± 0.33^{a}	

3.5.2. Catalase (CAT) activity

Concerning the activity of CAT (Figure 14), along the salinity exposure gradient, the three species presented the same trend. For all species, higher CAT activity was registered at low salinities and, at higher salinities, the activity of this enzyme was lower. In *V. corrugata*, a pronounced decrease in CAT activity was noticed from the lowest (7 and 14) to the highest salinities (21 and 28) tolerated by this species (cf. Figure 14). *V. philippinarum* and *V. decussata* demonstrated a similar behaviour, but with *V. philippinarum* presenting lower values. *V. decussata* and *V. philippinarum* presented significant differences along all salinity gradient, especially between the lowest (≤ 21) and the highest (> 21) salinities (cf. Figure 14).

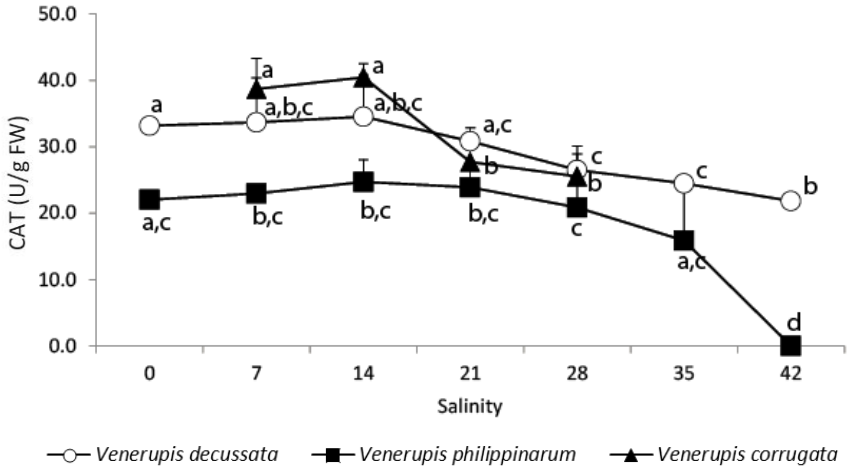

Figure 14. Catalase (CAT) activity (mU/g FW) in *Venerupis decussata*, *Venerupis corrugata* and *Venerupis philippinarum* after exposure to a range of salinities (0, 7, 14, 21, 28, 35 and 42). Values are the mean of three replicates ± standard deviation. For each species, different letters (a-d) represent significant differences (p≤0.05) among salinities.

The main differences between species was observed at salinity 42, where *V. philippinarum* presented a very low CAT activity (Table 8). *V. decussata* and *V. philipinarum* showed significant differences at salinities 0, 7, 21 and 42, while *V. corrugata* was significant different from *V. decussata* along the salinity range tolerated by the two species. At salinity 28, the three species did not show any significant differences (cf. Table 8).

Table 8. Catalase (CAT) activity (mU/g FW) in *Venerupis decussata*, *Venerupis philippinarum* and *Venerupis corrugata* along the salinity range (0, 7, 14, 21, 28, 35 and 42). Values are the mean of three replicates ± standard deviation. For each salinity, different letters (a-b) represent significant differences (p≤0.05) among species.

Salinity	*V. decussata*	*V. philippinarum*	*V. corrugata*
0	33.17 ± 0.90[a]	22.00 ± 1.05[b]	
7	33.65 ± 9.65[a]	22.97 ± 0.79[b]	38.68 ± 4.52[a]
14	34.54 ± 7.91[a]	24.71 ± 3.34[a,b]	40.45 ± 1.70[a]
21	30.82 ± 2.03[a]	23.89 ± 3.07[b]	27.72 ± 0.34[a,b]
28	26.51 ± 2.41[a]	20.85 ± 9.22[a]	25.54 ± 3.80[a]
35	24.48 ± 0.97[a]	15.88 ± 8.97[a]	
42	21.82 ± 0.11[a]	0.00 ± 0.11[b]	

3.5.3. Superoxide dismutase (SOD) activity

In the case of activity of SOD enzyme (Figure 15), the three species evidenced the highest activity at salinity 14. *V. decussata* showed a very pronounced increase from lower salinities (0 and 7) to salinity 14 and also an abrupt decrease to the highest salinities. Figure 15 shows that *V. philippinarum* and *V. corrugata* followed the same trend of SOD activity than *V. decussata*, but less pronounced. For *V. corrugata* it was observed the lowest value at salinity 7 and significantly higher values at salinities 14, 21 and 28. For *V. philippinarum* it was noticed a significant increase from salinity 0 to salinity 14 and a significant decrease up to salinity 42 (cf. Figure 15).

Between *V. decussata* and *V. philippinarum* significant differences along the salinity range were noticed, except for salinity 42 (Table 9). Significant differences were found between *V. corrugata* and *V. decussata* at all the salinities tolerated by *V. corrugata*. On the ther hand, *V. corrugata* only showed significant differences from *V. philippinarum* at salinities 7 and 28 (cf. Table 9).

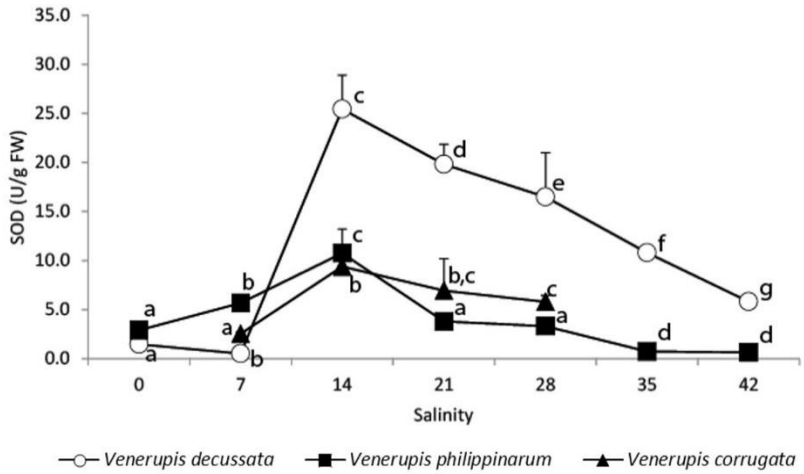

Figure 15. Superoxide Dismutase (SOD) activity for *Venerupis decussata*, *Venerupis corrugata* and *Venerupis philippinarum* after an exposure to a salinity range (0, 7, 14, 21, 28, 35 and 42). Values are the mean of three replicates ± standard deviation. For each species, different letters (a-g) represent significant differences (p≤0.05) among salinities.

Table 9. Superoxide dismutase (SOD) activity (U/g FW) in *Venerupis decussata*, *Venerupis philippinarum* and *Venerupis corrugata* along the salinity range (0, 7, 14, 21, 28, 35 and 42). Values are the mean of three replicates ± standard deviation. For each salinity, different letters (a-c) represent significant differences (p≤0.05) among species.

Salinity	*V. decussata*	*V. philippinarum*	*V. corrugata*
0	1.47 ± 0.33^{a}	2.90 ± 0.49^{b}	
7	0.51 ± 0.16^{a}	5.68 ± 0.64^{b}	2.53 ± 0.15^{c}
14	25.42 ± 3.45^{a}	10.75 ± 2.43^{b}	9.39 ± 1.28^{b}
21	19.82 ± 2.00^{a}	3.79 ± 0.85^{b}	6.94 ± 3.26^{b}
28	16.47 ± 4.54^{a}	3.32 ± 0.74^{b}	5.79 ± 0.67^{c}
35	10.79 ± 0.69^{a}	0.74 ± 0.11^{b}	
42	5.83 ± 0.23^{a}	0.64 ± 0.28^{a}	

3.5.1. Glutathione S-transferase (GSTs) activity

Regarding the activity of GSTs (Figure 16), the three clam species evidenced significant differences along the salinity range, but *V. philippinarum* showed a more stable trend. Between salinities 14, 21 and 28 and also between salinities 35 and 42, *V. philippinarum* showed no significant differences. *V. decussata* and *V. corrugata* showed more pronounced differences along the salinities tested. The first, presented a significant increase from salinity 0 to salinity 21 and a significant decrease up to salinity 42 (cf. Figure 16). For *V. corrugata* the lowest GST value was

found at salinity 21 and seems that this species followed the opposite trend of *V. decussata*, showing no significant differences between the lowest salinities tolerated by *V. corrugata* (7 and 14), where the values were significantly higher than at salinity 28 (cf. Figure 16).

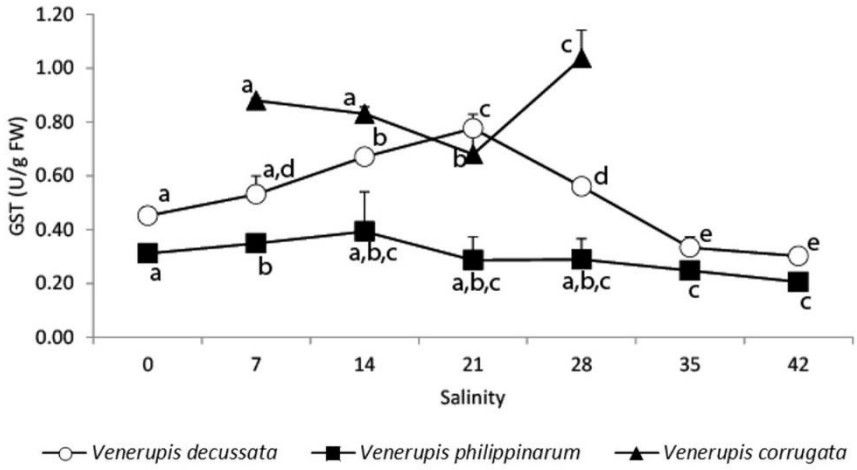

Figure 16. Glutathione S-transferase (GST) activity for *Venerupis decussat*a, *Venerupis corrugata* and *Venerupis philippinarum* when exposed to salinities (0, 7, 14, 21, 28, 35 and 42). Values are the mean of three replicates ± standard deviation. For each species, different letters (a-e) represent significant differences (p≤0.05) among salinities.

The data on Table 10, shows significant differences among the three species along the salinity range. Comparing *V. decussata* and *V. philippinarum*, significant differences were observed at all the tested salinities. *V. corrugata* also showed significant differences comparing with the other two species, except at salinity 21, where *V. corrugata* did not presented significant differences comparing with *V. decussata* (cf. Table 10).

Table 10. Glutathione S-transferase (GST) activity (U/g FW) in *Venerupis decussata*, *Venerupis philippinarum* and *Venerupis corrugata* along the salinity range (0, 7, 14, 21, 28, 35 and 42). Values are the mean of three replicates ± standard deviation. For each salinity, different letters (a-c) represent significant differences (p≤0.05) among species.

Salinity	***V. decussata***	***V. philippinarum***	***V. corrugata***
0	0.45 ± 0.03^{a}	0.31 ± 0.01^{b}	
7	0.53 ± 0.07^{a}	0.35 ± 0.01^{b}	0.88 ± 0.01^{c}
14	0.67 ± 0.02^{a}	0.39 ± 0.15^{b}	0.83 ± 0.02^{c}
21	0.78 ± 0.01^{a}	0.29 ± 0.09^{b}	0.68 ± 0.15^{a}
28	0.56 ± 0.03^{a}	0.29 ± 0.08^{b}	1.04 ± 0.10^{c}
35	0.33 ± 0.04^{a}	0.25 ± 0.00^{b}	
42	0.30 ± 0.01^{a}	0.01 ± 0.03^{b}	

3.5.2. Total glutathione (GSHt)

Concerning GSHt content (Figure 17), it was observed that *V. corrugata* was the species with lower levels without significant differences along salinities. However, a smooth decrease from the lowest (0 and 7) to the highest (21 and 28) salinities was noticed for this species. *V. decussata* maintained the GSHt content up to salinity 14, showing no significant differences between these salinities followed by a significant decrease up to salinity 35 (cf. Figure 17). *V. philippinarum* presented an slight increase up to salinity 21, followed by a decrease to salinity 35 and a significant increase to salinity 42. Both *V. decussata* and *V. philippinarum* showed a significant increase of GSHt content at salinity 42 (cf. Figure 17).

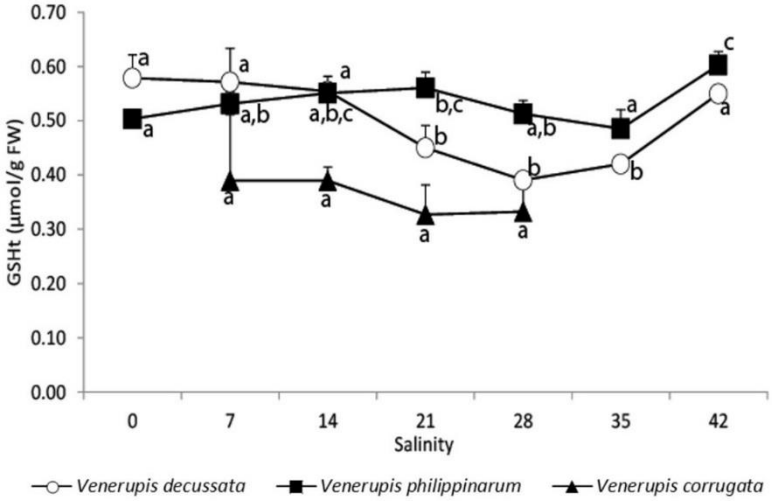

Figure 17. Total glutathione (GSHt) content (µmol/g FW) in *Venerupis decussata*, *Venerupis corrugata* and *Venerupis philippinarum*. Values are the mean of three replicates ± standard deviation. For each species, different letters (a-c) represent significant differences (p≤0.05) among salinities.

Among the three clam species greater significant differences were noticed at salinity 21 (Table 11). *V. decussata* and *V. philippinarum* showed significant differences at salinities ≥ 21, while *V. corrugata* presented significant differences at salinities 14, 21 and 28, when compared with *V. philippinarum* and, at salinities 14 and 21, comparing with *V. decussata* (cf. Table 11).

Table 11. Total glutathione (GSHt) content (µmol/g FW) in *Venerupis decussata*, *Venerupis philippinarum* and *Venerupis corrugata* along the salinity range (0, 7, 14, 21, 28, 35 and 42). Values are the mean of three replicates ± standard deviation. For each salinity, different letters (a-c) represent significant differences (p≤0.05) among species.

Salinity	*V. decussata*	*V. philippinarum*	*V. corrugata*
0	0.58 ± 0.04[a]	0.50 ± 0.02[a]	
7	0.57 ± 0.06[a]	0.53 ± 0.02[a]	0.39 ± 0.12[a]
14	0.55 ± 0.01[a]	0.55 ± 0.03[a]	0.39 ± 0.03[b]
21	0.45 ± 0.04[a]	0.56 ± 0.03[b]	0.33 ± 0.05[c]
28	0.39 ± 0.00[a]	0.51 ± 0.02[b]	0.33 ± 0.06[a]
35	0.42 ± 0.01[a]	0.49 ± 0.04[b]	
42	0.55 ± 0.00[a]	0.60 ± 0.02[b]	

3.5.1. Reduced glutathione (GSH)

The quantification of GSH (Figure 18) revealed significant differences for three species between the tested salinities. *V. decussata* presented a significant GSH increase up to salinity 14 and was also noticed a significant decreased from salinity 14 to salinity 28 and an increase up to the highest salinity tested (42), without significant differences comparing with other salinities (cf. Figure 18). *V. philippinarum* followed the same trend, except between salinities 21 and 35 where the GSH values were constant. *V. corrugata* presented a decrease at higher salinity, that this species tolerates (28), although with no significant differences between the remaining salinities.

Between *V. decussata* and *V. philippinarum*, except at salinity 28, no significant differences were found (Table 12). *V. corrugata* showed significant differences comparing with *V. decussata* at salinities 7 and 14 and when compared with *V. philippinarum* no significant differences were found. At salinity 21 no significant differences between the three species were registered (cf. Table 12).

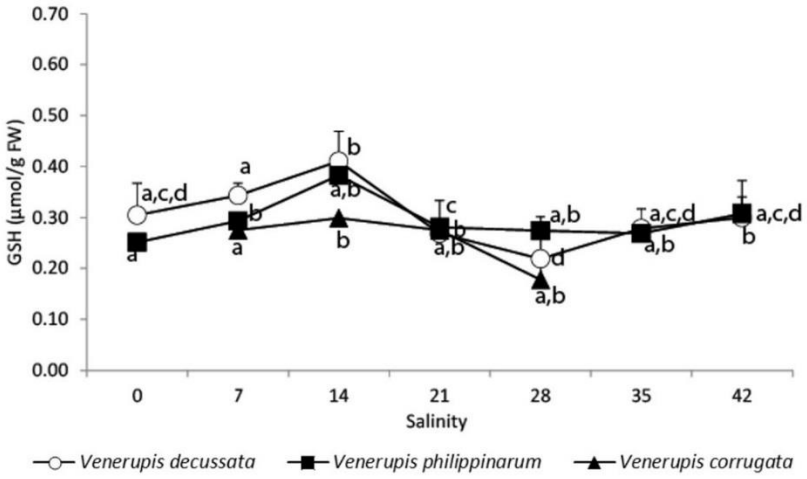

Figure 18. Reduced glutathione (GSH) content (µmol/g FW) for *Venerupis decussata*, *Venerupis corrugata* and *Venerupis philippinarum*. Values are the mean of three replicates ± standard deviation. For each species, different letters (a-d) represent significant differences (p≤0.05) among salinities.

Table 12. Reduced glutathione (GSH) content (µmol/g FW) in *Venerupis decussata*, *Venerupis philippinarum* and *Venerupis corrugata* along the salinity range (0, 7, 14, 21, 28, 35 and 42). Values are the mean of three replicates ± standard deviation. For each salinity, different letters (a-b) represent significant differences (p≤0.05) among species.

Salinity	*V. decussata*	*V. philippinarum*	*V. corrugata*
0	0.30 ± 0.06^{a}	0.25 ± 0.01^{a}	
7	0.34 ± 0.02^{a}	0.29 ± 0.02^{b}	0.28 ± 0.01^{b}
14	0.41 ± 0.01^{a}	0.38 ± 0.09^{a,b}	0.30 ± 0.00^{b}
21	0.27 ± 0.02^{a}	0.28 ±0.00^{a}	0.28 ± 0.06^{a}
28	0.22 ± 0.01^{a}	0.27 ± 0.03^{b}	0.18 ± 0.10^{a,b}
35	0.28 ± 0.04^{a}	0.27 ± 0.02^{a}	
42	0.30 ± 0.07^{a}	0.31 ± 0.03^{a}	

3.5.1. Ratio of reduced glutathione (GSH) / oxidized glutathione (GSSG)

The results concerning the ratio between GSH and GSSG, showed significantly higher values at salinity 14 for *V. decussata* and *V. philippinarum*, with significant differences between the remaining salinities (Figure 19). *V. corrugata* demonstrated a different behaviour, comparing with the two other species, presenting the higher GSH/GSSG value at salinity 21, followed by an abrupt and significant decrease up to salinity 28 (the highest salinity tolerated by this species).

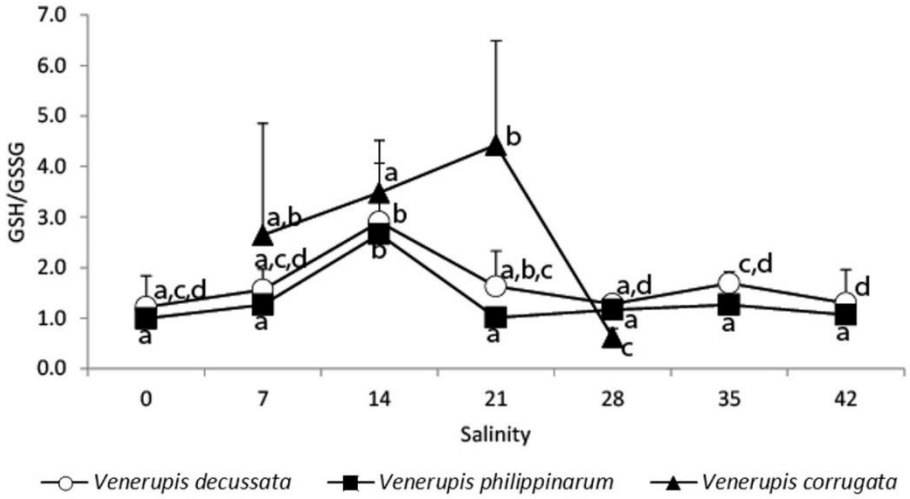

Figure 19. GSH/GSSG ratio for *Venerupis decussata*, *Venerupis corrugata* and *Venerupis philippinarum*. Values are the mean of three replicates ± standard deviation. For each species, different letters (a-d) represent significant differences (p≤0.05) among salinities.

Comparing the three species under the same salinity range, significant differences were only detected at salinities 21 and 28, where *V. corrugata* had a different behaviour from the two other species, presenting the highest value at salinity 21 and an abrupt decrease up to salinity 28 (cf. Figure 19 and Table 13).

Table 13. GSH/GSSG ratio in *Venerupis decussata*, *Venerupis philippinarum* and *Venerupis corrugata* along the salinity range (0, 7, 14, 21, 28, 35 and 42). Values are the mean of three replicates ± standard deviation. For each salinity, different letters (a-b) represent significant difference (p≤0.05) among species.

Salinity	***V. decussata***	***V. philippinarum***	***V. corrugata***
0	1.22 ± 0.62[a]	0.99 ± 0.03[a]	
7	1.56 ± 0.41[a]	1.26 ± 0.29[a]	2.64 ± 2.21[a]
14	2.91 ± 0.58[a]	2.66 ± 1.40[a]	3.48 ± 1.04[a]
21	1.63 ± 0.70[a]	1.01 ± 0.10[a]	4.43 ± 2.06[b]
28	1.28 ± 0.12[a]	1.16 ± 0.21[a]	0.62 ± 0.16[b]
35	1.69 ± 0.23[a]	1.27 ± 0.25[a]	
42	1.30 ± 0.66[a]	1.07 ± 0.23[a]	

3.6. Nuclear magnetic resonance (NMR) spectroscopy

3.6.1. Aqueous extracts

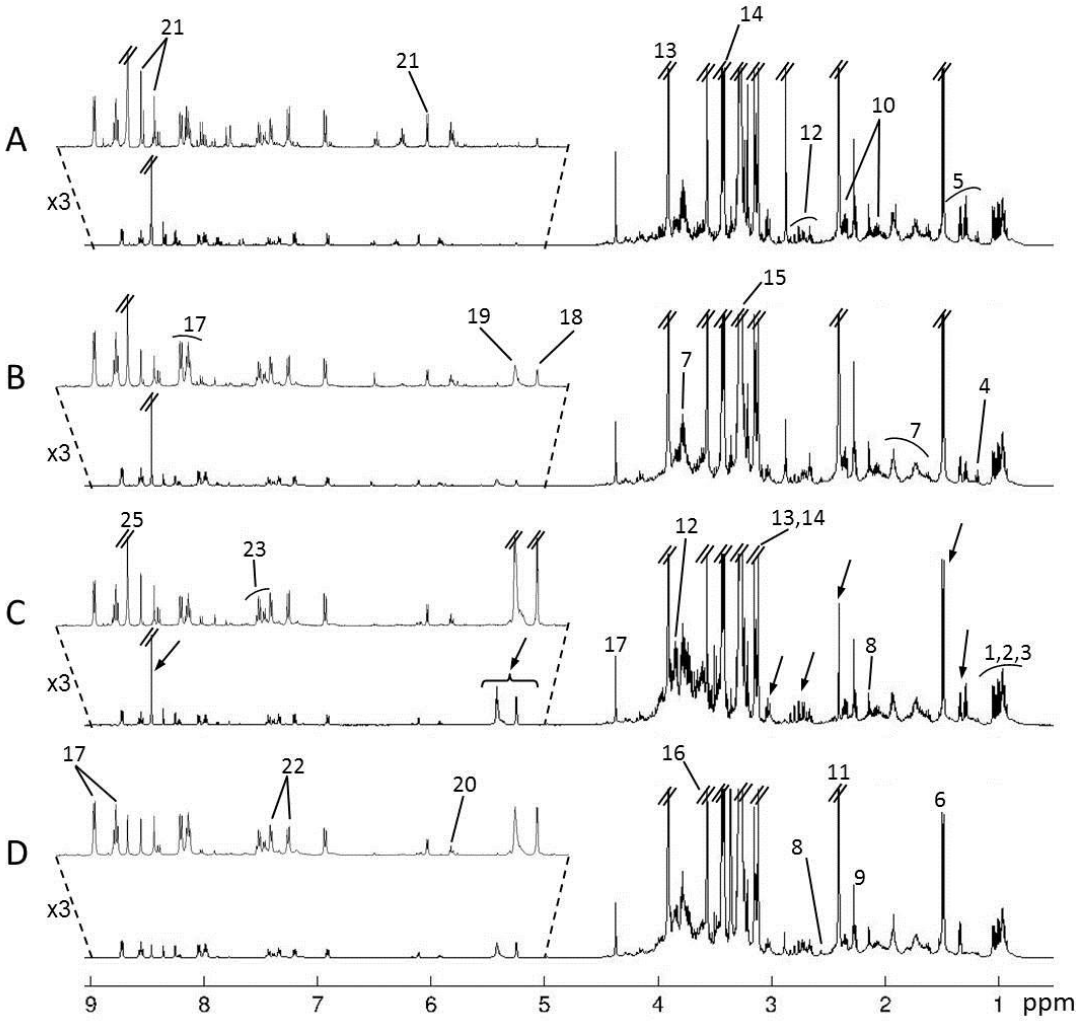

Figure 20. ^{1}H Nuclear Magnetic Resonance (NMR) spectra of aqueous extracts obtained from *Venerupis philippinarum* exposed to different salinities: A: 0, B: 7, C: 28, D: 42. Each spectrum represents the mean of the replicates (salinity 0, n=1; salinity 7, n=2; salinity 28, n=3; salinity 42, n=3). Legend: 1, 2, 3, isoleucine (Ile), leucine (Leu) and valine (Val); 4, ethanol (extraction solvent); 5, threonine (Thr)/lactate; 6, alanine (Ala); 7, arginine (Arg); 8, glutamine (Gln); 9 acetoacetate (tentative); 10, glutamate (Glu); 11, succinate; 12, asparagine (Asn); 13, betaine; 14, taurine; 15, hypotaurine; 16, glycine (Gly); 17, homarine; 18, glucose (an anomer); 19, glycogen (anomeric protons); 20, uridine; 21, inosine/adenosine; 22, tyrosine (Tyr); 23, phenylalanine (Phe); 24, hypoxanthine; 25, formate. Arrows indicate some of the differences noted by visual inspection of the spectra.

Figure 20 shows representative ^{1}H NMR spectra obtained for *V. philippinarum* species, when exposed to salinities 0, 7, 28 and 42. Due to the limited number of replicates, comparison of these spectra should be considered as exploratory. However, apparent spectral changes between different salinities may be noted by visual inspection of the spectra (cf. Figure 20), such as those regarding threonine (peak 5), alanine (peak 6), acetoacetate (peak 9), succinate (peak 11), glucose and glycogen (peaks 18 and 19) and formate (peak 25). Table 14 lists the variations noted in the integrals of some metabolites, at low salinities (0 and 7) and at the highest salinity (42), compared to 28, although most variations are qualitative at this stage and only formic acid showed a statistically relevant change. Regarding amino acids, deviation from the ideal salinity 28 (either towards low or high salinity) seems to be associated with generally higher amino acid levels (Thr, Ala, Glu, Gln, Gly, Tyr), with the exceptions of Asn (decreased non-specifically at three salinities) and Arg, which showed an apparently specific response to low (↓ Arg) and high (↑ Arg) salinities. In relation to organic acids, lower (0 and 7) and higher (42) salinities seem accompanied by increased acetoacetic acid (acetoacetate) and succinic acids (succinate) and a decrease for salinities 7 and 42 in formic acid (formate), the latter becoming significant at 42 ($p = 0.00121$). Other changes seem to be mostly non-specific to salinity, such as the decreases in taurine, betaine, glucose and glycogen and the increase in adenosine/inosine. On the other hand, apparent salinity-specific changes are noted either in terms of different magnitudes of change (namely for formic acid, hypotaurine and homarine) or of decrease or increase of change (for uridine, hypoxanthine and Arg, as mentioned above).

Table 14. Changes in metabolites as viewed by ^{1}H NMR spectroscopy of aqueous *V. philippinarum* extracts exposed at different salinities (0, 7 and 42) comparing to organisms of the same species exposed at salinity 28.

Labeling numbers	Compound	δ/ppm (multiplicity)[a]	Variation direction and magnitude (%) vs. Salinity 28 (n =3)		
			Salinity 0 (n=1)	Salinity 7 (n=2)	Salinity 42 (n=3)
	Amino acids				
1	Leucine	0.96 (t)	↓ (- 21.7 ± 12.5)	↑	↑
2	Isoleucine	1.01 (d)	↓	↑	↑
3	Valine	1.04 (d)	↓	↑ (19.5 ± 8.5)	↑ (20.6 ± 9.3)
5	Threonine/Lactate	1.34 (d)	↑ (15.2 ± 1.2)	↑ (11.1 ± 1.2)	↑
6	Alanine	1.49 (d)	↑	↑ (61.1 ± 25.6)	↑ (31.9 ± 11.8)
7	Arginnine	1.92 (m)	↓	↓	↑
10	Glutamate	2.35 (m)	↑ (15.8 ± 5.5)	↑ (55.8 ± 15.5)	↑
8	Glutamine	2.43 (m)	↑ (26.0 ± 3.6)	↑ (68.0 ± 8.0)	↑ (34.4 ± 15.2)
12	Asparagine	2.81 (dd)	↓ (- 23.5 ± 7.6)	↓	↓
16	Glycine	3.57 (s)	↑	↑	↑ (55.3 ± 19.9)
22	Tyrosine	6.91 (d)	↑	↑	↑ (25.8 ± 9.9)
23	Phenylalanine	7.38 (m)	↓	↑	↑
	Organic acids				
9	Acetoacetate[d]	2.27 (s)	↑	↑ (71.4 ± 45.0)	↑
11	Succinate	2.41 (s)	↑ (699.7 ± 10.9)	↑ (897.6 ± 88.0)	↑ (411.7 ± 130.9)
25	Formate	8.46 (s)	↑ (103.2 ± 5.8)	↓	↓ (- 80.2 ± 15.9)[b]
	Osmolytes				
15	Hypotaurine	2.66 (t)	↑	↑ (106.7 ± 31.6)	↑
14	Taurine	3.43 (t)	↓ (- 32.1 ± 12.7)	↓	↓
13	Betaine	3.91 (s)	↓ (- 20.3 ± 11.2)	↓	↓
17	Homarine (N-methylpicolinic acid)	8.72 (d)	↓	↑	↑ (46.1 ± 17.9)
	Carbohydrates				
18	Glucose	5.25 (d)	↓ (- 91.8 ± 15.2)	↓ (- 76.7 ± 22.7)	↓
19	Glycogen	5.42 (br)	↓ (- 95.2 ± 17.2)	↓ (- 76.6 ± 34.0)	↓ (- 48.4 ± 27.1)
	Others				
20	Uridine	5.92 (m)	↑ (103.6 ± 9.2)	↑	↓
21	Adenosine/inosine	6.10 (d)	↑ (40.0 ± 11.2)	↑	↑
24	Hypoxanthine	8.21 (s)	↓	↑	↓
	Unassigned[c]				
	Un1	1.29 (t)	↑	↓	↓ (-65.4 ± 35.7)
	Un2	2.25 (s)	↑	↑ (26.7 ± 10.1)	↑ (21.1 ± 12.3)
	Un3	2.26 (s)	↑	↑ (59.4 ± 28.0)	↑
	Un4	3.03 (t)	↑	↑	↓
	Un5	4.37 (s)	↑	↑	↑

Variations indicated with a single arrow should, at this stage, be regarded as qualitative only; for the remaining variations, the corresponding magnitude is indicated, although large deviations are noted (except for formate), probably due to biological variability. [a] Chemical shifts shown correspond to signals used for integration, in some cases part of the full spin system; s, singlet; d, doublet; t, triplet; m, multiplet; dd, doublet of doublets; br, broad; Un, unassigned resonance. [b] $p = 0.00121$. [c] Still unassigned NMR peaks. [d] Tentative assignment.

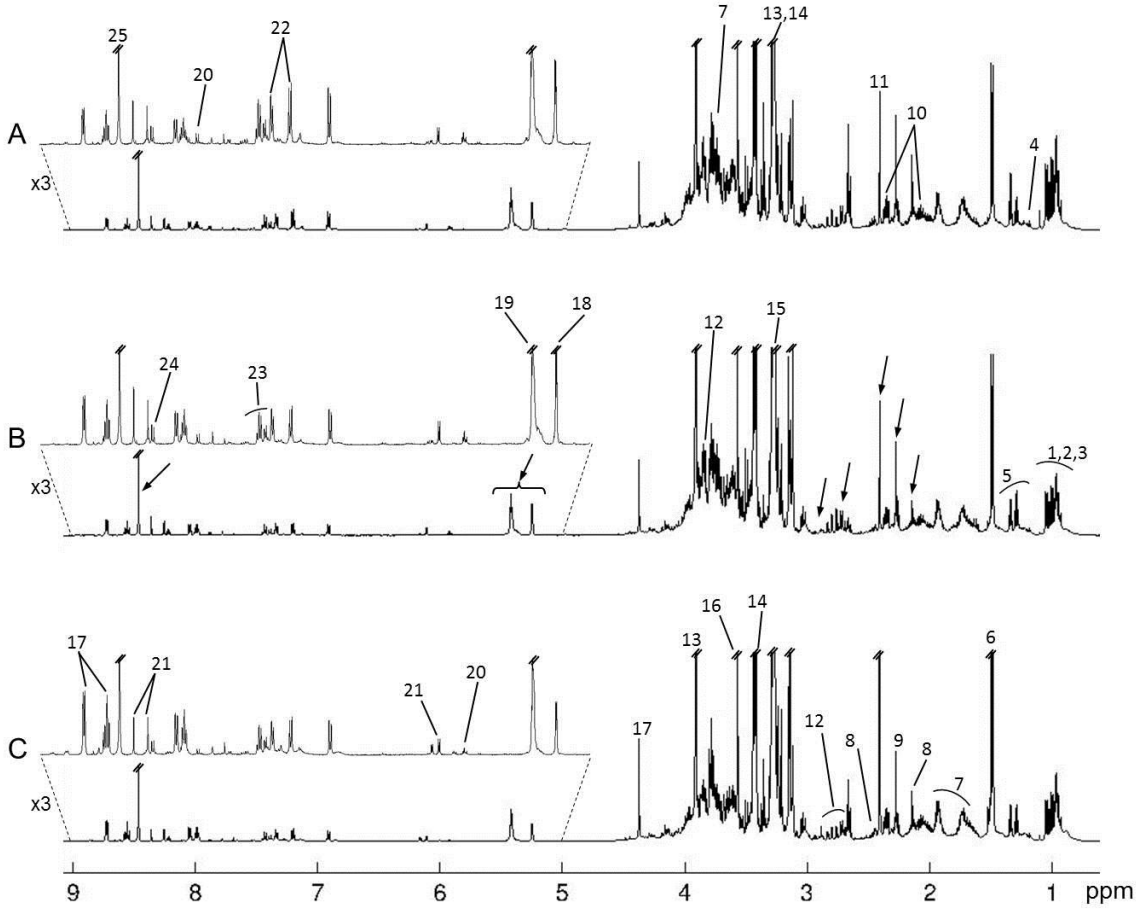

Figure 21. ^{1}H Nuclear Magnetic Resonance (NMR) spectra of aqueous extracts obtained from *Venerupis decussata* (A), *Venerupis philippinarum* (B) and *Venerupis corrugata* (C), exposed to salinity 28. Each spectrum represents the mean of three replicates. Legend: 1, 2, 3, isoleucine (Ile), leucine (Leu) and valine (Val); 4, ethanol (extraction solvent); 5, threonine; 6, alanine (Ala); 7, arginine (Arg); 8, glutamine (Gln); 9 acetoacetate (tentative); 10, glutamate (Glu); 11, succinate; 12, asparagine (Asn); 13, betaine; 14, taurine; 15, hypotaurine; 16, glycine (Gly); 17, homarine; 18, glucose (an anomer); 19, glycogen (anomeric protons); 20, uridine; 21, inosine/adenosine; 22, tyrosine (Tyr); 23, phenylalanine (Phe); 24, hypoxanthine; 25, formate. Arrows indicate some of the differences noted by visual inspection of the spectra.

Figure 21 shows the representative ^{1}H NMR spectra obtained for the *V. decussata*, *V. corrugata* and *V. philippinarum* species, when exposed to salinity 28. Comparison of these spectra is still exploratory, being required larger numbers of replicates in order to confirm these results. However, apparent spectral changes between the three species may be noted by visual inspection of the spectra (cf. Figure 21), such as those regarding glutamine (peak 8), acetoacetate (peak 9), succinate (peak 11), glucose and glycogen (peaks 18 and 19) and formate (peak 25). Regarding amino acids, *V. decussata* seems to be associated with generally higher amino acid levels (Leu, Ile, Val, Glu, Tyr). In relation to organic acids, *V. decussata* also showed to be the species with higher levels of acetoacetic (peack 9) and succinic acid (peak 11). Other changes seem to be mostly non-

specific of species, such as the differences in taurine, betaine, glucose and glycogen and in adenosine/inosine. On the other hand, apparent species-specific changes are noted in terms of different magnitudes of change or of direction of change (decrease or increase). Table 15 lists the variations noted in the integrals of some metabolites, of *V. decussata* and *V. corrugata* compared with *V. philippinarum*, although most variations are qualitative at this stage and any statistically relevant change was noticed. Regarding amino acids, *V. decussata* seems to show generally higher amino acids levels (Leu, Ile, Val, Thr, Glu, Gln, Gly, Tyr, Phe), while *V. corrugata* seems to present mostly lower amino acids levels (Leu, Ile, Val, Thr, Asn, Tyr, Phe). Glu (↑), Gln (↑), Asn (↓) and Gly (↑) present the same qualitative variation in *V. decussata* and *V. corrugata*. In relation to organic acids, only formic acid showed lower levels in *V. decussata* and an increase was reported in acetoacetic and succinic acids in both species, comparing with *V. philippinarum*. Osmolytes presented, in general, a decrease in *V. decussata* (taurine, betaine and homarine) and an increase in *V. corrugata* (hypotaurine, taurine and homarine). Glucose, uridinine, adenosine and hypoxanthine showed the same variation (↓) in *V. decussata* and *V. corrugata*.

Table 15. Changes in metabolites as viewed by ^{1}H NMR spectroscopy of aqueous extracts of *Venerupis decussata* and *Venerupis corrugata* comparing with *Venerupis philippinarum*, all exposed at salinity 28.

Labeling numbers	Compound	δ/ppm (multiplicity)[a]	Variation direction and magnitude (%) vs. *V. philippinarum* (n =3)	
			V. decussata (n=3)	*V. corrugata* (n=3)
	Amino acids			
1	Leucine	0.96 (t)	↑	↓
2	Isoleucine	1.01 (d)	↑	↓
3	Valine	1.04 (d)	↑	↓
5	Threonine/Lactate	1.34 (d)	↑	↓
6	Alanine	1.49 (d)	↓	↑ (191.8 ± 40.6)
7	Arginnine	1.92 (m)	↓	↑
10	Glutamate	2.35 (m)	↑	↑
8	Glutamine	2.43 (m)	↑	↑
12	Asparagine	2.81 (dd)	↓	↓
16	Glycine	3.57 (s)	↑ (32.0 ± 18.8)	↑ (40.9 ± 18.3)
22	Tyrosine	6.91 (d)	↑	↓
23	Phenylalanine	7.38 (m)	↑	↓
	Organic acids			
9	Acetoacetate[c]	2.27 (s)	↑	↑
11	Succinate	2.41 (s)	↑	↑ (173.2 ± 87.7)
25	Formate	8.46 (s)	↓	↑
	Osmolytes			
15	Hypotaurine	2.66 (t)	↑ (248.0 ± 15.0)	↑ (149.7 ± 31.2)
14	Taurine	3.43 (t)	↓	↑
13	Betaine	3.91 (s)	↓	↓
17	Homarine (N-methylpicolinic acid)	8.72 (d)	↓	↑
	Carbohydrates			
18	Glucose	5.25 (d)	↓	↓
	Others			
20	Uridine	5.92 (m)	↓	↓
21	Adenosine/inosine	6.10 (d)	↓	↓
24	Hypoxanthine	8.21 (s)	↓	↓
	Unassigned[b]			
	Un1	1.29 (t)	↓	↑
	Un2	2.25 (s)	↑	↑
	Un3	2.26 (s)	↓	↑
	Un4	3.03 (t)	↑	↑
	Un5	4.37 (s)	↓	↑

Variations indicated with a single arrow should, at this stage, be regarded as qualitative only; for the remaining variations, the corresponding magnitude is indicated, although large deviations are, probably due to biological variability. [a] Chemical shifts shown correspond to signals used for integration, in some cases part of the full spin system; s, singlet; d, doublet; t, triplet; m, multiplet; dd, doublet of doublets; br, broad; Un, unassigned resonance . [b] Still unassigned NMR peaks. [c] Tentative assignment.

3.6.2. Lipid extracts

Figure 22 shows the representative ^{1}H NMR spectra of lipid extracts obtained for *V. philippinarum*, when exposed to salinities 0, 7, 28 and 42. Due to the limited number of replicates available, at this stage, comparison of these spectra should be considered as exploratory. However, apparent spectral changes between different salinities may be noted by visual inspection of the spectra (cf. Figure 22). $C_{26}H_3$, $C_{27}H_3$ and $C_{21}H_3$ in cholesterol (peaks 2, 3 and 4) seemed to be associated with higher levels at different salinities from 28. Changes in $(CH_2)_n$ in fatty acids (peak 6), comparatively to the amount presented at salinity 28 (Figure 22 C) was apparently related with higher amount at lower salinities (0 and 7, figures 22 A and B, respectively). A large increase of $-CH_2-CH=CH-$ environments (peak 10) was noticed at salinity 0 (Figure 23A), comparatively to salinity 28 (Figure 22C). At salinity 7 (Figure 22B) an increase in intensity of the $-CH_2CH_2COOC-$ resonance (peak 9) and C_1H_2, $C(3)H_2$, in glycerol (peaks 15 and 16), were observed, when compared with salinity 28.

The representative ^{1}H NMR spectra obtained for the *V. decussata*, *V. corrugata* and *V. philippinarum* species, when exposed to salinity 28 are showed in the Figure 23. Comparison of these spectra is still exploratory, being required larger numbers of replicates in order to confront the herein observed results. Apparently, An increase in the resonance of $(CH_2)_n$, compared to the CH3 peak indicates an increase in average chain length if the fatty acids being produced, differentiating *V. philippinarum* (Figure 23B) from *V. decussata* and *V. corrugata* (Figures 23 A and C, respectively), showing lower levels in *V. philippinarum*. On the other hand, the obtained results suggested that *V. corrugata* differ itself from the other two species, revealing higher levels of $-CH=CHCH_2-CH=CH$ environments in fatty acids (peak 12) and -CH=CH-, C(2)H in glycerol (peaks 17 and 18). An increase in unsaturated environments compared to CH3 indicates a change in the average unsatuiration degree of the fatty acids being produced.

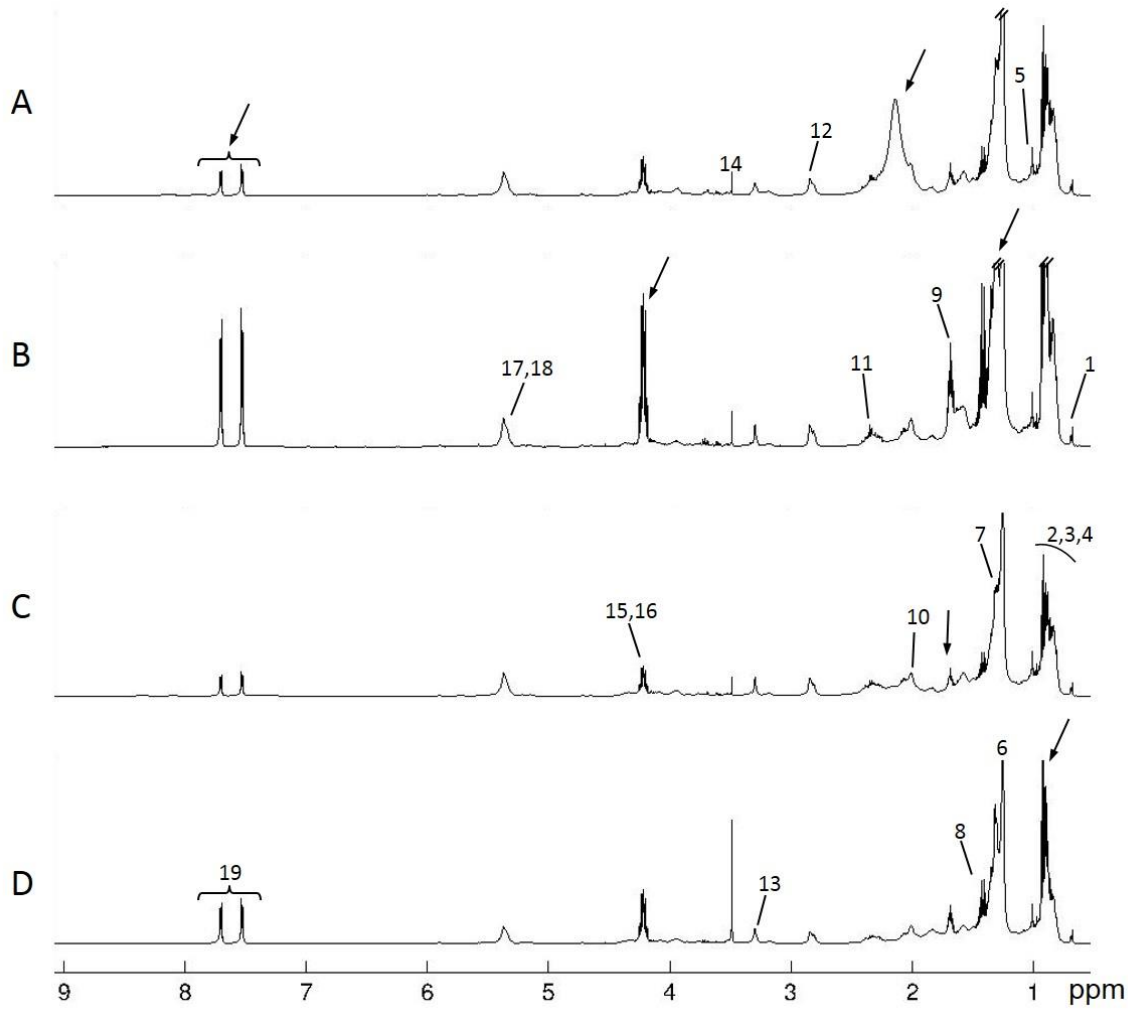

Figure 22. ^{1}H Nuclear Magnetic Resonance (NMR) spectra of lipid extracts obtained from *Venerupis philippinarum* exposed to different salinities: A: 0, B: 7, C: 28, D: 42. Each spectrum represents the mean of the replicates (salinity 0, n=1; salinity 7, n=2; salinity 28, n=3; salinity 42, n=3). Legend: 1, $C_{18}H_3$ in cholesterol; 2, 3, 4, $C_{26}H_3$, $C_{27}H_3$, $C_{21}H_3$ in cholesterol; 5, $C_{19}H_3$ in cholesterol; 6, $(CH_2)_n$ in fatty acids; 7, =CH-CH_2-$CH_2(CH_2)$ in fatty acids; 8, CO-CH_2-CH_2 in fatty acids; 9, -CH_2CH_2COOC- in fatty acids; 10, -CH_2-CH=CH- in fatty acids; 11, CH_2-COOC in fatty acids; 12, -CH=CHCH_2-CH=CH in fatty acids; 13, phospholipids choline head group $N(CH_3)_3$; 14, methanol (extraction solvent); 15, 16, C_1H_2, C_3H_2 in glycerol; 17, 18, -CH=CH-, C_2H in glycerol; 19, still unassigned NMR peaks. Arrows indicate some metabolites where differences were noted by visual inspection of the spectra.

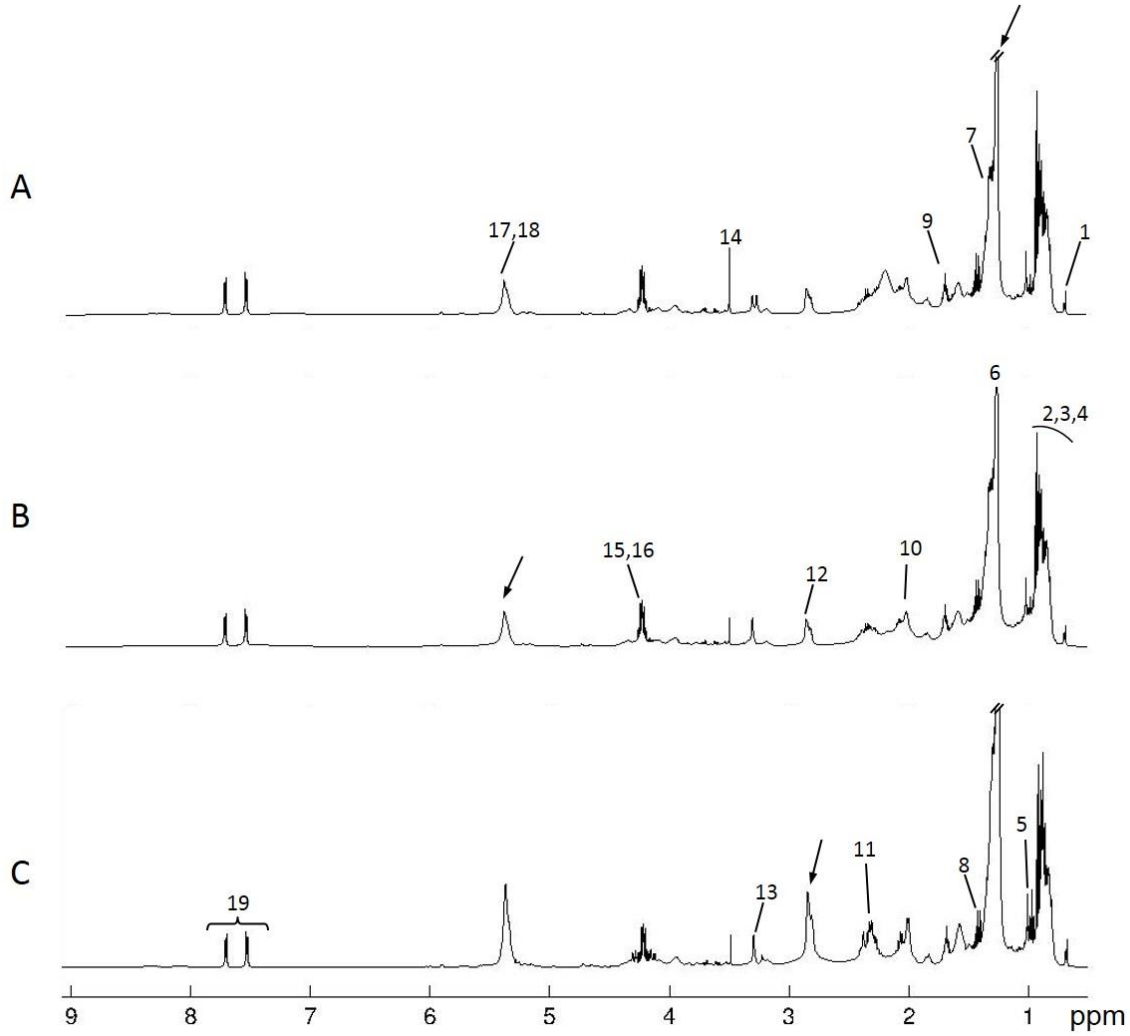

Figure 23. ^{1}H Nuclear Magnetic Resonance (NMR) spectra of lipid extracts obtained from *Venerupis decussata* (A), *Venerupis philippinarum* (B) and *Venerupis corrugata* (C), exposed to salinity 28. Each spectrum represents the mean of three replicates. Legend: 1, $C_{18}H_3$ in cholesterol; 2, 3, 4, $C_{26}H_3$, $C_{27}H_3$, $C_{21}H_3$ in cholesterol; 5, $C_{19}H_3$ in cholesterol; 6, $(CH_2)_n$ in fatty acids; 7, $=CH\text{-}CH_2\text{-}CH_2(CH_2)$ in fatty acids; 8, $CO\text{-}CH_2\text{-}CH_2$ in fatty acids; 9, $-CH_2CH_2COOC-$ in fatty acids; 10, $-CH_2\text{-}CH=CH-$ in fatty acids; 11, $CH_2\text{-}COOC$ in fatty acids; 12, $-CH=CHCH_2\text{-}CH=CH$ in fatty acids; 13, phospholipids choline head group $N(CH_3)_3$; 14, methanol (extraction solvent); 15, 16, C_1H_2, C_3H_2 in glycerol; 17, 18, $-CH=CH-$, C_2H in glycerol; 19, still unassigned NMR peaks. Arrows indicate some of the differences noted by visual inspection of the spectra.

3.7. Data analysis

The PCO analysis (Figure 24) revealed that PCO1 explained 37.9 % of the total variation among conditions, separating the three species, with *V. corrugata* in the negative axis spaced from the other two species, whose most conditions were in the positive axis. PCO2 described 19.5 % of the total variation separating the lower (in the negative axis) from the higher salinity conditions (in the positive axis). The physiological and biochemical descriptors superimposed on PCO, showed that glycogen presented high positive correlation with *V. corrugata* at salinities 21 and 28.

Lowest salinities for *V. corrugata* (7 and 14) and salinity 21 for *V. decussata* showed strong correlation with the activity of the enzymes GST and CAT. The lowest salinities for *V. decussata* (0 and 7) and for *V. philippinarum*, (7, 14 and 21, with the exception of salinity 0) showed strong correlation with the antioxidants GSHt and GSH (cf. Figure 24).

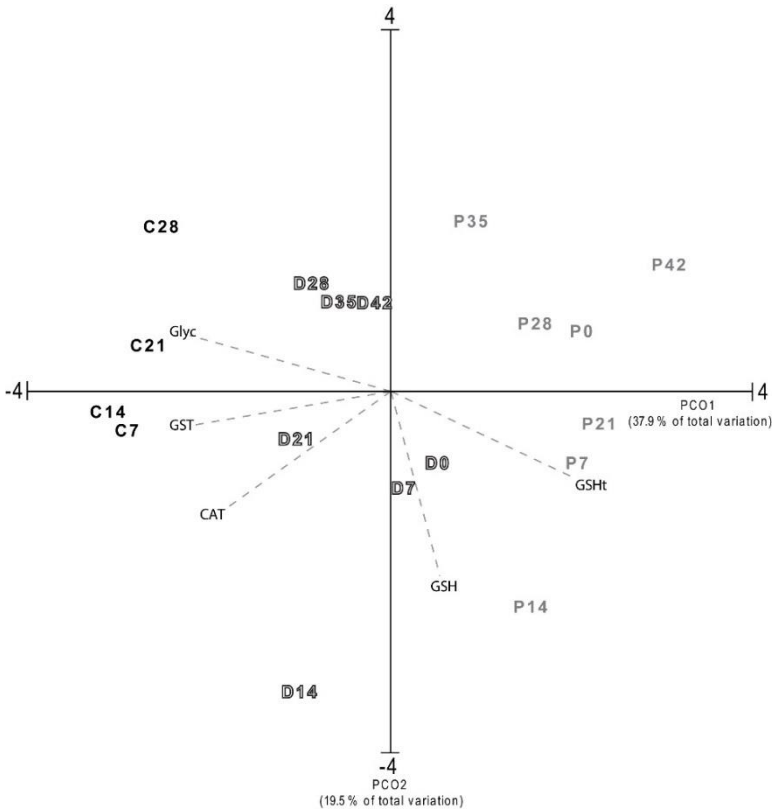

Figure 24. Centroids ordination diagram (PCO, Principal Coordinates analysis) based on the physiological and biochemical responses of the three species when exposed to different salinities. Pearson correlation vectors are superimposed as supplementary variables, namely physiological and biochemical data (r > 0.75). Legend: PCO1, first principal component; PCO2, second principal component; D, *Venerupis decussata*; C, *Venerupis corrugata*; P, *Venerupis philippinarum*; numbers (0, 7, 14, 21, 28, 35 and 42) correspond to the tested salinities; Glyc, glycogen; GST, glutathione-S-transferase; CAT, catalase; GSH, reduced glutathione; GSHt, total glutathione.

The corresponding PCA for aqueous extracts (Figure 25A) showed the clear separation between *V. corrugata* and the other two species at salinity 28 (in black) on PC2. It was also possible to observe a separation of samples of *V. philippinarum* at salinity 42 (grey squares) from salinity 28 on PC1. The PLS-DA scores plot (Figure 25B) confirm the separation between *V. corrugata* and both, *V. decussata* and *V. philippinarum* at salinity 28 on LV2, with same separation. Although the separation of salinities 42 and 28 in *V. philippinarum* was less clear, it was still possible to observe this separation on LV2. Any other clear separation was detected.

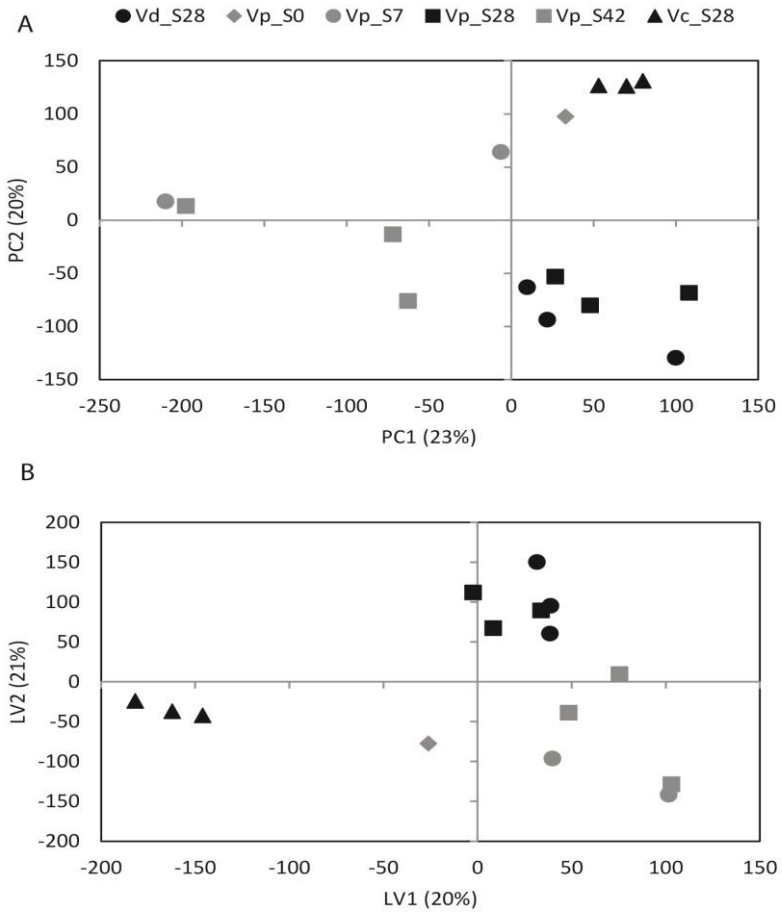

Figure 25. MVA including all aqueous extracts tested in NMR spectroscopy, UV-scaled data. A, PCA scores plot; B, PLS-DA scores plot (R^2x=0.418; R^2y=0.373 Q^2=0.114). Legend: PC1, first component of PCA; PC2, second component of PCA; LV1, first component of PLS-DA; LV2, second component of PLS-DA; Vd, *Venerupis decussata*; Vp, *Venerupis philippinarum*; Vc, *Venerupis corrugata*; S0, S7, S28 and S42 correspond to salinities 0, 7, 28 and 42 respectively.

The MVA described for lipid extracts did not show any clear separation between the tested conditions. The corresponding PCA (Figure 26A) only suggested a separation between the salinity 42 in *V. philippinarum* (grey squares) and the other salinities on PC2. In PLS-DA scores plot (Figure 26B) the same separation was clearer on LV1 and it emphasized the separation of the salinity 42 from de salinity 7 in the same species.

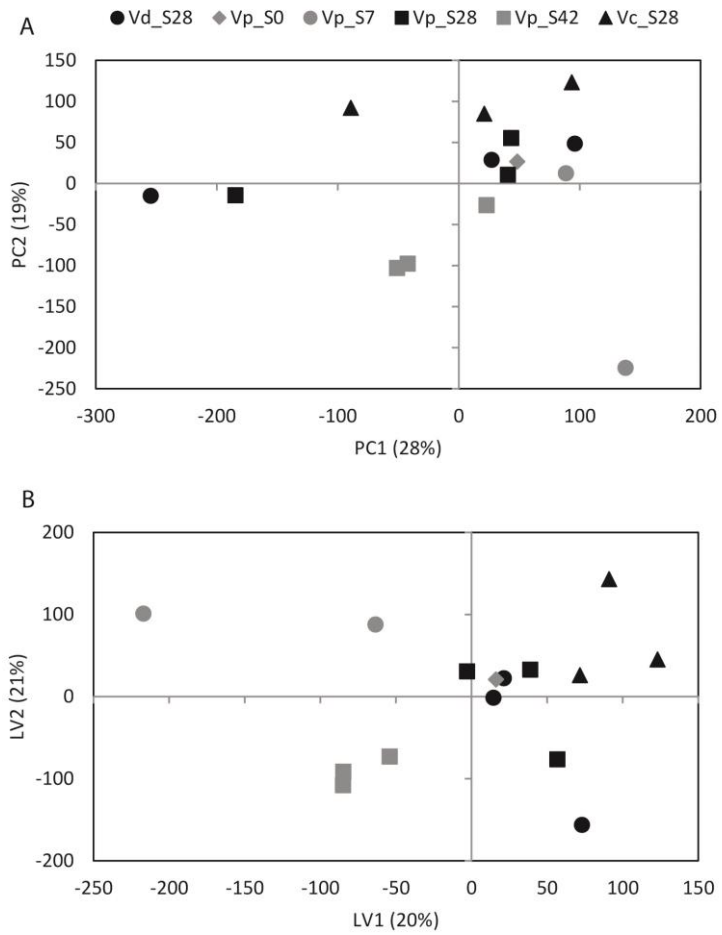

Figure 26. MVA including all lipid extracts tested in NMR spectroscopy, UV-scaled data. A, PCA scores plot; B, PLS-DA scores plot (R^2x=0.409; R^2y=0.3 Q^2=-0.008). Legend: PC1, first component of PCA; PC2, second component of PCA; LV1, first component of PLS-DA; LV2, second component of PLS-DA; Vd, *Venerupis decussata*; Vp, *Venerupis philippinarum*; Vc, *Venerupis corrugata*; S0, S7, S28 and S42 correspond to salinities 0, 7, 28 and 42 respectively.

4. Discussion

4.1. Context

Climate changes have been forcing organisms to rapidly adapt to a new conditions on the environment. These changes may be related to strong precipitation events with hyposaline stress conditions or associated to longer hot seasons, causing an increase in salinity. Organisms living in estuaries, such as bivalves, have constantly to deal with these fluctuations on salinity and indeed, the alterations will be more pronounced and longer with worsening on the climate changes.

Since the salinity is one of the most important abiotic factors that affect marine organisms limiting their spatial distribution in the environment (Widdows and Shick, 1985) and having high influence in the fishery and culture of bivalves (Matozzo et al., 2007; Hamer et al., 2008), it is very important to understand how salinity changes affect aquatic organisms. When these abiotic stressors are combined with biotic, like biological invasions, the adaptations to the new environment can be more difficult to the native species.

Thus, the present study aimed to compare the survival capacity and the physiological, biochemical and metabolomic alterations of three clams (*Venerupis corrugata*, *V. decussata* and *V. philippinarum*) inhabiting the same coastal area, exposed to a range of salinities (0, 7, 14, 21, 28, 35 and 42) with the objective of understand the effects of salinity shifts on these species.

4.2. Mortality

The results obtained showed that the native species *V. corrugata* was the species with lower survival capacity, presenting 100 % of mortality rates at the extremes of salinity tested (0, 35 and 42) and higher mortality percentage at other salinities (7, 14, 21, 28) when comparing with other two species, *V. decussata* and *V. philippinarum*. The lowest percentage of mortality for this species was detected at salinity 21, which may indicate optimal conditions for *V. corrugata* survival. With 100 % of survival at all salinities, except at 0, *V. decussata*, one of the native species in the study area, was the species more capable to tolerate a wide salinity range. With exception to lower salinities tested (0 and 7), *V. philippinarum* also showed a great capacity to survive under different salt concentrations, presenting 0 % of mortality at salinities higher than 7.

At this stage, it becomes clear that the three species used in this study have different performances when under salinity stress. The three species showed different tolerances to salinity changes, presenting different mortality rates, especially at low salinities. These differences

may be explained by the osmotic, physiological, biochemical and metabolic alterations provoked in each species, as will be discussed.

4.3. Osmotic balance

Euryhaline organisms, those capable of living at different salinities, present a life-dependent on several adaptations. Osmoregulation based on active ion transport mechanisms is one of these adaptations (Berguer and Kharazova, 1997). Wu et al. (2013) reported that hypo osmotic stress can significantly reduce food intake, driving organisms to severe starvation.

The survival of marine organisms, namely bivalves, is dependent on osmotic balance (Bianchini et al., 2008; Romano and Zeng, 2012) and this balance is mainly achieved with Na in marine environments. With the obtained results it is possible to observe that in *V. decussata*, *V. corrugata* and *V. philippinarum* intracellular Na levels were strongly dependent on the external salinity in the range between 0 and 42, increasing along the increasing salinity exposure. Berger and Kharazova (1997) demonstrated that in *V. philippinarum* Na concentrations varied according to the alterations on salt concentration opposed to K levels, which maintained quite constantly at the same considered salinity gradient. The results obtained in the present study also showed a difference between Na and K concentrations. *V. decussata* and *V. philippinarum* presented constant levels of K along the entire salinity range, while the levels of this ion in *V. corrugata* increased along with the increase of salt concentration. Like Berger and Kharazova (1997) proposed, these results suggested that Na plays an important key role in osmotic balance on the tested organisms, since the rise of Na concentrations seems to be a mechanism to protect cells from the influence of extremely high salinities. The same authors suggested that Na ions diffuse into the cell when the salinity is high, and when salinity decreases Na is actively removed. At low salinities, variation in Na concentrations are probably insufficient to maintain osmotic regulation. The higher levels of Ca at low salinities (0, 7 and 14) in *V. decussata* and *V. philippinarum* may indicate that osmotic regulation is compensated with this ion instead of Na. *V. corrugata* did not follow the trend of the other two species. The levels of Ca at the lowest salinity that this species can tolerate (7) may justify the imbalance of osmoregulation, since the mechanisms developed by this species are not capable to compensate the low values of Na. Oxidized products, resulting from oxidation of phospholipids membranes, leads to the permeability of the membranes, making easier the input of Ca ions, which may conduct to cellular death (Manduzio et al., 2005). Thus, the

higher levels of Ca registered for *V. decussata* and *V. philippinarum* at lower salinities (0 and 7), could represent a higher oxidative stress at these salinities.

Works conducted by Elston et al. (2003) boosted the hypothesis that low salinity (10) forced *V. philippinarum* to strongly close their shells as a defence response to the changes in the surrounding environment. Shumway (1977a) also concluded that the concentrations of Na, Ca and Mg in hemolymph of bivalves are similar to the surrounding environment as long as the organisms maintain their valves opened. The same author demonstrated that the tested bivalves close their valves when the salinity dramatically decreases and turn to open when the salinity is tolerable to them. In the same study, when the seawater varied from 100 % to 30 %, finishing the cycle after 12 h when the sweater return to 100 %, on the second cycle the levels of Na, Ca and Mg stopped to follow the percentage of seawater. This may indicate that if the salt concentrations were maintained lower for a longer period of time, probably the clams would not be able to maintain their valves closed and at the same time, keep a balanced osmoregulation. The present thesis further demonstrated that Mg levels were proportional to the salinity range used. In other words, the concentration of Mg in clams was increased along salinity exposure, being similar to the surrounding environment. This data confirm that the three species under the present study, are osmoconformers.

In fact, osmoregulation is a high-cost energy process (Nelson and Cox, 2005) and marine organisms, namely bivalves, under stressful conditions, like changes in surrounding salt concentrations, are forced to spend more energy resources trying to maintain their euryhaline characteristics. According to Yancey (2005), some osmoconformer organisms are able to balance their osmotic pressure using organic osmolytes. The results obtained by ^{1}H NMR spectroscopy in the present study, showed an increase of isoleucine, leucine and valine, which are also aminoacids, at salinity 7, comparatively to salinity 28. However, the obtained results showed that taurine and betaine decreased at lower (7) and higher salinities (42) comparing to salinity 28 in *V. philippinarum* samples analysed on NMR approach, which do not comply with the regulation of osmoregulation achieved by osmolytes. These declines are in agreement with Elston et al. (2003) works, which also found that the decrease in amino acids was one of the stressful metabolic alterations of *V. philippinarum*, when exposed to an altered environment with lower salt concentrations.

4.4. Physiological performance

The decrease of functional activity is the most usual reaction of marine molluscs to changes in salinity (Berger and Kharazova, 1997). Kim et al. (2001) suggested that the shell closure and consequent reduction of oxygen comsumption rate, works as a defence mechanism conserving energy somewhat, a way of reducing energy expenditure on respiration processes and activity when the organisms were exposed to lower salinities. This defence mechanism can explain the results obtained regarding energy reserves in the three species. In fact, the present thesis revealed that the glycogen and glucose content in *V. philippinarum* were lower at salinities below 28, which may indicate that this species protected itself from lower salinities, being forced to call up reserve energies. In *V. decussata* the differences in energy content between lower (< 28) and higher salinities (> 28) were less significant than in *V. philippinarum*. This may indicate that *V. philippinarum* close their valves sooner than *V. decussata* when the surrounding environment decreases the concentration on salt. On the other hand, *V. corrugata* was not responsive in terms of glycogen content. The glycogen content was maintained along the range of salinities tolerated by *V. corrugata*, which may indicate that this species keeps the normal filtration, with no need to resort energy reserves, like glycogen. However, the glucose content was significantly lower at salinities below and above 21 in the pattern presented for *V. corrugata*. It is possible that this species was appealing to glucose reserves when the environmental conditions were not favourable to it normal biological functioning.

The results obtained evidenced that clams mobilize stored energy (glycogen) and may also use protein breakdown to cope with extreme salinity levels. The valve closure not only induces hypoxiabut also reduces food intake as well. At a limiting situation, energy resources are exhausted and osmotic imbalance may arise, inducing water influx into the cells, and causing swelling and cellular rupture (Coughlan et al., 2009). These effects may explain the high mortality of *V. philippinarum* at 0 and 7, of *V. decussata* at salinity 0 and of *V. corrugata* at low salinities (0, 7 and 14) observed in the present work, revealing that the three species have different limit for tolerance to changes in salt concentrations. Also Patrick et al. (2005) and Anacleto et al. (2013) showed that low glycogen contents were associated with mortality events.

Although clams can close their shell valves during long periods of time, this behaviour will induce hypoxia (Kim et al., 2001), which will have significant effects on cell metabolism. Low O_2 concentrations in cells will decrease the oxidative phosphorylation of ATP, which will induce the accumulation of metabolites that feed the respiratory chain and the activation of alternative

metabolic pathways of ATP production. Since these routes produce ATP less efficiently than oxidative phosphorylation and osmoregulation is an energetically expensive process, glycogen stores have to be rapidly consumed and cells have to resort to protein catabolism as an alternative source of energy.

Structural and functional changes in proteins are considered stress-related effects as well (Risso-de Faverney et al., 2000). In fact, our results showed that protein content is increasingly affected by salinity decrease. The results on the diagrams of protein expression, showed that the majority of variations occur at lower salinities (≤ 21) in the three species and this can be related to a decrease in the expression of new proteins, a higher breakdown or both. The results herein presented revealed that most alterations in protein expression at salinities < 28 for the three species under analysis, are represented by their repression. Navarro and Gonzalez (1998), in contrast, reported that when the scallop *Argopecten purpuratus* was transferred to a lower salinity (they expanded from south to centre and north of Chile, where salinity is lower) an increase of protein catabolism and the subsequent increase of amino acids were observed.

The lower activity of the electron respiratory chain decreases the oxidation of amino acids obtained by protein catabolism, leading to their accumulation, or the accumulation of their degradation intermediate metabolites, such as succinic acid (in isoleucine, threonine and methionine metabolism) or formic acid (in serine metabolism). This is consistent with the NMR results of *V. philippinarum* exposed to 7 and 42, which have shown higher levels of most amino acids and their oxidation intermediates (succinic and formic acids), compared to control (28). An exception is made for asparagine but this amino acid can be converted into glutamate and glutamine (which are increased) with ATP production. Other amino acids (leucine, lysine, phenylalanine, tryptophan and tyrosine) may be degraded into ketone bodies (Nelson and Cox, 2005). This is confirmed in the present work by the observed increase in acetoacetic acid with formation of glutamate (seen to increase). Liu et al. (2011b) reported that alanine and succinic acid are responsible for most of the end products of glucose and amino acid breakdown in anaerobic metabolism. Pierce et al. (1992) also found elevated levels of alanine in salinity-stressed bivalves. Thus, *V. philippinarum* also seems to obtain energy by anaerobic metabolism. The results obtained evidenced that low salinity appears to increase nucleotides in *V. philippinarum*. Indeed, hypoxanthine, product of adenine/inosine (purine) metabolism and uridine (pyrimidine) increased at lower (7) salinity, compared to control (28). Dykens and Shick (1988) suggested that anoxia tolerance may be achieved by the predominance of xanthine dehydrogenase over xanthine oxidase activity, leading to hypoxanthine accumulation. Uridine increase was also reported to be

related to hypoxia (Harkness and Lund, 1983). Thus, both hypoxanthine and uridine changes suggest that at low salinity *V. philippinarum* experiences anoxic conditions, which may arise from the closure of shell valves as a mechanism to tolerate salinity.

The ability of these animals to sustain prolonged periods of hypoxia is linked with a coordinated suppression of many metabolic processes including enzymes, protein synthesis, and the movement of ions across membranes. Kim et al. (2001) suggested that reduced OCR, due to shell closure in the Manila clam, could function as a way of "energy conservation" to a certain extent by reducing energy expenditure on respiration and activity when exposed to lower salinities. This mechanism can explain why the three species analysed in the present study did not decrease the energy reserves, such as glycogen, when exposed to lower salinities compared to glycogen content found at the "optimal salinity conditions" (between 21 and 28). Besides glycogen be considered the main energy reserve, lipids could also be consider as energy reserve in bivalves, particularly when feed activity is insufficient to maintain their normal metabolism, providing even more energy reserve than glycogen (Beninger and Lucas, 1984). In fact, our results showed a decrease in some fatty acids at lower salinities (0 and 7), in metabolic performance assessed by ^{1}H NMR spectroscopy for *V. philippinarum*. This could be an evidence that lipids were being used as energy reserve when they close their valves to protect themselves of stressful surrounding environment, limiting the filtration rate.

4.5. Oxidative stress

The overproduction of reactive oxygen species (ROS), represent an important challenge to organisms, normally leading to oxidative stress, which will cause different cellular dysfunctions and several adaptive responses (Manduzio et al., 2005; Antunes et al., 2013). Physiologically stressful conditions, such as salinity changes can increase cellular damage in marine invertebrates due to an overproduction of ROS, leading to the oxidation of the lipid membranes (Abele et al. 2002, Abele and Puntarulo (2004). Some studies have concluded that clams are capable to deal with metal contamination, activating defence systems, like antioxidant enzymes, to eliminate the overproduced ROS and, consequently, reducing the oxidative damage, such as decreasing the lipid peroxidation (LPO) levels (Figueira et al., 2012). The present study also showed that at the salinities outside the optimal concentrations for the studied species (between 21 and 28, salinities causing lower mortalities), clams tend to significantly increase lipid peroxidation, which results from the higher ROS production. Significantly higher levels of LPO were observed in *V. corrugata*,

which may reveal a stronger oxidative stress out of the preferred salinity (21). Although the differences along the salinity range in *V. decussata* and *V. philippinarum* were less marked comparatively to *V. corrugata*, it was possible observe the same trend, with salinities 21 and 28 presenting the lowest values of lipid peroxidation. Since LPO has been considered the main cause of the loss of the cell function, when it was in an oxidative stress situation (Storey, 1996; Freitas et al., 2012b; Figueira et al., 2012; Carregosa et al, 2014b), these results suggests lower levels of oxidative stress at salinities between 21 and 28.

The induction of the activity of antioxidant enzymes, like SOD (an enzyme scavenging superoxide anion) and CAT (an enzyme that catalyses the decomposition of H_2O_2), also result from the overproduction of ROS in an oxidative stress situation (Freitas et al., 2012b; Figueira et al., 2012). Thus, lower levels of these two enzymes, represent lower levels of oxidative stress. The present study, revealed a significant decrease of SOD' activity from salinity 14 up to salinity 42 for both *V. philippinarum* and *V. decussata* and up to salinity 28 for *V. corrugata*, fighting against the superoxide anion which indicate an increase of oxidative stress at lower salinities. However, low levels of SOD activity were registered at the lowest salinities (0 and/or 7) for three species. Monari et al. (2005) showed that anoxia, due to shell closure, significantly decreased total haemocyte count as well as SOD activity, in the clam *Chamelea gallina*. The results obtained in the present work are in agreement with such findings since at the lowest tested salinities (0 and 7) the three species presented the lowest activity of SOD due to their tendency to remain their valves closed at low salinities. On the other hand, this decrease in SOD activity may indicate a response to the provoked stress. As Geret et al. (2003) suggested a decrease of antioxidant systems can represent a first response to stress caused by pollutants. In *V. decussata*, the activity of SOD presented lower levels at low salinities (0 and 7) than at higher salinities (35 and 42), which can be explained by the overproduction of ROS. The extreme high amount of ROS interfere with these enzymes, inhibiting them, with consequent increase of oxidative stress, possibly meaning that the cells were in apoptosis. This is an evidence that the tested organisms are experiencing a very high stressful environment, justifying thus the mortality rates at low salinities for the three species.

Several authors have demonstrated the positive relationship between CAT and SOD (Geret et al., 2002; Geret and Bebianno, 2004; Maria and Bebianno, 2011; Wang et al., 2012). The present study further revealed that at salinity 14 the three species increased the activity of the antioxidant enzyme CAT, suggesting a little increase of oxidative stress at this salinity. It was not observed an extreme decrease of the activity of this enzyme at salinities lower than 14, as well as

in the activity of SOD, maybe because H_2O_2 levels were lower that superoxide anion, which allowed the functioning of CAT. Since the SOD could be inactivated at extreme lower salinities, $O_2\bullet^-$ was not reduced to H_2O_2, whose levels were possibly maintained and CAT was able to perform its function. Significantly lower levels of CAT, confirm lower oxidative stress at higher salinities (> 21) comparing with salinities lower than 28. In fact, the increase in the SOD activity contributed to the strong decrease of the LPO levels, especially at salinities 14 and 21. At higher salinities (35 and 42) the activity of these antioxidant enzymes significantly decreased contributing to the increase in the LPO levels. Also Silva et al. (2005) showed that CAT activity in the oyster *Crassostrea rhizophorae* was higher at salinity 9 decreasing with the increase of salinity (15, 25 and 35).

GSTs catalyse the conjugations of glutathione and the result-compounds of cell injury (lipid peroxidation) (Storey, 1996). The obtained results allowed to observe the occurrence of significant differences along the salinity gradient and between the three clam species in relation to GSTs activity. For *V. decussata*, the highest values were found in salinity 21 and, consequently, in the remain salinities the activity of GST was lower. According to Hayes et al. (2004), the inhibition of GST activity may be an indicator of cell damage and toxicity and on opposite its induction can be related to an adaptive response to an altered environment. The behaviour observed for *V. corrugata*, could represent this adaptive response, since the activity of GST was induced outside of salinity 21. Although *V. philippinarum* showed slight differences between salinities under and above 21, this species did not revealed pronounced differences as in two other species, meaning that this enzyme was not highly responsive to salinity alterations in *V. philippinarum*. The present work also evidenced that in *V. decussata* and *V. philippinarum* the higher GSTs activity was accompanied by lower LPO levels, but *V. corrugata* did not show the same behaviour. GSTs are a major Phase II detoxication enzymes found mainly in the cytosol and function as a substrate of antioxidant enzymes to eliminate the reactive oxygen induced by xenobiotic compounds providing protection against electrophiles and products of oxidative stress (Hoarau et al., 2002). Thus, the elevation of GSTs activity between salinities 14 and 28 in *V. decussata* may strongly contributed to the lower LPO levels found at these salinities. Furthermore, the decrease in GSTs activity, accompanied by the decrease in the activity of the antioxidant enzymes SOD and CAT in *V. decusssata*, may be responsible for the increase in the LPO levels at the highest tested salinities (35 and 42). For *V. philippinarum*, the same relationship was suggested in the results obtained for SOD and CAT activity (decreasing from salinities 14 and 21, respectively, up to salinity 42) and LPO levels (increasing at salinities higher than 28). The

increase registered at salinity 28 for LPO levels of *V. corrugata*, may be explained by the decrease observed in activity of CAT. Concentration of MDA (Malondialdehyde) is the reflection of unsaturated fatty acids composition in cell, in proportion with the lipid peroxidation levels (Wheatley, 2000). As LPO is an indicator of oxidative damage, it is possible to suggest that under different conditions from those at salinities 21 and 28, for *V. decussata* and *V. philippinarum* and 21 for *V. corrugata*, the cell damage could occur and tend to worsen whenever the changes were higher. Membrane's function is affected by the presence of lipid hydroperoxides, derived from lipid peroxidation, which consequently, leads to the leak of some ions into the cell, like Ca^{2+}, resulting from the decrease of fluidity of the membrane (Storey, 1996). In fact, the results showed higher amounts of Ca at lower salinities (0 and 7), which may be related with the higher permeability of the membranes.

Glutathione (GSHt), a tripeptide of glutamate, cysteine and glycine, playing as a detoxification agent and it has been considered important in osmotic and oxidative stresses (Figueira et al., 2005; Manduzio et al., 2005). Along the increasing salinity gradient the three studied species tend to decrease the GSHt content, up to salinity 35 for *V. decussata* and *V. philippinarum* and up to 28 for *V. corrugata*. Similar findings were found by Anthony and Patel (2000) who demonstrated that at higher salinities (32) glutathione significantly decreased compared to salinity 16 in the clam species *Anadora granosa*.

Reduced glutathione (GSH) ensures the cellular status redox, working as a cofactor in the response to several toxic compounds, being thus considered an important defence against ROS (Antognelli et al., 2006). In normal redox status of cell, i.e. when the surrounding environment do not present any stress, high levels of intracellular glutathione are registered, which control the effects of reactive oxygen species before the oxidative stress occurs (Storey, 1996). Thus, higher levels of GSH would mean lower levels of oxidative stress. However, GSH presented higher values at salinity 14 for *V. decussata* and *V. philippinarum*, which did not seem to be in agreement with the values of SOD and CAT, for example. This increase may be achieved by the higher activity of SOD, which were decreasing superoxide anion and consequently, the oxidative stress. On the other hand, this increase of GSH in *V. decussata* and *V. philippinarum*, can indicate a deficient performance of glutathione peroxidase (GPx). In other words, the higher levels registered for this antioxidant at salinity 14, could indicate that, despite de oxidative stress was higher than at salinities higher than 14, GSH was not used by GPx. The results of GSH for *V. corrugata* showed that this species was less responsive than the other two species, *V. decussata* and *V. philippinarum*.

The GSH/GSSG ratio is considered to be an index of cellular redox status, indicating the level of oxidative stress in cell (Storey, 1996; Ault and Lawrence, 2003). When the levels of GSSG increase due to higher amount of oxyradicals, this ratio decreases, meaning higher oxidative stress in cells (Storey, 1996). *V. corrugata* showed significantly lower levels of this ratio at salinity 28, probably meaning a higher oxidative stress. The higher value of GSH/GSSG for this species was found at salinity 21, which may indicate lower oxidative stress in cells, being in agreement with other markers, like LPO and GST. The results obtained for GSHt showed a significant increase at salinity 42 for *V. decussata* and *V. philippinarum* and for the ratio GSH/GSSG a slight decrease at the same salinity was observed. This may indicate that GSSG is increased. A similar increase found in GSH, was registered at salinity 14 for both, *V. philippinarum* and *V. decussata* in ratio between reduced and oxidized glutathione. Along the salinity range, the values were maintained around 1, which may indicate that GSH and GSSG were balanced. These results do not comply other markers, such as LPO, SOD and GST which allowed to deduce higher oxidative stress out of salinities 21 and 28.

5. Conclusions

5.1. Conclusions

As bivalves are very important resources for costal populations around the world, in economically terms, this kind of studies (assessing the health of the organisms and the effects of natural stressors) can provide important information about the physiological status of the animals in a climate change scenario and could be a useful tool for assessing the environmental quality to potential bivalve farming areas.

The results herein presented, revealed that *V. corrugata* was the most sensitive clam to salinity changes, with high mortality rates at the lowest (0 and 7) and the highest (35 and 42) salinities tested. On the other hand, *V. decussata* and *V. philippinarum* were able to tolerate all salinities higher than 7 and up to salinity 42. The present work showed that clams experiencing changes in salinity altered their biochemical mechanisms to cope with these stressful conditions. The mortalities registered at low salinities, may indicate that in fact, the clams' metabolic performance is affected and the organisms are not capable to lead with such alterations. The mortality rates, clearly showed that extremely low salinities represent higher stress to this three species studied.

This study also evidences that *V. decussata* and *V. philippinarum* can survive at salinities between 14 and 42 for some days, which is a time interval consistent with changes in salinity caused by heavy rainfall periods, or short episodes of heat.

Surviving organisms can also evidence the effects of exposure to salinities shifts. In fact, organisms showed alterations in the levels of glucose, glycogen and ions with important biological functions such as Ca and Mg. These differences will certainly be reflected in the growth performance of clams and will imply lower productivity in those areas of the ecosystem where sub-optimal salinities for these three species arise repeatedly.

V. philippinarum tolerates a wide range of salinities, through an apparent mechanism of Na regulation. At extreme salinities (0, 7 and 42), the ionic osmoregulation seems to be achieved by Ca increase and shell valve closure, since the metabolites related to the anaerobic metabolism of glucose and amino acid breakdown are accumulated and the metabolites related to hypoxia conditions are increased especially at low salinities. The alteration of the metabolite profile, as viewed by NMR spectroscopy, seems to be a consequence of hypoxia and not of osmotic adjustment since the accumulation of compatible osmotic compounds, such as betaine and taurine, decreased relatively to the salinity 28 in *V. philippinarum*. The overall profile changes

means that the NMR-visible profile is sensitive to salinity and, hence, further studies should be carried out.

The results give evidence that clams mobilize stored energy (glycogen) and may also use protein breakdown to cope with extreme salinity levels. The valve closure not only induces hypoxia but also reduces food intake as well and in a limit situation, the osmotic imbalance may increase, leading to swelling and cellular rupture. These effects may explain the mortality rates of *V. decussata* at salinity 0, of *V. philippinarum* and *V. corrugata* at 0 and 7, observed in the present work.

Also, LPO, SOD and GST showed to be very useful biomarkers to salinity stress, with a strong correlation with the increasing salinity gradient. The clams used in the present study demonstrated that the optimal salinity range varied between 21 and 28, where these species presented lower LPO levels and therefore lower mortality.

Studies of the environmental stress in marine organisms are particularly important, specially to assess the health condition of those species cultivated for human consumption. For this, the assessment of stress responses related with oxidative stress in marine organisms, furnish important information useful to examination of the environmental quality. The results here presented and discussed, with bivalve species from the Ria de Aveiro, indicate that salinity fluctuations can cause substantial changes in their antioxidant defence systems and oxidative injury levels.

The biomarkers tested in this study, allow to infer that although tested organisms are considered euryhaline, they are not capable to adapt to extremely low salinities. This is a particularly interesting finding, since the comparison of these three clam species allowed to conclude that, despite they are living together in same areas, they have distinct responses to salinity alterations. This information is of major importance for the management of this resource and should be taken into account when defining areas and intensity of capture.

The invasive species used in this study, *V. philippinarum*, showed to be less tolerant to changes in salinity than *V. decussata*, one of the native species studied. Comparing these two species, *V. philippinarum* presented higher mortality rate and lower values of almost all of the physiological and biochemical parameters tested. In a scenario of great salinity changes in areas where these species live together, might mean a higher problem for *V. philippinarum*, than for *V. decussata*, especially when the changes represent a decrease in salinity. The other native species under analysis, *V. corrugata*, showed to have very different responses, compared with *V.*

decussata and *V. philippinarum*. Thus, this kind of changes will certainly have impacts on the occurrence of *V. corrugata*, since the invasive species presented higher survival capacity under salinity alterations. In fact, local fishermen testify the difficulty of finding this species in Ria de Aveiro. Although one of the native species (*V. decussata*) showed higher capacity to deal with these alterations, comparatively to the exotic species (*V. philippinarum*), they continue to live in simpatry in same areas, with higher abundance of the invasive species, according to the local fishermen. These facts indicate that changes in salinity have different impacts in native and invasive species, getting worse the competition in the field for those with higher difficulties to deal with these alterations, as *V. corrugata*.

5.2. Future considerations

Studies related with salinity fluctuations in marine bivalves should be performed in the future approaching metabolic alterations by NMR spectroscopy with enough samples to use multivariate analytical tools to statistically evaluate the alterations registered comparing to biological variability, since there is not any studies related with this issue.

Regarding to GC x GC – ToFMS data, these should be processed and analysed in order to understand the alterations in terms of volatile metabolites, which also was not studied yet, subjecting these three species to a natural stressor, as salinity fluctuations.

It is clear that salinity, is not the only stress that influences the biological functioning of the tested organisms and others living in the same or similar ecosystems. Thus, field studies shoud be performed, especially in farming zones, assessing salinity and other environmental conditions with the aim to found the ideal conditions to better health of the organisms. Also, a combination of natural stressors and anthropogenic pollution requires further research as it results in several adverse effects.

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7. Annexes

7.1. Papers on peer reviewed journals (Science Citation Index® (Thompson ISI))

Carregosa V., Figueira E., Gil A.M., Pereira S., Pinto J., Soares A.M.V.M, Freitas R., 2014. Tolerance of *Venerupis philippinarum* to salinity: osmotic and metabolic aspects. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 171, 36-43. **IF 2012: 2.167, Percentile 84%**

http://dx.doi.org/ 10.1016/j.cbpa.2014.02.009

Carregosa V., Velez C., Pires A., A.M.V.M Soares, Figueira E., Freitas R. (2014). Physiological and biochemical responses of the Polychaete Diopatra neapolitana to organic matter enrichment. Aquatic Toxicology 155, 32-42. **IF 2012: 3.730, Percentile 97%**

http://dx.doi.org/ 10.1016/j.aquatox.2014.05.029

Carregosa V., Velez C., Soares A.M.V.M., Figueira E., Freitas R., 2014. Physiological and biochemical responses of three Veneridae clams exposed to salinity changes. Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology, *In Press*. **IF 2012: 2.069, Percentile 82%**

http://dx.doi.org/ 10.1016/j.cbpb.2014.08.001

7.2. Participation in International Meetings

7.2.1. Oral Communications

Carregosa V., Figueira E., Gil A., Freitas R., 2013. Tolerance and response of native (*Ruditapes decussatus* and *Venerupis pullastra*) and invasive (*Ruditapes philippinarum*) clams to salinity changes. PRIMO'17, Pollutant responses in marine organisms. 5-8 May 2013, Faro, Portugal.

7.2.2. Posters

Carregosa V., Figueira E., Soares A. M. V. M., Freitas R., 2014. Salinity variation: effects on two clam species, *Venerupis decussata* and *Venerupis philippinarum*. ICEH CISA, International Congress on Environmental Health. 24-26 September 2014, Porto, Portugal. *Accepted*

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TOLERANCE AND RESPONSE OF NATIVE (*RUDITAPES DECUSSATUS* AND *VENERUPIS PULLASTRA*) AND INVASIVE (*RUDITAPES PHILIPPINARUM*) CLAMS TO SALINITY CHANGES

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Unlike the concern that has been growing in relation to the impacts of contamination in marine benthic populations, the responses of aquatic organisms to natural alterations, namely changes in salinity, have received little attention. In fact, salinity is one of the dominant environmental factors that most affect marine bivalves, limiting their spatial distribution in the environment. The ebb and flood of the tide combined with fresh water inputs, from rivers or heavy rainy periods, and extreme dry seasons can dramatically alter the salinity of water, causing alterations in the benthic populations, namely intertidal bivalves. Furthermore, salinity of a given environment will restrict the spatial distribution of the species, which is especially important when assessing the spread of an invasive species into a new environment. In order to understand how native (*Ruditapes decussatus* and *Venerupis pullastra*) and invasive (*R. philippinarum*) clam species cope with salinity changes, biochemical and metabolomic patterns were investigated. The results obtained showed that *Ruditapes* species presented high mortality at lower salinities (0, 7) but tolerate high salinities (35, 42). On the other hand, *V. pullastra* presented high mortality rates both at low (0, 7) and high salinities (35, 42). The quantification of Na and K content revealed that, along the salinity gradient, *R. decussatus* was the species with higher ability to maintain the ionic homeostasis. The biochemical parameters also showed that *R. decussatus* was the clam that best cope with salinity changes and *V. pullastra* was the most sensitive. Metabolomic patterns were obtained by ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy of clam extracts. Multivariate analysis of the NMR spectra enabled metabolite changes to be observed in relation to clam exposure to different salinity concentrations. The relevance of these metabolite change s, in relation to salinity response and resistance metabolic signatures, is discussed.