Universidade de Aveiro Departamento de Biologia
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Gonçalo Alexandre Jacinto Domingues

A pressão hidrostática na toxicidade de cádmio em *Palaemon varians*

Hydrostatic pressure on cadmium toxicity in *Palaemon varians*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia Aplicada, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro, Investigadora Auxiliar do Departamento de Biologia (dbio) e Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro, e co-orientação do Doutor Ricardo Jorge Guerra Calado, Investigador Principal do Departamento de Biologia (dbio) e Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro.

Dedico este trabalho aos meus avós, Raul e Deolinda, que sempre vou lembrar com saudade

o júri

Presidente	Prof. Doutora Ana Maria de Jesus Rodrigues Professora Auxiliar do Departamento de Biologia & CESAM da Universidade de Aveiro
Arguente	Doutor Carlos Alexandre Sarabando Gravato Investigador Principal do Departamento de Biologia & CESAM da Universidade de Aveiro
Orientador	Doutora Susana Patrícia Mendes Loureiro Investigadora Auxiliar do Departamento de Biologia & CESAM da Universidade de Aveiro
Co-orientador	Doutor Ricardo Jorge Guerra Calado Investigador Principal do Departamento de Biologia & CESAM da Universidade de Aveiro

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palavras-chave Biomarcadores, condições abióticas, oceano profundo, temperatura, mineração marinha

resumo

Recentemente têm sido identificados alguns perigos relativamente ao ambiente no mar profundo, como atividades de mineração marinha, e que exigem uma melhor compreensão sobre as características únicas destes ecossistemas. A alta pressão hidrostática (HHP) é uma das condições abióticas mais importantes para a vida neste ambiente; no entanto os seus efeitos nos processos e estruturas dos organismos são pouco compreendidos. Enquanto a realização de testes com organismos do fundo do mar é muito desafiadora e cara, avaliar os efeitos da HHP utilizando espécies de águas rasas parece ser a melhor abordagem. O camarão *Palaemon varians* é uma espécie com estreita relação filogenética com algumas espécies-chave do mar profundo. No presente estudo, *P. varians* foi exposto a uma gama de diferentes HHP (10, 20, 30 e 40 MPa) e a duas temperaturas diferentes (4 ºC e 20 ºC), e diversos marcadores bioquímicos (as actividades de AChE, GST e CAT e os níveis de LPO) foram medidos a fim de avaliar a utilização desta espécie como modelo para estudos a desenvolver em laboratório. Todos os animais expostos a HHP acima de 20 MPa morreram durante a exposição. Embora nenhuma interação nos marcadores bioquímicos medidos tenha sido encontrada entre HHP e temperatura, os animais expostos a 20 MPa e 4 ° C também morreram durante a exposição. Os níveis de LPO e a atividade da GST aumentaram a temperaturas baixas, e por isso a utilização desta espécie a essas temperaturas requer uma investigação mais aprofundada. Devido à ausência de resposta de todos

os biomarcadores medidos, esta espécie parece ser adequado para ensaios laboratoriais com pressões de 10 MPa. A pressões de 20 MPa, foram observadas algumas alteração nos níveis de LPO e AChE após 8 horas de recuperação da exposição sendo por isso necessário aprofundar o estudo destes efeitos. Além disso, a mortalidade registada a temperaturas baixas faz com que o uso desta espécie em tais pressões seja limitada. Na segunda parte deste trabalho, a fim de avaliar o efeito da pressão sobre a sensibilidade desta espécie para a exposição a cádmio, *P. varians* foi exposto a várias concentrações de cádmio durante 96 h, juntamente com diferentes regimes de pressão: pressão atmosférica; simultaneamente, durante 8 horas, com uma pressão de 20 MPa; e pré-expostos a 20 MPa durante 8 h. Os valores de LC50 calculados foram semelhantes para os diferentes regimes de pressão, o que indica que a esta pressão não são observados efeitos sobre a toxicidade do cádmio para *P. varians*. Embora esta seja uma avaliação importante dos efeitos tóxicos do cádmio a pressões elevadas, são necessários estudos adicionais sobre outras espécies e outros produtos químicos que também são propensos a aparecer no fundo do mar.

keywords Biomarkers; abiotic conditions, deep-ocean, temperature, marine mining.

abstract

The recent rising threats to the deep-sea, as deep-sea mining, require a better understanding about the unique characteristics of these ecosystems. High hydrostatic pressure (HHP) is one of the most important abiotic conditions to life in this environment; however its effects on processes and structures of the organisms are very poorly understood. While experimentation with organisms from the deep-sea is very challenging and expensive, assessing the effects of HHP using shallow-water species seem to be best approach. The caridean shrimp *Palaemon varians* is a species with close phylogenetic relation with some key species in the deep-sea. In the present study, *P. varians* was exposed to a range of different HHP (10, 20, 30 and 40 MPa) and to two different temperatures (4 ºC and 20 ºC), and biochemical markers (AChE, GST, CAT activities and LPO level) were measured in order to evaluate the use of this species as a model for future laboratory research. All animals died during exposure at HHP above 20 MPa. While no interaction between HHP and temperature on biochemical markers were found, animals at 20 MPa and 4 ºC also died during the exposure. LPO levels and GST activity increased at low temperatures, and the use of this species at such temperatures requires further investigation. Due to the absence of response of all biomarkers measured, this species seems to be suitable for laboratory assays with pressures of 10 MPa. At 20 MPa, some small alteration in LPO and AChE levels after 8 hours post exposure may require further investigation. Also, the mortality

registered at low temperatures makes the use of this species at such pressures limited. In order to evaluate the effect of pressure on the sensitivity of this species to cadmium, *P. varians* were exposed to several concentration of cadmium during 96h, along with different pressure regimes: at atmospheric pressure; simultaneously, for 8h, with a pressure of 20 MPa; and pre-exposed to 20 MPa for 8 h. Only slightly differences were found between the calculated LC50 for the different pressure regimes, indicating that at this pressure no effects on the toxicity of cadmium to *P. varians* are observed. Although this is an important input regarding hazard assessment at higher pressures, additional studies are needed regarding other species and other chemicals that are also prone to appear in the deep sea.

INDEX

LIST OF ACRONYMS AND ABBREVIATIONS

HHP	High Hydrostatic Pressure
HC	Hyperbaric Chamber
GST	Glutathione S-transferase
LPO	Lipid Peroxidation
CAT	Catalase
AChE	Acetycholinesterase
ROS	Reactive Oxygen Species
PMS	Post-mitochondrial supernatant

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PART 1 GENERAL INTRODUCTION

1.1 THE DEEP-SEA

The deep-sea covers an area of 360 x 10^6 Km^2, representing around 90% of the world oceans and around 50% of the Earth's surface. With an average depth of 3800 m and a volume of 1368 x 10^6 Km^3, is by far the largest ecosystem on earth (Ramirez-Llodra et al. 2011). The beginning of the deep-sea is marked by a clear transition between the shallow-water fauna of the continental shelf to the deep-water fauna at about 200 m depth (Sanders et al. 1965). Because of this 200 m water layer, all existing and needed information for a comprehensive understanding of structure and functions of the deep-sea depends on modern and expensive technology, being often a very slow process. Due to these difficulties, in 1844, Edward Forbes, after a period of extensive sampling in the Mediterranean in which no sign of life was observed, proposed the Azoic Theory, stating that little or no life could be found bellow 600 m (Anderson and Rice 2006). However, during the late 19th century, the necessity of laying telegraphic cables in the ocean bottom pushed by the industrial revolution led to a series of important expeditions, especially the worldwide cruise HMS Challenger (1872-1876) that found deep-sea organisms in all the samples recovered from different abyssal plains (Glover et al. 2010; Ramirez-Llodra et al. 2011; Thistle 2003). After this expedition, a growing interest for the deep-sea led to a period of intense exploration, culminating in the Galathea expedition which proved that animals could be found at all depths (Ramirez-Llodra et al. 2011). After this period of intense exploration the deep sea was viewed as a quiescent environment, a vast undisturbed and invariant dark sedimented plain with low biodiversity and no seasonality (Tyler 1988). Nowadays, this idea has been strongly rejected. Newly developed technology, such as cameras and remote operated vehicles, allow having a broader vision of the deep-sea environment. It is now known that benthic storms are an example of a dynamic process that can re-suspend sediment in hours or the sink of a

whale carcass can provide food for a variety of specialized organisms, changing the community composition of a given site for years (Gage and Tyler 1992). Extensive sampling using corers, trawls and sledges has shown a much higher biodiversity that originality thought and recent studies put the biodiversity of several taxonomic groups present in the deep-sea above several hotspots of diversity, such as the coral reefs and rain forests (McClain and Schlacher 2015). The extreme conditions of the deep-sea seem to be a counterpoint to such high biodiversity, and the reasons behind it are not fully understood (López-García et al. 2001; McClain and Schlacher 2015).

To explain the adaptation of deep-sea organisms, the importance of this ecosystem in the global environment and the threats and ways to protect it, further investigation is needed in order to understand the condition that rule life in the deep-waters.

1.1.1 Characterization and abiotic conditions

The deep-sea has very unique set of conditions that makes life-traits very different from any other ecosystem.

Temperature is relatively stable in deep-water systems when compared to other environments. In shallow-water, temperature changes with seasonality and latitude, varying between almost 0 ºC in high latitudes to ≈27 ºC in low latitudes (Thistle 2003). However, temperature drops with increased depth, reaching to -1 to 4 ºC in abyssal plains, while variation due to latitude and seasonality can be considered almost negligible (Gage and Tyler 1992; Mantyla and Reid 1983). Hydrothermal vents are the exception; in those the temperatures can reach up to 400 ºC, heating the water and constituting "warm oasis" in the frigid waters of the deep-sea (Lutz and Kennish 1993).

Pressure is probably the most predictable condition in marine ecosystems. It has a linear relationship with depth and increases about 1 MPa for every 100 m of water. In the deepest parts of ocean, like in the Mariana Trench, it can reach almost 110 MPa. These pressures can crush any terrestrial and shallow-water organism, although many animals and bacteria have adapted to live in such conditions (Somero 1992).

Slow descent surface water keeps the salinity of the deep-sea at 35, very close to those observed in marine shallow-water (Thistle 2003). Few exceptions occur, for example in the Mediterranean Sea, where the deep-water have a considerably higher salinity of about 38 (Tsimplis and Baker 2000).

No photosynthetic useful light reaches depths below 250 m, even in clear water. Therefore photosynthetic processes do not occur in the deep-ocean. Chemosynthetic processes are considered the basis of food chains in some isolated communities, like hydrothermal-vents and cold seeps, but in most of the abyssal plains, food is imported from shallow-water and land communities (Thistle 2003). The occasional sinking of a large carcass (e.g., whales) constitute an enormous input of food that can last for decades, while the continuous sinking of small particles as crustacean molts and faecal pellets are also very important in terms of nutrient re-cycling (Gage 2003). Despite this absence of photosynthesis, the deep-sea water is kept oxygen saturated by the descent of surface waters which form currents across the ocean floor (Thistle 2003).

Most of the deep-sea is covered by small particulate sediment, mostly originated by the weathering of rocks on land and by particles produced by planktonic organisms such as the silica shells from diatoms (Thiel 2003). In some places, bare rock is also present, which constitutes an important fixation point for several organisms (Glover et al. 2010).

The abiotic conditions in the deep ocean are so different from other habitats that it is often considered an extreme environment. But the definition for “extreme environments” is related to the distance from the “optimum conditions”. Therefore it usually reflects a terrestrial or shallow water organism “point of view”. For both deep-sea and shallow water species, the homeostatic effort required to maintain internal conditions when exposed to environmental conditions beyond optimum is increased and poses a challenge to their survival (Brown and Thatje 2014; Treude et al. 2002).

1.1.2 Biological effects of pressure

Any physiological or biochemical process that involve a change in the system’s volume is affected by changes in pressure (Somero 1992). The effect of high hydrostatic

pressure (HHP) in a system can be analyzed by the follow equation (Gross and Jaenicke 1994; Macdonald 1997):

$$\left(\frac{\partial \ln k}{\partial p}\right)_T = \frac{-\Delta V}{RT}$$

Where k is the reaction rate coefficient, p the pressure, T the absolute temperature, R the gas constant and ΔV is the difference between final and initial volume of the reactants. At constant temperature, the reaction rate coefficient (k), which determines the direction in which a chemical reaction shifts, is affected by the pressure and by changes in volume. Hence, the dependence of k on pressure can be written:

$$\left(\frac{\partial \ln k}{\partial p}\right)_T = \frac{-\Delta V^{\neq}}{RT}$$

Where $\Delta V^{\neq}$ represents the activation volume of a reaction. Thus any reaction with a decrease (negative) change in volume will be accelerated and any reaction with an increase (positive) in volume will be slowed down. In table 1 are listed the volume change associated with some important biochemical reaction that are affected by pressure. For example, the formation of ionic interaction in the water molecule occurs associated with an increase in the volume. Hence an increase in pressure favors the dissociation of the ionic interaction and an increase of the free $[H]^+$. This leads to a decrease in the pH of pure water by 0.3 when pressure is increased from 0.1 MPa to 100 MPa (Gross and Jaenicke 1994).

Several proteins and biomolecules are affected by pressure. In the table 1 we can see that the denaturation of myoglobin is induced by an increase in the pressure. This dissociation by pressure occurs in most of the multisubunit proteins, with sensitivity to pressure being as higher as the rate of the volume change associated to its dissociation (Cioni and Strambini 1996; Somero 1992). Even when partial, this denaturation exposes nonpolar groups of the protein to water causing its inactivation (Balny et al. 2002). Diverse processes in the organism can be affected by this sensitivity of proteins to HHP, among others, enzymes catalysis, protein mediated signalization and transport of biomolecules (Chong et al. 1985; Ruan and Weber 1989; Siebenaller et al. 1991). The cell

morphology can be also changed due to the dissociation of tubulin and actin caused by HHP (Brown and Thatje 2014; Swezey and Somero 1985).

Table 1 Reaction volume associated with some biochemical important reactions at 25 ºC (adapted from Gross and Jaenicke 1994)

Reaction	Example	ΔV (ml/mol)
Protonation	$H^+ + OH^- \rightarrow H_20$	+21.3
	Protein-COO^- + H^+ → protein-COOH	+10
Hydrogen-bond formation	Poly(A+U) (helix formation) in DNA denaturation	+1
Hydrophobic hydration	$(CH_4)_{hexane} \rightarrow (CH_4)_{water}$	-22.7
Hydration of polar groups	n-propanol → $(\text{n-propanol})_{water}$	-4.5
Protein dissociation/association	Tubulin propagation; ΔV per subunit	+90
	Ribosome association (*E. coli* 70S)	≥200
Protein denaturation	Myoglobin (pH 5, 20 ºC)	-98

Almost all studied processes played by the cell membrane were reported to be affected by changes in pressure (Macdonald 1997; Pradillon and Gaill 2007; Somero 1992). This reduction in the membrane functionality is explained by the reduction in the fluidity of the phospholipid bilayer (Sébert et al. 1997). Changes in the fluidity highly affect the transmission potential in neural cells of organisms of shallow waters (Oliphant et al. 2011; Wann and Macdonald 1980). These malfunctions cause the high pressure neurological syndrome in terrestrial and shallow water organism's when exposed from slightly too severe changes in pressure. Virtually, all neural and muscular functions on the organism are affected by this change in pressure causing tremors, lack of movement coordination and other neurological malfunctions (Oliphant et al. 2011).

Transmembranar ion flux across the membrane is drastically affected by changes in pressure (Macdonald 1997). For example, Roer and Shelton 1981 reported a reduction of sodium uptake by crayfish of 80% when exposed to a pressure of 50 to 100 atm. Several

proteins on the membrane that play key roles on the osmoregulation are also highly sensitive to pressure changes. One of the most important proteins in the osmoregulation process, the Na^+ K^+ - adenosine thriphosphatase (Na^+ - K^+ ATPase), was shown to be very sensitive to the change in the fluidity of the surrounding cell membrane being highly inhibited by HHP (Chong et al. 1985).

With all these effects on the function of proteins and membranes, it is not surprising that complex processes, such as the transcription and transduction of DNA into mRNA and proteins, involving several proteins and different cell compartments, are shown to be highly affected (e.g. Hardon and Albright 1974; Hildbrand and Pollard 1972). For example, Hildebrand and Pollard (1972) showed that an increase in pressure of about 640 atm caused 95% inhibition of the synthesis of polyphenylalanine.

However, it is important to point out that volume change can occur not only in molecules directly involved in the processes itself, but also by the pressure ability to change the density and viscosity of the involving aqueous environment. Therefore, some processes are disrupted not by a shift in the protein responsible for it, but by changes in fluidity of the surrounding membrane (Chong et al. 1985; Somero 1992). Hence it is important to look at the whole system and not only a process or a chemical reaction alone.

1.1.3 High pressure and low temperature, a biological bottleneck

Despite the mechanism of action being different, studies have shown that both high pressures and low temperature have similar effect on biological systems (Pradillon and Gaill 2007; Ravaux et al. 2013). While pressure effects result only from volume changes, temperature affects both volume and energy in the system (Pradillon and Gaill 2007).

For the cellular membrane, both low temperature and high pressure cause an increase in the phospholipid bilayer order and also a decrease in fluidity (Macdonald and Cossins 1984). A temperature increase of 2.8 ºC has been reported to reverse the effect on the fluidity of the membrane caused by an increase in pressure of 10 MPa (Brown and

Thatje 2014; De Smedt 1979 et al.). The reduction in membrane functionality and fluidity and of the proteins associated caused by both high pressure and low temperature difficult the transport of ions and biomolecules from and within the cell, leading to a higher demand of energy to keep the internal homeostasis (Brown and Thatje 2015; Somero 1992). Survival under such conditions is time limited, because the higher demand of oxygen to keep the increased mitochondrial activity is not always matched by an increase on the ventilation and circulation capacity on the organism (Brown and Thatje 2014; Cottin et al. 2012). Despite adaption being needed in other compartments and organs, the capacity of an organism to keep the aerobic function, appear to be the key for survival at low temperatures (Sommer and Pörtner 2002).

Like the exposure to high pressures, low temperature also causes denaturation on the structure of diverse proteins, putting more pressure on the mitochondria's capacity to deliver energy for the production of chaperones to counteract this process (Brown and Thatje 2015; Privalov 1990). This denaturation is related with the unfolding on the structure caused by the hydration of nonpolar groups by water (Privalov 1990). This unfolding open the normally compact structure of the protein, exposing other nonpolar groups to water and causing its inactivation (Balny et al. 2002; Privalov 1990). This relation between the water content of the body tissues and the resistance to exposures to pressure and temperature outside of the normal ranges can be observed in tardigrades. Tardigrades are small terrestrial microorganisms that when exposed to extremely dry environments become immobile and shrink, losing almost all the water in their body and stopping most of the biological processes (Seki and Toyoshima 1998). This state allows them, among other things, surviving to extremely low temperatures and high pressures. While on his normal state they only support pressures until 200 MPa and temperatures of about -196 ºC, at the dehydrated state they can survive pressures above 600 MPa and temperatures as low as -253 ºC for long periods of time (Hengherr et al. 2009; Seki and Toyoshima 1998).

Given the fact that both high-pressure and low temperature promote similar effects in the organisms structures and metabolism, it is not surprising to observe a similar response in organism exposed to low temperatures or HHP. Several cold shock response

proteins usually found in response to cold temperatures can also be found in organisms exposed to high pressures (Wemekamp-Kamphuis et al. 2002). Wemekamp-Kamphuis et al. (2002) reported that the pathogen *Listeria monocytogenes* increases his resistance to high pressure from 200 MPa to 300 MPa when prior incubated at 10 ºC and then transferred to 37 ºC, when compared to those always kept at 37 ºC. Avrova (1984) reported an increased presence of mono-unsaturated fatty acids and low saturated fatty acids (a well-known adaptation to cold temperatures) on the deep-living fish *Antimora rostrata* when compared to his shallow-water cousins adapted only to cold temperatures.

The synergistically effect of cold temperatures and high pressures indicate that these abiotic conditions can be the major barrier that shallow water living organism had to overcome in the colonization of the deep-sea (Brown and Thatje 2015; Cottin et al. 2012; Mestre et al. 2014). The colonization of deep-sea may therefore occurred during the Mesozoic and early Cenozoic periods, when the ocean was colder and the difference in temperatures between upper and bottom of the water column were smaller (Cottin et al. 2012). Evolution of deep-sea life at high altitudes, where the differences in the temperature of the upper layers of water and the water below is also a possibility (Thatje et al. 2005).

Some taxonomical groups appear to be originated from the deep-sea. For example, Lindner et al. (2008) found that stylasterid corals, the second most diverse group of extant hard corals, appeared in shallow water environment through three distinct invasions from the deep-sea. Despite this and other examples, the invasions of deep-sea animals to shallow-water environment appear to be relatively few (Brown and Thatje 2014). This can be explained by the low ability of deep-sea organisms to compete with shallow-water species by resources. De Long and Yayanos (1986) reported a lower capacity of transport of glucose in deep-sea bacteria when exposed to shallow-water pressures. Several adaptation allow the deep-sea organisms to survive under-pressure, however those same adaptation cripple their ability to compete in shallow-water environments. Deep-living species not only are adapted to the high pressure, but they also perform better under it and sometimes even require these conditions to survive and perform basic life functions like reproduction and growing (Harper et al. 1987; Yayanos

1986). Therefore organisms that colonized the deep-sea had adapted to low temperatures, HHP and total absence of light (Somero 1992).

1.1.4 Mining activity and other treats

The vastness and remoteness of the deep-sea have always kept it away from public consciousness. Due to its size and the covering water layer, it is perceived as safe from anthropogenic pressures that endanger almost all the other habitats. When dumping waste on land is considered unethically, unaesthetically or inconvenient, it is common sinking it on the sea. This “away from sight, away from mind” idea led to the presence of diverse materials in the bottom of the sea. Despite several conventions that legislate and prohibit the dumping of litter from ships, being the International Convention for the Prevention of Pollution from Ships (MARPOL) the most important (www.imo.org), United Nations Environment Programme (UNEP) estimates that about 6.4 million tons are still being dumped in the sea every year from vessels only (UNEP, 2009; Strand et al. 2015). Despite the vastness of the deep-sea, this dump of materials can have a huge impact on the ecosystem. For example, in the age of steam power, transatlantic ships used to dump clinker to the sea. In many spots of the deep-sea, usually associated with old common routes, this residuum of burnt coal can form more than 50% of the hard substratum with unknown consequences (Kidd and Hugget 1980).

Like the visible waste, several contaminants such as synthetic organochlorine compounds (Iwata et al. 1993), chlorofluorocarbons (Doney and Bullister 1992), pharmaceuticals, sewage and radioactive materials (Ramirez-Llodra et al. 2011) also reach the deep-sea. The downwelling of dense water masses is the main entry of these contaminants (Thiel 2003), which can also sink alone adsorbed to small particles (Mason and Fitzgerald 1992; Thiel 2003).

Another source of pressure that has not been taken into account for several years is the effect of global changes in the deep-sea. The deep-sea is not immune to the fast global changes that are presently occurring, due to anthropogenic activity. Climate change is predicted to imply acidification of the seawater, temperature changes,

expansion of hypoxic zones, destabilization of the slopes and changes in the nutrient cycle (Glover et al. 2010; Ramirez-Llodra et al. 2011). Unlike other more studied ecosystems, effects in the deep-sea are very hard to predict and evaluate, thus very few data is available, and changes in the community composition are very hard to evaluate (Glover et al. 2010).

Recently, a new threat to the deep-sea ecosystem has been also described. The idea of exploring the mineral resources of the deep-sea is not recent, as the first dredge of minerals with commercial interest was performed in 1873 (Thiel 2003; Thiel et al. 2013). Between the years of 1977-81, very intensive exploitation was performed, but the mining project did not advance due the value loss of metals in the market (Thiel et al. 2013). Several impact studies were carried out, however the lack of technology and the market conditions were not favorable and several projects were delayed. In the recent years however, the rising price of the minerals in the global markets and the development of new advanced technologies that permit cheaper and effective ways of exploration had caused an exponentially increase in the interest for this resources (Thiel et al. 2013). Three forms of mineral resources are the mainly target for mining exploitation: manganese nodules, cobalt rich crusts and massive polymetallic sulphide deposits (Ramirez-Llodra et al. 2011). The problem with these resources is that most of them are localized in hydrothermal vents and on other spots of high biodiversity and their removal is expected to cause a high impact on the communities (Dover 2014). The full extensions of impacts are far from being completely understood, and the recovery rates of communities can take several years (Mestre et al. 2014). Due the high value of the resources several projects have already started (Nautilus and Coffey 2008; Lamarche and Clark 2012; ISA 2011). Mining cause several direct impacts on the previously undisturbed deep-sea communities, like the loss of habitat by removing the hard subtract which is essential for organisms fixation (Coffey and Nautilus 2008). The minerals are mechanically removed and then dragged to the surface and filtered to recover particles >8 µm, with the remaining mud being then returned to the sea, and forming a plume that buries sessile organisms from areas of several Km^2 (Coffey and Nautilus 2008; Dover 2014; Montagna et al. 2013). These processes can re-suspend a lot of metals in sea water, some

of them with a recognized higher toxicity, that are then transported for several kilometers by the bottom currents of the deep-sea (Mestre et al. 2005; ISA 2011; Thiel 2003). In the impact studies conducted in the Red Sea, between 1977 and 1981, several trace metals, such as cadmium, nickel, mercury, lead, and cobalt were released to the water (Thiel 2003). The first toxicological tests with mine tailings, demonstrated a threshold level of approximately 10 mg solids per liter. Considering the typical recommended safety factor of 0.1% to exclude chronic effects, content above 0.01 mg L^{-1} would be unacceptable (Thiel 2003). Concentrations above this levels are however likely to occur in the plume associated with the removal of minerals (ISA 2011; Mestre et al. 2014).

Originated by either inland sources, such as industry and sewage, by vessels or from mining, man-made contaminants are today ubiquitous in the deep-ocean (Thiel 2003). Several studies have shown that different abiotic factors can change the toxicity of many compounds (see Holmstrup et al. 2010, for review), therefore it is important to understand the role of the unique environmental conditions in the deep-sea on the toxicity of the compounds. Providing this data can be essential to the elaboration of complete impact studies of the mining in the deep-sea and to the well informed take of conservation and management actions. In addition, finding good test species to assess hazard in the deep-sea environment can be challenging. Not only all the systems apparatus for capturing, transport and maintenance of the organisms in laboratory are really expensive, but also it is changeling to capture a sufficient number of animals to allow a representative amount of replicates required for a robust laboratory assay (Mestre et al. 2014; Ravaux et al. 2013; Treude et al. 2002). Finding suitable shallow-water organisms that can be used as model for this type of assays is of crucial importance.

1.2 *PALAEMON VARIANS*

1.2.1 Taxonomy and morphology

The crustacean *Palaemon varians* (Leach 1814), referred in old literature as *Palaemonetes varians* (Grave and Ashelby 2013), commonly called Atlantic Ditch shrimp, is a shrimp from the order Decapoda and infraorder Caridea.

Like other caridean shrimps, the body of *P. varians* is elongated with a very hydrodynamic form which allows fast locomotion while swimming. His body can be divided in two major parts, the cephalothorax, constituted by the head, two stalked eyes, thorax, and the abdomen. The body is covered by an exoskeleton, a thin, transparent and very flexible organ that confers support and protection.

The cephalothorax is protected by a calcified carapace and contains almost all the shrimp vital organs such as the brain, the stomach and the heart (fig. 1). The abdomen is almost totally constituted by muscle, adapted for their swimming activity, and also holds the ventral nerve cord and the hindgut (Bauer 2004).

Figure 1 Adult specimen of *Palaemon varians*.

Like most decapods, *P. varians* has sexual dimorphism. The second pair of pleopods is modified in the males and used for mating. Females do not present this modification and usually have a bigger body compared to the males. During the reproductive season, that takes place from late may until early september, females have a different molt called breeding dress that is characterized by the presence of extra setae, with the purpose of

egg bearing (Bouchon 1991; Frelon et al. 1993). This molt allows the fixation and incubation of embryos in the abdomen, for approximately 30 days, and posterior release.

1.2.2 Habitat, ecology and relevance

P. varians can be generally found in inland coastal ponds, like estuaries and salt marshes with abundant vegetation that provides shelter and food. Normally it is found in turbid and shallow waters, with salinities varying from very low (but never in freshwater) to salinities above the salinity of the seawater (>35). Besides salinity, several studies have found that this species has a high capacity to adapt to different water temperatures, oxygen concentrations, and even pressure (although it is never found on deep waters), characteristics that allow *P. varians* to have a wide range of distribution in Western Europe (Healey 1997; Oliphant et al. 2011; Palma et al. 2009). It can be found from the coasts of Norway in the North Sea and in Baltic Sea, down to the coasts of southern Europe and north-western Africa until Tunisia in the Mediterranean basin (Aguzzi et al. 2005; Cuesta et al. 2012; Gonzales-Ortégon and Cuesta 2006; Healey 1997).

P. varians is very important in the transference of nutrients across various tropic levels. Escaravage and Castel (1990) reported an increase in abundance of nematodes, insect larvae's and copepods when *P. varians* is present in the community. Due to its detritivorous habits, it promotes the mechanical breakdown of organic matter, such as macroalgae and plants, into small particles causing their resuspension (along with bacteria and other organisms) in the water column, making them available to the microfauna (Escaravage and Castel 1990). Despite its diet consisting mainly of small algae and plant parts, *P. varians* is also an active predator of microfauna, such as nematodes and mosquito larvae, across the entire water column (Aguzzi et al. 2005; Escaravage and Castel 1990).

Being one of the few native species of shrimps from the Iberian Peninsula with commercial value, *P. varians* is commonly captured in Portugal and Spain (Palma et al. 2009; Pinto 2010). Due to its high economic value and seasonal fluctuation in terms of

availability, *P. varians* is produced in intensive/semi-intensive systems, like abandoned salt ponds in southern Spain and Portugal (Aguzzi 2005; Encarnação 2013; Pinto 2010). According to the records of FAO (Food and Agriculture Organization of the United Nations), *P. varians* is the only species of crustacea produced in Portugal. The production started in 2005 and in 2013 it was registered the highest production with a total of about 5 tons produced. Despite being a small shrimp, *P. varians* is used in human consumption and as live bait in fishery (Palma et al. 2009). *P. varians* is also used as live feed in aquaculture, with special importance to the production of commercial species with higher demand on live feeds (Domingues et al. 2003; Domingues et al. 2009; Palma et al. 2008; Sykes et al. 2006). Domingues et al. 2003 and Sykes et al. 2006 reported good growth rates of cuttlefish *Sepia officinalis* when fed with *P. varians*. Palma et al. 2008 reported the same results for the seahorse *Hippocampus guttulatus* when feed with a mixture of *P. varians* and mysids shrimps.

Although not so extensively used as other species, *P. varians* can be considered a good test-species to be used in ecotoxicological assays (e.g. Boisson et al. 2003) due to its relatively short life cycle, ease to handle, maintain and reproduce in laboratory. Also, this species has a lower sensitivity to changes in abiotic conditions, such as salinity (from 0 to salinities above the seawater level), temperature (between 0 ºC and 33 ºC, Oliphant et al. 2011) and hypoxia (Nielsen and Hangerman 1998).

1.3 CADMIUM

1.3.1 Sources and uses

Cadmium (Cd), as others trace metals, occurs naturally in the environment, in geological ores and in background levels in the marine environment (OSPAR 2010). Its distribution is ubiquitous trough the environment, being present in water, soil and air (Flick et al. 1971). In freshwater cadmium is normally present in its ionic form, Cd^{2+}, very toxic to organisms. In saltwater, however, it forms complexes with chloride, reducing its

toxicity (Simpson 1981). When compared with other trace metals, cadmium and his compounds are relatively more soluble in water and in organic solvents (Nordic Council of Ministers 2003). Despite the existence of cadmium ores (greenockite), their abundance is not sufficient to justify mining and cadmium for industrial purposes is produced as a by-product from mining, smelting and refining of other minerals, primarily sulphide ores of zinc (ECB 2007).

Cadmium is used in NiCd batteries, as intermediate and catalyst for electroplating, as a pigment in paint, as stabilizer for plastic, in photographical processes and in dyes (ECB 2007; OSPAR 2010). However, due to its toxicity new legislation has been adopted in the last decades, restricting cadmium use and making its price drop; nowadays its production is more dependent on zinc refinement than on market demand (Nordic Council of Ministers 2003).

In the European Union, cadmium use has been reduced in the last decades and banned in several products as cosmetics, packing and toys. Its use is regulated by the Annex XVII of Regulation (EC) 1907/2006 of December 2006 on registration, evaluation, authorization and restriction of chemicals (REACH), which has been applied in all member states since the 1st June 2009.

The most important sources of cadmium and its compounds to the environment are emissions from combustion processes in industry and power plants, and in fertilizers, where cadmium is present as an impurity in the phosphate minerals used during production (ECB 2007; OSPAR 2010). Despite the lower toxicity of cadmium in its metallic form, when in the environment it transforms into soluble ions Cd^{2+} which are much more toxic/hazardous (ECB 2007; Sunda et al. 1978).

1.3.2 Biological effects

Cadmium was included in 2013 in the Priority List of Hazardous Substances and considered the seventh more hazardous substance for human health by the Agency for Toxic Substances & Disease Registry. For the aquatic ecosystem, Abel (1989) had listed cadmium as the second most toxic metal to aquatic life, only surpassed by mercury.

Cadmium is harmful to cells primarily due to his affinity to sulfur, present in several amino acids, binding to the proteins and preventing them to interpret their metabolic role (Rainbow 2002; Sarkar et al. 2013). Moreover, aquatic invertebrates, algae, fish and other species, as humans, bioaccumulate cadmium in their tissues and contrary to the majority of trace metals, evidences of possible cadmium biomagnification were reported in a microbiological food web and in freshwater food webs (Croteau et al 2005; Gray 2002; Rainbow 2002; Werlin et al. 2010). All crustaceans, as *P. varians*, are not able to regulate cadmium concentration in their body, being accumulated in the cytosol in a detoxified form, primarily bound to methallothioneins, without potential excretion (Engel and Fowler 1979; Rainbow 2007). This accumulation of cadmium in the cells can induce multiple effects, like inhibition of several transport mechanisms in the cell membrane, changing the cellular and plasma concentration of other metals, such as calcium and sodium, whose concentrations are essential for the normal function of the cell (Dhavale et al. 1988; Verbost et al. 1989). As other toxic compounds, accumulation of cadmium is also linked with overproduction of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and hydroxyl radicals (•OH) (McGeer et al. 2011). Despite the short half-life, ROS can cause multiple damages in several structures of the cell, such as proteins, lipids and DNA (Gobe and Crane 2010). The cell has several enzymes which catalyze reaction to control the presence of ROS, such as catalase that converts H_2O_2 into water and oxygen (Patra et al. 2011). Cadmium binds to the complex III of the mitochondrial electron transport chain, causing an increased formation of ROS, and disrupting the balance between antioxidant enzymes and the amount of ROS (Chen et al. 2011; McGeer et al. 2011; Wang et al. 2004).

Some marine diatoms, like *Thalassiossira weissflogii*, in the absence of zinc, can produce a specific carbonic anhydrase that replaces zinc by cadmium in its active site. This enzyme catalyzes the conversion between carbonic acid and carbon dioxide, essential for the algae metabolism (Lane & Morel 2000; Lane et al. 2005). Due to the low concentration of zinc in the ocean and the high density of microalgae in the aquatic environment when compared to plants, despite its toxicity, cadmium may play an essential role in the global cycle of carbon (Lane et al. 2005). However, no other biological function is known for cadmium and still being generally considered a non-essential metal for organisms.

1.4 BIOMARKERS

Biomarkers are tools which allow the evaluation of an exposure to a toxic agent, the extent of the response and that can also be used to predict a likely response (Timbrell 1998). Biomarkers can be divided in 3 types: i) biomarkers of exposure are the actual chemical, or molecules that results from his metabolization than can be measured in the body, and/or in its excretions. This type of biomarkers is particularly useful in biomonitoring assays (e.g. Eason and O'Halloran 2003). ii) Biomarkers of effect are the quantifiable changes caused by a chemical compound or abiotic factor, and are important in lab-based bioassays because they can provide information about the mechanism of toxicity of a certain compound (e.g. Oliveira et al. 2013). iii) Biomarkers of susceptibility are genetic factors that turn some organisms more sensitive than others to a certain compound.

Several enzymes are involved in the response to a toxic compound. The super family of soluble GSTs is a ubiquitous superfamily of multi-functional enzymes that occur mainly in the cytosol (Hayes et al. 2005); These group of enzymes neutralize a broad range of toxic compounds and endogenous metabolic by-products in the detoxification process, conjugating electrophilic xenobiotics, such as chemical carcinogens and environmental pollutants, with glutathione (Hayes et al. 2005; LaCourse et al. 2009).

The exposure to several toxic compounds causes the production of reactive oxygen species (ROS), which, although with short half-life, are highly reactive and have the capacity to damage several structures of the cell (Winston and Di Giulio 1991). Lipid peroxidation (LPO) is one of the major effects of oxidative damage caused by ROS, and leads to loss of function of several cell structures, and its increase is a sign of a disequilibrium in the mechanism of the cell to prevent ROS induced damage (Ruas et al. 2008; Santi et al. 2011). Catalase is one of those enzymes involved in the protection of the cell against ROS by catalyzing the reduction of H_2O_2 into oxygen and water (Thannickeal and Fanburg 2000). The activity of catalase and other enzymes such as superoxide dismutase (which reduce O_2^- to H_2O_2) is very important in the prevention of damages caused by ROS and play crucial roles in maintaining the cell homeostasis (Diaz-Albiter et al. 2011; Kopecka-Pilarczyk and Coimbra 2010).

Acetylcholinesterase (AChE) is an enzyme that catalyzes the hydrolysis of acetylcholine in the cholinergic synapses in the central nervous system and in neuromuscular synapses, and its activity terminates the synaptic transmission (Santi et al. 2011). It was extensively used as a biomarker indicator of exposure to organophosphorus and carbamate compounds (Colovic et al. 2013), although nowadays it is widely known that this enzyme is inhibited by other chemicals such as metals (e.g. Frasco et al. 2005). The inhibition of AChE causes accumulation of acetylcholine that leads to hyperstimulation of nicotinic and muscarinic receptors, disrupting the neurotransmission (Colovic et al. 2013).

Extensive work has been performed to address effects of toxic compounds using several biomarkers (e.g. Dewes et al. 2005; Ferreira et al. 2015; Oliveira et al. 2012). However, several biomarkers have been reported to be affected by environmental conditions such as temperature (Vinagre et al. 2014), HHP (Low and Somero 1975) and salinity (Rodrigues et al. 2012), among others.

1.5 RATIONALE AND AIMS

Collection, transport and stocking of deep-sea organisms is a challenging and expensive task and toxicity testing using them as models imply a lot of technical constraints. Therefore, it should be possible to employ shallow-water species as models to evaluate the potencial effects of HHP. Caridean shrimps from the species *P. varians* appear to be suitable candidates as they are phylogenetically related to important and emblematic deep-sea species (Mestre et al. 2014). Additionally, previous studies indicate *P. varians* as a species with a high capacity to adapt to different environmental conditions, such as pressure (Cottin et al. 2012; New et al. 2014; Oliphant et al. 2011). This species is also very common and easy to capture and manage in the laboratory.

This thesis is divided in two major parts. With the objective of determining how HHP affected *P. varians* and how suitable this species is to laboratory testing in pressure assays, several biomarkers were measured upon exposure to two different temperatures and five different pressure regimes. The biomarkers used were the activity of AChE, CAT and GST and LPO.

In the second part of this work, the influence of HHP on the toxicity of Cd was studied. Despite being far from human presence, contaminants released in the environment by anthropogenic activity can easily reach the deep sea. Also, deep-sea mining will lead to presence of metals, such as cadmium, in concentrations exceeding the safety levels currently considered. For this purpose, *P. varians* was exposed to a range of cadmium concentrations, at different HHP regimes, and survival was recorded. The suitability of this species for this kind of assays was discussed.

In the two parts of this work, a pressure system was assembled to simulate HHP closer to those of the deep-sea. All procedures and details are included in this thesis in Annex 1.

The main objectives of this work were: i) to evaluate the effect of HHP and low temperature in *P. varians*; and ii) how HHP affects the sensitivity of *P. varians* to Cd.

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PART 2: HIGH HYDROSTATIC PRESSURE (HHP) AND PHYSIOLOGICAL CHANGES IN *PALAEMON VARIANS*: A BIOCHEMICAL APPROACH

2.1 ABSTRACT

The recent rising threats to the deep-sea require a better understanding about the unique characteristics of these ecosystems. High hydrostatic pressure (HHP) is one of the most important abiotic conditions to life in this environment; however its effects on processes and structures of the organisms are very poorly understood. While experimentation with organisms from the deep-sea is very challenging and expensive, assessing the effects of HHP using species with strong phylogenetic relation with deep-sea organisms seem to be the best approach. The caridean shrimp *Palaemon varians* is a species with close phylogenetic relation with some key species in the deep-sea. In the present study, *P. varians* was exposed to a range of different HHP (10, 20, 30 and 40 MPa) and to two different temperatures (4 ºC and 20 ºC), and biochemical biomarkers (AChE, GST, CAT activities and LPO level) were measured in order to evaluate the use of this species as a model for future laboratory research in HHP assays. All animals exposed at HHP above 20 MPa died during exposure. While no interaction in biochemical biomarkers was found between HHP and temperature, animals exposed at 20 MPa and 4 ºC also died during the exposure. LPO levels and GST activity increased at low temperatures, and the use of this species at such temperatures requires further investigation. Due to the absence of response of all the biomarkers measured, this species seems to be suitable for laboratory assays with pressures of 10 MPa. At 20 MPa, some alterations in LPO level and AChE activity after 8 hours post exposure may require further investigation. Also, the

mortality registered at low temperatures makes the use of this species at such pressures limited.

2.2 INTRODUCTION

The deep sea covers more than half of the earth's surface. Starting in the edge of the continental shelf (Sanders et al. 1965; Thistle 2003), the deep ocean is protected from the surface conditions by a 200 m water layer. Because of this water layer, the deep-sea presents completely different conditions than those observed in shallow-water ecosystems, as very high pressures and lower temperatures. These extreme conditions had posed a challenge for its exploration and therefore most of the processes that occur are unknown. However, recent advances in the modern industry and science allow an exploration of deep-sea resources, like minerals and fish, which was impossible a few decades ago, disturbing these previous relatively stable ecosystems (Ramirez-Llodra et al. 2011). In addition, the growth of the world population had led to a mass production of several materials which often found their final destination in the deep sea. While visible waste tends to settle in the ocean floor, chemical compounds like metals and pesticides are transported during long-term periods by the same global currents that supply oxygen to the deep-sea ecosystems (Thiel 2003). A model study performed by Stemmler and Lammel (2013) suggested that this horizontal and vertical transport can eventually led to a re-rise of these contaminants to shallow-water, in important fisheries sites, disturbing alongside shallow-water ecosystems. Moreover, several of these contaminants have the ability to bioaccumulate, disrupting the trophic chains, and changing the deep-sea ecosystem, and all the services provided (Thiel 2003; Thurber et al. 2014). Therefore, developing techniques for monitoring the state of the deep-sea ecosystems are of crucial importance in the near future, being the first step the detailed knowledge and characterization of its good status.

Several biomarkers have been used as indicators of cellular stress due to environmental contamination in several environmental compartments. Several

environmental conditions, as temperature (Vinagre et al. 2014) or salinity (Rodrigues et al. 2012), have also been reported to affect the levels of several biomarkers; therefore the influence of those factors must be taken into account in pollution monitoring programs, to avoid biased results. One of the abiotic factors that should be considered when evaluating chemicals effects in the deep sea is HHP, whose effects are still poorly understood. Kopecka-Pilarczyk and Coimbra (2010a) found no (to few) effects of HHP on several biomarkers tested in the silver eel *Anguilla Anguilla,* a species in which part of its life cycle occurs in the deep-sea. Hochachka (1974) reported adaptation of acetylcholinesterase (AChE) extracted from the brain of deep-sea fish, which appear to have a smaller binding region in the anionic site, as high pressure favors no-hydrophobic bindings; therefore effects of HHP in AChE can be expected in shallow-water organisms. Both HHP and low temperatures have been reported to have several similar effects in organisms (eg. Oliphant et al. 2011), however no studies evaluating the effects of both conditions in biochemical biomarkers have been performed.

Due to the constraints regarding the evaluation of hazardous effects in deep-sea ecosystems, one proposed way is the use of species that can tolerate or can adapt to high pressures (Mestre al. 2014). The brackish shrimp *P. varians* has a close phylogenetic relation with some deep-sea shrimps such as *Rimicaris exoculata*, *Chorocaris chacei* and *Mirocaris fortunata* (Gonzalez-Rey et al. 2008; Mestre et al. 2014; Olhiphant et al. 2011). Despite *P. varians* being only found in shallow-water ecosystems, it has the capacity to support a wide range of temperatures (between 0 ºC and 33 ºC) and HHP (up to 30 MPa) (Oliphant et al. 2011).

The objective of this work was to evaluate if *P. varians* can tolerate HHP and low temperatures and therefore be used as a test-species for hazardous assessment simulating the deep-sea particular conditions. For that the effects of HHP and/or low temperature were measured in biochemical biomarkers. The following rationale was followed: low to no changes in metabolism caused by pressure can indicate *P. varians* to be a good model for deep sea ecotoxicological testing. The biomarkers tested were catalase (CAT), glutathione S-transferase (GST), lipid peroxidation (LPO) and acetycholinesterase (AChE).

2.3 MATERIALS AND METHODS

2.3.1 Organisms' collection and acclimation conditions

Adult grass shrimps (*P. varians*) were collected from a salt marsh (N 40º38'39"; W 8º39'51") at the Ria de Aveiro estuary in Aveiro, Portugal in January 2015. Shrimps were collected with a net and afterwards transported to the laboratory at the University of Aveiro, in 20 L buckets containing water from the collection point (±12 ºC and salinity 17). Animals with a carapace length between 4.7 and 5.2 mm were selected and acclimatized during 48 hours to the laboratory conditions. After this period, animals were transferred in a density inferior of 3 shrimps per liter, to 20 L tanks containing clean synthetic sea water at a salinity of 35 prepared with Tropic Marin® Sea Salt and freshwater purified by a reverse osmosis unit. Tanks were then kept at 20 ºC in a temperature controlled room and 16 h: 8 h light : dark cycle for a minimum of 10 days prior to testing. Once a day, 20% water was renewed in each tank. Ammonia levels and pH were monitored using API® colorimetric tests once a day. Constant aeration was provided by bubble stones and oxygen monitored using a probe Oxi 330i/set WTW®. Food was provided twice a day consisting of decapsulated Artemia cysts (*Artemia salina*) and seaweed food pellets ®Hikari Marine A. After one hour the reminiscent food was removed from the system.

2.3.2 Pressure system

The hydrostatic pressure system used in this work is described in annex 2.

2.3.3 Pressure exposure

Animals were not fed 24 h prior and during the tests. For the pressure exposure, two animals were placed in 140 mL plastic vessels filled with synthetic saltwater at a salinity of 35. All vessels were filled up till the top and closed with a lid. Four replicates/vessels were placed in per hyperbaric chamber. Each chamber was then filled with water at 20 ºC, closed and set to a desired pressure: 0, 10, 20, 30 and 40 MPa. Two

chambers were used per pressure, being one left at 20 ºC and the other at 4 ºC. The experimental control was additionally set to simulate optimal conditions, using the same exposure medium, two animals were place in four vessels with 250 mL synthetic salt water, at 20 ºC with aeration and light.

After eight hours, all chambers were depressurized for 5 minutes, and one shrimp from each replicate treatment was transferred to a 2 mL cryotube and deep frozen in liquid nitrogen. The remaining individuals from each replicate were then transferred to the control conditions (250 mL of aerated clean medium with aeration and light, at 20 ºC). For shrimps exposed in the chambers at 4 ºC, the water in the control conditions was pre-cooled to the same temperature prior to the transfer of the shrimp to avoid a thermal shock, and then left in the room at 20 ºC. Organisms were left to recover another eight hours in optimum conditions, and were then deep frozen as above. Exposure conditions as pH, temperature and dissolved oxygen were also measured at the beginning and at the end of each exposure. All animals were stored at -80 ºC until biochemical analyses.

2.3.4 Chemicals

Acetylthiocholine iodide, 5,5'-Dithiobis(2-nitrobenzoic acid), bovine γ-globulin, reduced L-Gluthathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), trichloroacetic acid (TCA), thiobarbituric acid (TBA), 2,6-Di-tert-butyl-4-methylphenol (BHT), diethylenetriaminepentaacetic acid (DTPA) and hydrogen peroxide (H_2O_2) were obtained from Sigma-Aldrich Europe (Netherlands). All the other chemicals used were purchased from Merck (Germany).

2.3.5 Biomarkers analysis

A) Sample preparation:

Prior to homogenization, shrimps' exoskeletons and locomotors appendices were removed with the help of a tweezers and a scalpel under a stereo microscope.

The eyes plus stalks were then separated from the rest of the body and homogenized in 0.5 mL of ice-cold k-phosphate buffer (pH 7.2, 0.1 M) and then

centrifuged at 3824 g during 3 minutes at 4 ºC. The resulting supernatant was used to measure AChE. Regarding the previous results by Veldesema-Currie (1973) eyes and stalks were used to measure AChE activity as these organs alone represent a high percentage of the total acetylcholinesterase activity (14±3.3%).

The remaining tissues were rapidly weighted and homogenized in k-phosphate buffer (pH 7.4, 0.1 M). A 150 µL aliquot of the homogenate was separated and 2.5 µL of a solution of BHT at 4% was added to prevent the formation of lipid oxidation artifacts (Torres et al. 2002) and the aliquot stored at -80 ºC up to a maximum of 24 h before lipid peroxidation assessment. The remaining homogenate was centrifuged at 10 000 g during 20 minutes at 4 ºC to obtain post-mitochondrial supernatant (PMS). An aliquot of the PMS was then diluted in k-phosphate buffer (0.05 M, pH 7.0) for CAT determination and a second aliquot diluted in k-phosphate buffer (0.1 M, pH 6.5) for GST determination. Both aliquots were stored up to a maximum of 1 week at -80 ºC prior to analysis.

All measurements were conducted using a Thermo Scientific Multiskan® Spectrum microplate reader.

B) Lipid peroxidation determination

LPO was determined by measuring the thiobarbituric acid reactive substances (namely malondialdehyde, a product of lipid peroxidation) as described by Ohkawa (1979) and Bird and Draper (1984). Briefly, to each sample aliquot prepared 500 µL of 12% TCA, 400 µL of Tris-HCL (60 mM) with DTPA (0.1 mM) and 500 µL of 0.73% TBA were added to the homogenate. The samples were then incubated during 60 minutes at 100 ºC and centrifuged at 14000 g during 5 minutes, at 25 ºC. From the supernatant, 300 µL of sample was then transferred to the microplate and read at 535 nm. LPO was calculated using an extinction coefficient of 1.53×10^5 M^{-1} cm^{-1} for the formation of TBARS and expressed in nmol TBARS g^{-1} of tissue.

C) Protein determination

Protein quantification of all samples was performed according to Bradford (1976) methodology as adapted to microplate by Guilhermino et al. (1996). In summary, 10 µL of

the sample was incubated with 250 µL of Bradford reagent for 15 minutes. Absorbance was then read at 595 nm. Bovine γ-globulin was used as protein standard.

D) Acetylcholinesterase activity determination

AChE activity was determined by Elman's technique (Elman et al. 1961) adapted to microplate by Guilhermino et al. (1996). The sample was diluted 10 times, and the protein in the samples used was 0,251±0.067 mg/ml. Briefly, for four replicates of 50 µl sample added, 250 µl of reaction mixture (30 ml of k- phosphate buffer 0.1 M, pH 7.2, 0.2 ml of acetylthiocholine iodide 0.075 M and 1 ml of DTNB 10 mM) was added and the absorbance at 414 nm was measured at 10, 15 and 20 minutes. After reading absorbance, the AChE activity was calculated using an extinction coefficient for DTNB of 13.6 M^{-1} cm^{-1}. For normalization, the result was divided by the total amount of total protein and the enzymatic activity of AChE was expressed in nmol of substrate hydrolyzed per minute per mg of protein.

E) Catalase activity

CAT activity was measured as described by Clairborne (1985) adapted to microplate. Briefly, for each PMS aliquot previously prepared (total protein of 0.787±0.332), 15 µL of sample was added in quadruplicate in a microplate and 135 µL of K-phosphate buffer (pH 7.0, 0.05 M) and 150 µL of H_2O_2 were added. H_2O_2 consumption was then measured at 240 nm during 1 minute. After reading the absorbance, CAT activity was calculated using a molar extinction coefficient for the conversion of H_2O_2 of 50 M^{-1} cm^{-1}. For normalization purpose, the result was divided by the total amount of protein and catalase activity was expressed in µmol min^{-1} mg^{-1} of protein.

F) GST activity

GST activity was determined by measuring the conjugation of reduced glutathione with CDNB at 340 nm as described by Habit et al. (1974) adapted to microplate. In brief, 200 µL of this reaction solution (14.85 mL of K-phosphate buffer 0.1M, pH= 6.5, 0.45 mL

of CDNB 10 mM and 2.7 mL of GSH) was added to 100 µL of PMS previously prepared (with a total protein of 0.630±0.183 mg/mL of PMS) and measured at 340 nm during 5 minutes. After reading the absorbance, GST activity was calculated using a molar extinction coefficient for CDNB of 9.5 mM^{-1} cm^{-1}. For normalization purpose, the result was divided by the total amount of protein and the GST activity was expressed in nmol min^{-1} mg^{-1} of protein.

2.3.6 Statistical analysis

A Shapiro-Wilk test was performed in order to evaluate the normality distribution of data. Appropriate data transformations were performed when necessary to perform further analysis. A two-way analysis of variance (ANOVA) was performed to compare pressure (atmospheric and 10 MPa) and temperature (4 ºC and 20 ºC) (α=0.05). A one way-ANOVA was performed to evaluate organisms exposed to different pressures (atmospheric, 10 MPa and 20 MPa) at 20 ºC. Post hoc, multiple comparisons of factors were carried out using Holm-Sidák method. Different t-tests were performed in all the different treatments between value after the 8 hours exposure and after 8 hours recovery. All statistics were performed using SigmaPlot version 11.0.

2.4 Results

All animals exposed to 30 and 40 MPa of HHP died during the exposure. In addition, at 4 ºC the animals exposed to 20 MPa of HHP also died. All the other animals used during the test survived both the exposure and after 8 hours of recovery. When removed from the chambers, animals exposed to 20 MPa showed high activity when compared to all other treatments, but with some erratic movements. After five minutes in the recovery vessel, animals start presenting an apparently normal behavior, resting in the bottom of the vessel without loss of equilibrium.

No statistical difference were found ($p>0.05$) between animals at optimal conditions (20 ºC, aeration and light) and those inside chambers, with no pressure set, at 20 ºC (fig. 2).

No interaction between pressure and temperature was found in all molecular biomarkers measured, both after exposure and after the 8 hours recovery ($p>0.05$).

LPO levels (fig. 3) after the 8 hours exposure at 20 ºC with atmospheric pressure and at 10 MPa (258.06±154.82 nmol TBARS g^{-1} wt^{-1} and 160.28±65.09 nmol TBARS g^{-1} wt^{-1}) were significantly lower than animals exposed to 4 ºC at the same pressures (437.91±157.55 nmol TBARS g^{-1} wt^{-1} and 333.02±95.03 nmol TBARS g^{-1} wt^{-1} respectively) (Two way ANOVA, $F_{20,23}= 8.694$; $p< 0.01$). However, after 8 hours recovery, no differences due temperature or pressure were observed (Two way ANOVA, $F_{20,23} = 0.303$; $p>0.05$). For animals exposed at 20 MPa, no differences in LPO levels were observed after the 8 hours exposure. However, after 8 hours recovery, LPO level of organisms exposed at 20 MPa (468.24±63.8 nmol TBARS g^{-1} wt^{-1}) were significantly higher (One Way ANOVA $F_{13,15}=4.547$; $p<0.05$) than those kept at atmospheric pressure (252.05±126.14 nmol TBARS g^{-1} wt^{-1}).

Lower temperature also caused a significant higher activity of GST (fig. 4) (Two way ANOVA, $F_{24,27}= 6.868$; $p<0.05$), which does not occur after 8 recovery (Two way ANOVA, $F_{20,23}=0.047$; $p>0.05$). In organisms exposed to 10 MPa at 20 ºC, GST activity were significantly higher after 8 hours recovery (12,32±0.73 nmol min^{-1} mg^{-1} protein) when compared to those measured in shrimps which were not allowed to recover (8.51±1.68 nmol min^{-1} mg^{-1} protein) ($t=-3.817$; $p<0.01$).

No significant difference was observed in AChE levels (fig. 5) after the 8 hours exposure to pressure and temperature. However, after 8 hours recovery, animals previous exposed to 20 MPa at 20 ºC had a significant (1 way ANOVA, $F_{13,15} =5,141$; $p<0.05$) higher activity of AChE (91.57±33.78 nmol min^{-1} mg^{-1} protein) when compared to shrimps exposed to atmospheric pressure (51.55±17.56 nmol min^{-1} mg^{-1} protein) and 10 MPa (54.96±7.94 nmol min^{-1} mg^{-1} protein).

No significant differences were found in catalase activity (fig. 6) in all the conditions measured ($p<0.05$). In some animals, it was not possible to detect any enzymatic activity.

Oxygen and pH levels measured during the assay are presented in annex 1.

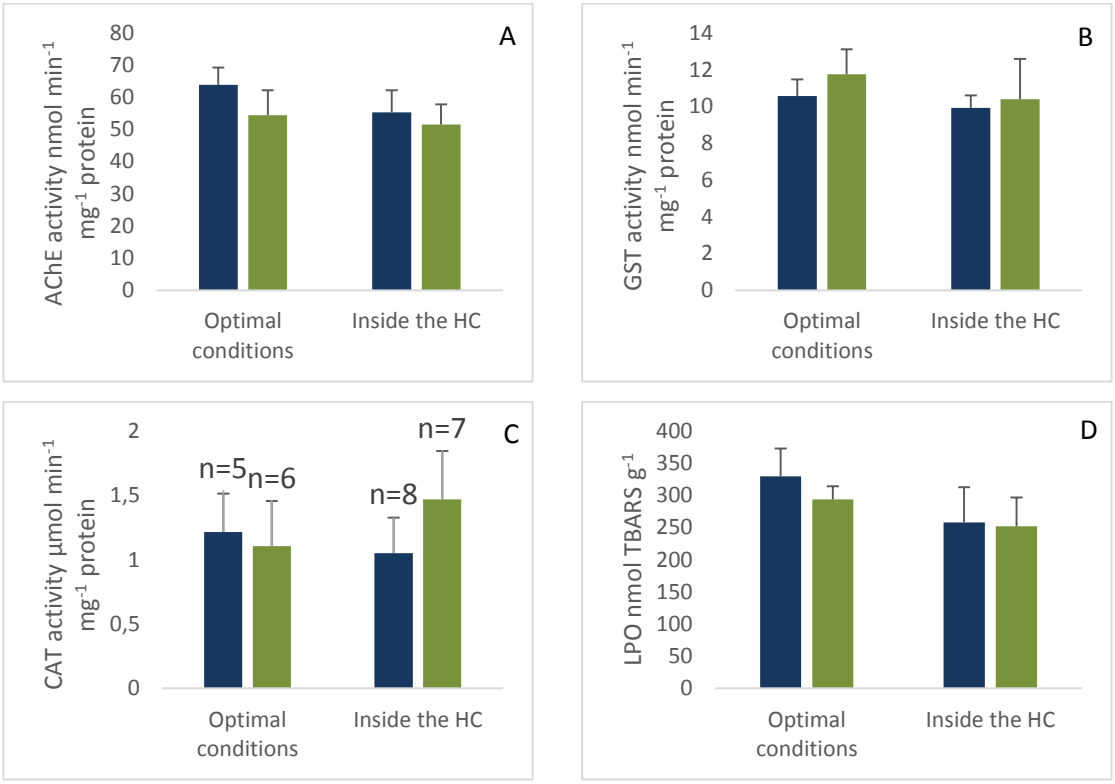

Figure 2 AChE (A), GST (B), CAT (C) activities and LPO levels (D) measured at optimal conditions (20 ºC, aeration and light) and in *Palaemon varians* that were inside the hyperbaric chamber (20 ºC, no aeration and no light) with standard error bars. The blue bars represent values in animals frozen immediately after the exposure and green bars represent animals that were allowed to recovery during 8 hours. In the graphic (C), the n represents the number of animas in which catalase activity was possible to assess.

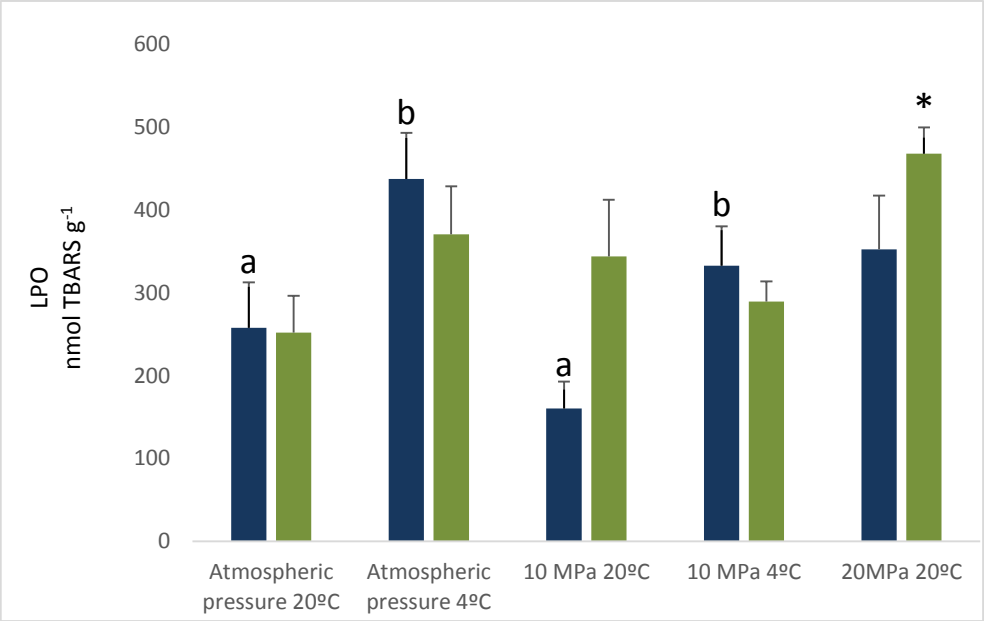

Figure 3 LPO levels (nmol TBARS g^{-1} tissue) of *Palaemon varians* exposed during 8 hours to different pressures (atmospheric pressure, 10 and 20 MPa) and temperature (20 ºC and 4 ºC) with standard error bars. The blue bars represent LPO values in animals frozen immediately after the exposure and green bars represent animals that were allowed to recovery during 8 hours. Different letters represent a statistical difference between organisms exposed at 20 ºC and 4 ºC. Statistical difference from the control is marked by an *.

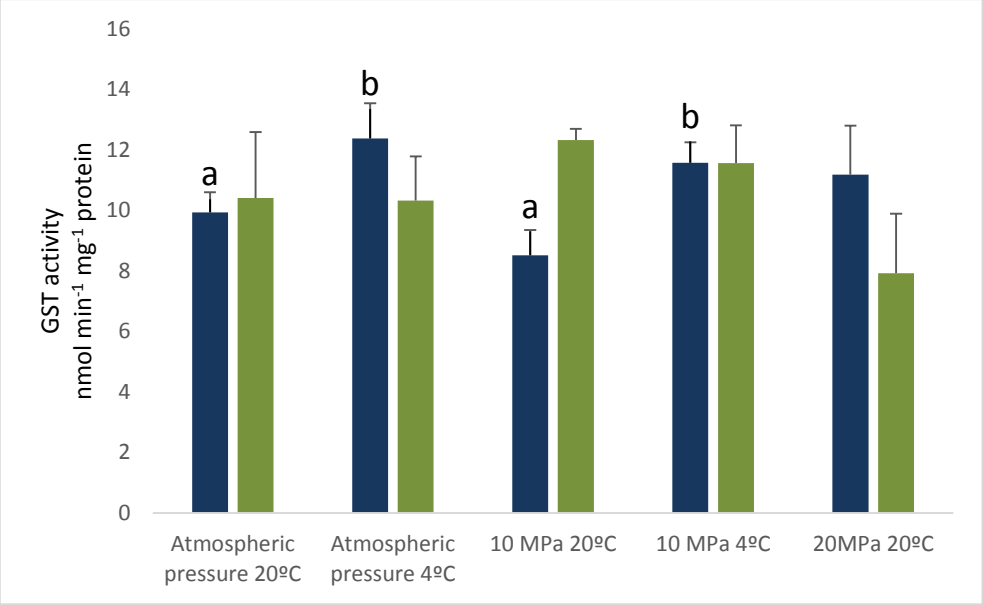

Figure 4 GST activity (nmol min^{-1} mg^{-1} protein) measured in *Palaemon varians* exposed during 8 hours to different pressures (atmospheric pressure, 10 and 20 MPa) and temperature (20 ºC and 4 ºC) with standard error bars. The blue bars represent values in animals frozen immediately after the exposure and green bars represent animals that were allowed to recovery during 8 hours. Different letters represent a statistical difference between organisms exposed at 20 ºC and 4 ºC.

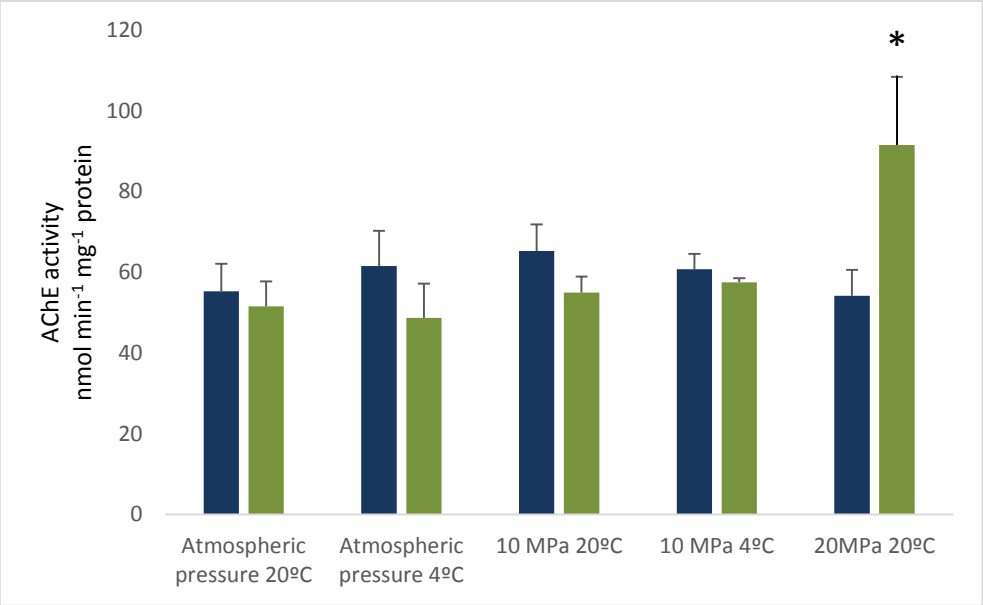

Figure 5 AChE activity (nmol min^{-1} mg^{-1} protein) measured in *Palaemon varians* exposed during 8 hours to different pressures (atmospheric pressure, 10 and 20 MPa) and temperature (20 ºC and 4 ºC) with standard error bars. The blue bars represent values in animals frozen immediately after the exposure and green bars represent animals that were allowed to recovery during 8 hours. Statistical difference is marked by a *.

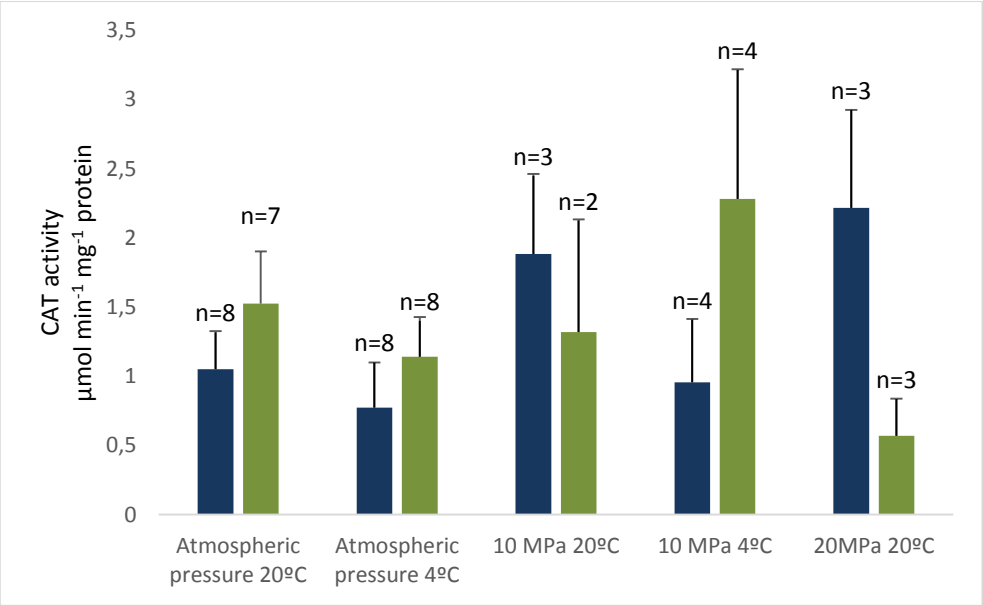

Figure 6 CAT activity (µmol min^{-1} mg^{-1} protein) measured in *Palaemon varians* exposed during 8 hours to different pressures (atmospheric pressure, 10 and 20 MPa) and temperature (20 ºC and 4 ºC) with standard error bars. The blue bars represent values in animals frozen immediately after the exposure and green bars represent animals that were allowed to recovery during 8 hours. The number of animals in which CAT activity was possible to assess is shown above each bar.

2.5 DISCUSSION

In the deep-ocean pressure can reach levels above 100 MPa (Glover et al. 2010) and several mining resources can be found at depths higher than 2000 m (Thiel et al. 2015). Due to the mortality observed in the present work, it is not recommended to use *P. varians* in assays with the objective to simulate pressures at depths beyond 2000 m, where HHP raises above 20 MPa.

While all the eight shrimps survived while exposed to 20 MPa at 20 ºC, all animals exposed to the same pressure at 4 ºC died, showing a synergistic effect between these temperature and pressure. Oliphant et al. (2011) and Cottin et al. (2012) observed a higher disruption of *P. varians* behavior caused by HHP at lower temperatures and a higher capacity to keep a normal function at HHP exposed to higher temperatures. Compared to these studies, *P. varians* showed less resistance to HHP in this work, thus all the shrimps exposed to 30 MPa died during the experiment. This fact may have occurred because the system used in the present study does not allow a slower and controlled acclimation to HHP, similar to those used in other works. New et al. (2014) reported an increase in HHP tolerance of *P. varians* when animals where acclimated to low temperatures or HHP.

Contrary to the results observed in mortality at higher pressures and to several other processes and structures that are synergistically affected by HHP and low temperature (Pradillion and Gail 2007; Somero et al. 1992), no interaction between 10 MPa pressure and low temperature were found in the measured biomarkers. HHP did not have an effect on the levels of all the biomarkers measured till 20 MPa. This means that *P. varians* can possible be a suitable candidate for ecotoxicological testing in order to evaluate the effects of pressure at 10 MPa. However, changes in proteins function and structure are sometimes reversible after a short period after the end of exposure to HHP (Gross and Jaenicke 1993; Macdonald 1997). In the future, a possible way to test the function of the metabolism, and in particular, the effects of HHP in the function of the enzymes involved in the detoxification processes can be the evaluation of the damages

caused by a toxicant while exposed to HHP in comparison to those caused in animals at atmospheric pressure.

As in the present work, Vinagre et al. (2014) and Wang et al. (2001) also have reported increased in LPO levels in organisms exposed to low temperatures. Despite the fact that low temperature causes a reduction on the metabolism, and therefore a smaller production of ROS could be expected, the increased effort to maintain the homeostatic internal conditions of the cell and the stress caused by the cold shock, can cause an increase in the release of ROS which cause peroxidation of the lipids (Abele and Puntarulo 2004; Wang et al. 2011). Also, antioxidant enzymes like CAT and GST of animals from warm waters have lower activity at low temperatures, and therefore a higher accumulation of ROS is possible (Abele 2002; Regoli et al. 1997). The significant increase in GST probably occurred due the increased level of hydroperoxides formed as secondary metabolites during the peroxidation of the polyunasaturaded fatty acids (Hayes et al. 2005). Vinagre et al. 2014 reported than different temperatures can change the levels of GST in shrimps. However Verlecar et al. (2007) did not found a clear increase in the GST activity in organism exposed to a cold-shock. It is important to note that the temperature used in this work in the cold exposure is much lower than those used by Verlecar et al. (2007) and (Vinagre et al. 2014).

Levels of catalase obtained in the present study (1.05 ± 0.78 µmoles min^{-1} mg^{-1} protein) were similar than those obtained in other studies, like the one of Gonzalez-Rey et al. (2007) where an activity of 2 ± 0.5 µmoles min^{-1} mg^{-1} protein in *P. varians* was reported. The absence of activity in some shrimps probably occurred due errors in the experimental work.

Hochachka (1974) reported high disruption of AChE activity by pressure. The Km of AChE isolated from *Electrophorus electricus* increased about fourfold upon exposure to 20 MPa. In the present work already no effect of exposure to pressure and temperature was observed in the AChE activity. While it is unlikely that the AChE of *P. varians* presents similar adaptation to the one found in deep-sea organisms by Hochachka, the absence of response in this work is a possible indicator that if in any time the activity of AChE

decreases while under pressure, it has the ability of rapidly regains its function while at normal temperature and pressure.

Like in the present work, Kopecka-Pilarczyk and Coimbra (2010a) did not find a significant effect of HHP in CAT and GST activity levels in silver eel (*Anguilla anguillar*). A significant reduction on LPO levels upon exposure to 5 MPa was observed. However, it is important to notice that contraty to P. varians, this specie is commonly found in some parts of its life cycle in the deep-sea. Also, these results were observed after 3 and 7 days of exposure, a longer-term period when compared to the one performed in this work. In *Pagellus bogaraveo* no effect of HHP was found in GST, CAT, AChE activities and LPO levels after 14 days exposure at 5 MPa (Kopecka-Pilarczyk and Coimbra 2010b).

No significant difference were found between animals placed inside the HC and those exposed at optimal conditions, therefore this chambers can be used in this kind of assays. Although due the impossibility of renew the medium or provide oxygen, the duration of the test cannot exceed short periods of time with big invertebrates.

Based on the absence or response of all the biomarkers measured in this work, *P. varians* appear to be a suitable candidate to assess chemical hazard under pressures up to 10 MPa, corresponding to depths of 1000 m. Although no effects were observed immediately after the exposure, the shifts on the levels of LPO and AChE after an eight hour recovery should be investigated in order to use *P. varians* as a model for pressures between 10 MPa and 20 MPa. The changes in the LPO and GST levels due low temperature can also restrain the use of *P. varians* as a model to evaluate stressors at deep-sea conditions. Although, a significant part of deep-sea mineral resources is located near hydrothermal vents, (Coffey and Nautilus 2008; Ramirez-Llodra et al. 2011), with these habitats displaying a high abundance and diversity of species (Thistle 2003). In this way, performing assays at higher temperature than those on the surrounding deep-sea may be of special relevance to access potential impacts of deep-sea mining in these environments.

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PART 3 EFFECT OF HIGH HYDROSTATIC PRESSURE (HHP) ON CADMIUM TOXICITY IN *PALAEMON VARIANS*

3.1 ABSTRACT

The release of several metals from deep-sea mining can represent serious hazards to organisms from the deep sea. While the toxicity of these metals is very well described for terrestrial and shallow-water organisms, few information is available regarding deep-sea organisms. Therefore, there is a need to develop or adapt ecotoxicological assays with model organisms, considering pressure and lower temperatures into account when assessing the effects of these metals to the deep-sea organisms. While the use of deep-sea species represents a challenge, shallow-water species, like *Palaemon varians*, can represent a good model organism due to their phylogenetic similarity. But understanding the effects of high hydrostatic pressure is essential to infer their use for this purpose. In the present work, *P. varians* were exposed to several concentration of cadmium during 96h, along with different pressure regimes: at atmospheric pressure; simultaneously, for 8h, with a pressure of 20 MPa; and pre-exposed to 20 MPa for 8 h. Only slightly differences were found between the calculated LC50 for the different pressure regimes, indicating that at this pressure no effects on the toxicity of cadmium to *P. varians* are observed. Although this is an important input regarding hazard assessment at higher pressures, additional studies are needed regarding other species and other chemicals that are also prone to appear in the deep sea.

3.2 INTRODUCTION

Covering an area of 360 million Km^2, equivalent to about 50% the surface of the Earth, the deep-sea is by far the largest ecosystem on earth. Protected by a water layer of about 200m, the deep waters were perceived as highly buffered from the abiotic changing conditions, or anthropogenic exploration and pollution which characterize terrestrial and shallow water environments (Ramirez-Llodra et al. 2011). However this vision of a stable environment has changed since the late XX century. The deep-sea is now seen has a very rich biodiverse ecosystem with unique characteristic, with numerous mass extinction in the past and exposed to several changes and treats caused by human activity (Glover et al. 2010; Grassle & Maciolek 1992; Thuy et al. 2012).

The major anthropogenic threats to the deep-sea ecosystem are chemical pollution, resources exploitation, like fisheries and minerals, and climate changes (Brown and Thatje 2015; Montagna et al. 2013; Ramirez-Llodra et al. 2011; Yasuhara et al. 2014). The development of new technologies enabled the exploration of diverse forms of inaccessible high commercial value mineral resources, such as gold, silver and zinc, which are now passive to be explored (Ramirez-Llodra et al. 2011). The future mining activity is considered to be the major threat to several endemic ecosystems in the deep sea (Dover 2014). The removal of the hard-substrate will cause not only a loss of habitat for the marine fauna fixation, but also a considerable/potential exposure to high amounts of metals, like mercury, cooper and cadmium, at concentrations exceeding the safety limits (Mestre et al. 2014; Thiel et al. 2015). The formation of sediment plumes can spread these metals across time and space (ISA 2011; Nautilus and Coffey 2008), causing hazard to deep-sea organisms. Several studies have shown the high toxicity of these metals to different marine fauna (e.g. Ahsanullarh et al. 1981). However no experimental work has been performed in order to test their effects in the deep-sea abiotic conditions.

Hydrostatic pressure is a continuous condition in the ocean, increasing about 1 MPa for each 100 meters of depth in the water column. Pressure changes several processes in the organisms, like membrane-based functions, conformation of proteins and the function and affinity for the enzymes' substrate (Brown and Thatje 2014; Somero 1992).

Exposure of organisms to pressures out of the normal range can cause perturbation in the neural function, motor impairment, immobilization and ultimately death (Brown and Thatje 2014; Macdonald 1997; Pradillon and Gaill 2007). However, the deleterious effects of metals and other compounds at high pressure conditions are almost completely unknown. The only work addressing this issue was performed by Edward and Ehrlich (1977) where several colonies of deep-sea bacteria were exposed to manganese, copper, cobalt and nickel. Their results indicated evidences of interaction between metal toxicity and the applied pressure. Depending on the tested metal, pressure had no effect or synergistic or antagonistic effects when combined with a metal on the cell yield of the colonies.

Testing the effect of pressure ranges in the toxicity of compounds in deep-sea organisms can be a hard and expensive task. The need of expensive equipment to keep the animals at high pressure at all moments, during transport and maintenance (Pradillon and Gaill 2007), and the high number of animals required for an ecotoxicological assay can be an obstacle. In addition, working with deep-sea organisms is challenging and perhaps impossible. Within this scope, and in the need of several impact studies to take pressure into account when accessing the effects of these compounds in water, the use of shallow-water organisms with close phylogenetic relations with deep-sea species is can be considered as a potential solution to overcome current constraints (Mestre et al. 2014).

The aim of this work was to test the effects of high hydrostatic pressure on the toxicity of cadmium for the shallow-water shrimp *Palaemon varians*, Leach 1814. However, due to technical constrains, it was not possible to apply pressure for the full exposure to cadmium. The effect of cadmium was tested in animals exposed to two different regimes of HHP during 8 hours (in animals exposed simultaneously to cadmium and pressure and in animals exposed to pressure prior to metal exposure).

3.3 MATERIALS AND METHODS

3.3.1 Organisms' collection and acclimation conditions

Adult grass shrimp, *P. varians*, were collected using traps with bait from a salt marsh (N 40º38'39"; W 8º39'51") near the Ria de Aveiro estuary in Aveiro, Portugal in March 2015. Collected shrimps were transported to Universidade de Aveiro in 20 l buckets containing water from the collection point. Animals with a carapace length between 3.5 and 4 mm were then selected. After an acclimation of 48 hours, shrimps were placed, in a density inferior of three shrimps per liter, in 20 L tanks containing synthetic sea water prepared with Tropic Marin® Sea Salt and freshwater purified by a reverse osmosis unit. Tanks were then kept in a temperature controlled room at 20 ºC and 16 h: 8 h light : dark cycle for 20 days prior to testing. Constant aeration was provided by bubble stones and oxygen was measured once a day with a probe Oxi 330i/set WTW®. Once a day, 20% water was renewed in each tank and ammonia levels and pH were measured using API® colorimetric tests. Food was provided 2 times a day consisting in decapsulated artemia cysts (*Artemia salina*) and seaweed food pellets ®Hikari Marine A. Excessive food was removed after 1 hour.

3.3.2 Cadmium exposure

The protocol EPA OPPTS 850.1035 Ecological Effects Guidelines for Mysid Acute Toxicity Test was used as a background and adapted for this work using *P. varians*.

A stock solution of 1 g Cd/L was prepared by dissolving cadmium chloride anhydrous (CAS No. 10108-64-2, Sigma-Aldrich, Germany) in ultra-pure water (Millipore® Academic Milli-Q system). To perform the dilutions necessary for the exposure tests, synthetic sea water was used which was previously filtered with a 0.45 µm mesh. Cadmium concentrations chosen for the test were 0.625, 1.25, 2.5, 5, 10 and 20 mg/L. These concentrations were based on preliminary studies (data not shown).

In the beginning and at the end of the tests, 50 mL of the medium sampled, from a pool of the different replicates for each concentration was sampled, and filtered with a

0.45µm mesh. Samples where then acidified with nitric acid 60% and stored, prior to chemical analysis (according to EPA (1993)). Cadmium concentration in samples were analyzed by ICP-OES spectrometer Horiba® Jobin Yvon, model Activa M with a autosampler JY AS500. The measurements were performed following the norm ISO 11885, using a potency of 1200 W, with the following conditions: Power generator 1200W, argon plasma flux of 13 L min^{-1}, a Burgener Miramist nebulizer with an aspiration rate of 1 mL min^{-1} and a conical spray chamber.

3.3.3 Acute toxicity test

For the acute toxicity test performed without changes in pressure, all organisms were placed in pairs in 140 mL plastic vessels containing clean synthetic sea water. Each flask was filled until top and closed with a lid. All vessels were then placed in the hyperbaric chambers, without pressure being applied and stored in a room acclimatized at 20 ºC. Water used to fill the chambers was also at 20 ºC. After eight hours the animals were removed, and randomly transferred for 250 mL plastic boxes containing the test medium. Three replicates were set for each tested concentration plus a control with six replicates (clean water). Three shrimps were randomly placed in each box and then closed with a lid. Continuous aeration was provided by a bubble stone placed in each box. Room temperature was kept at 20 ºC with photoperiod of 16 h : 8 h light : dark cycle. The number of survivors was counted every 24 h, and dead organisms removed; 20% of the medium was renewed every 24 h during the test duration. No food was provided during the test.

Dissolved oxygen using a probe Oxi 330i/set WTW® and pH were monitored using a probe pH 330/SET WTW® and temperature was controlled with a thermometer at the beginning and end of the test to pressure and as well as for the 96h exposure test.

3.3.4 Acute toxicity test with different exposures to pressure

For the acute toxicity test with pre-exposure to pressure, the same procedure described above was performed. The only difference was that 20 MPa pressure was applied in the HC during eight hours. After this time, each HC was depressurized for five minutes and the vessels containing the shrimps in clean water removed. Shrimps were then transferred for the plastic boxes containing the test medium.

Regarding the simultaneous exposure to cadmium and pressure, shrimps were placed in 140 mL plastic vessels containing the cadmium exposure medium, using the six different cadmium concentrations mentioned above plus a control with clean medium. Five vessels for each concentration were filled up until top, and two shrimps placed per replicate, and then closed with a lid. The vessels were then randomly placed in the HC and the chambers closed. Pressure was then set to 20 MPa and kept in a controlled room at 20 ºC for eight hours. After this period all HC were depressurized for 5 minutes and the shrimps randomly divided in groups of three and transferred to plastic boxes containing 250 mL of new medium with the same concentrations which they were exposed in the HC. As above three replicates for each treatment were established plus a control with six replicates. One individual for each concentration was discarded.

The rest of the procedure was the same as described above for the shrimps pre-exposed and not exposed to pressure.

3.3.5 Statistical analysis

Mortality was used as endpoint and LC50 values calculated by Probit analysis using Minitab® Release 14.12.0.

3.4 RESULTS

The measured concentration of dissolved cadmium used in the tests is presented in table 2. The LC50 values calculated were based on the real concentrations measured.

Table 2 Nominal cadmium concentration and the respective dissolved concentrations obtained in the chemical analysis (mg/L).

Nominal concentration (mg/L)	Dissolved concentration measured (mg/L)
0.625	0.60
1.25	1.17
2.5	2.22
5	4.40
10	9.09
20	17.90

No mortality was observed in the control animals, for all the pressure tests, during the full duration of the test. In addition, all animals were alive after the 8 hours of exposure to a pressure of 20 MPa in control and Cd treatments. However, animals presented some erratic movements after the decompression, as swimming backwards and sometimes in circles. After 5-10 minutes, no behavior differences were observed between animals exposed and not exposed to pressure and the animals appeared to be recovered from decompression.

The LC_{50} values were calculated for every 24 hs for *P. varians* exposed to cadmium under different pressure conditions using the Probit analysis and are presented in table 3. After 48 h of exposure, animals pre-exposed to 20 MPa pressure showed a lower LC50 value (LC50= 9.46 mg/L) when compared with the other treatments. However, after 96 h, all LC50 values were similar. LC50 values for 24 h could not be calculated because no dose-response curve could be attained.

Figures 7, 8 and 9 show the survival rates of *P. varians* at the different cadmium concentrations for 48 h, 7 2h and 96 h, respectively, for the three different pressure assays. While sensitivity to the higher cadmium concentrations (9.09 and 17.9 mg/L) were almost the same for the different pressure regimes, a higher mortality was observed in animals not exposed to pressure at 1.17, 2.22 and 4.40 mg/L cadmium concentrations. No mortality was observed at the lowest concentration of Cd (0.60 mg Cd/L).

Oxygen levels measured after the exposure to pressure in the HC were above recommended levels (60%). Oxygen levels in the post-exposure were above 90% in all replicates during the whole experiment. No significant variation in pH levels was observed and all samples had values between 8.1 and 8.2.

Table 3 LC50 values (± 95% confidence limits) in mg/L for 96 h calculated for *Palaemon varians* exposed to cadmium (value calculated for dissolved concentration of cadmium).

Pressure treatment	48 h LC50 (mg/L)	72 h LC50 (mg/L)	96 h LC50 (mg/L)
Cadmium exposure at atmospheric pressure	12.14 (8.73 – 15.55)	4.04 (2.80 – 5.28)	2.50 (1.77 – 3.22)
Cadmium exposure after a 8h pre-exposure to 20MPa (in clean media)	9.46 (7.05 – 11.87)	6.19 (4.60 – 7.78)	2.84 (2.15 – 3.53)
Simultaneous exposure of cadmium and 20 MPa (8h), followed by Cd exposure till 96h	12.11 (9.46 - 14.75)	4.69 (3.30 - 6.08)	2.88 (2.09 - 3.66)

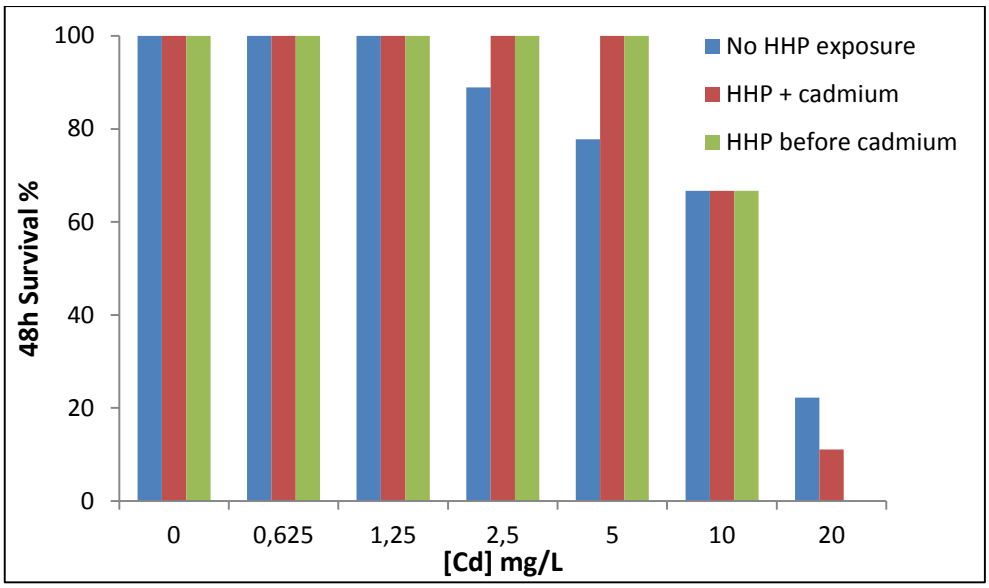

Figure 7 Total survival of *Palaemon varians* exposed to cadmium for 48 h. Data is presented as % of survival. The blue bars represents animals exposed to atmospheric pressure, the green bars represents animals pre-exposed to pressure before cadmium exposure, and the red bars the animals exposed to both pressure and cadmium simultaneously.

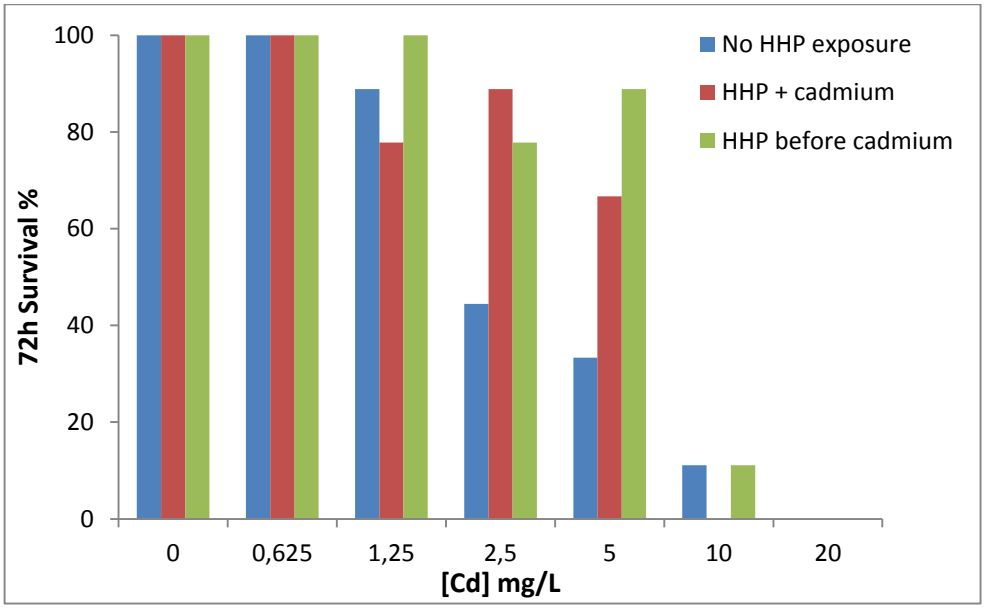

Figure 8 Total survival of *Palaemon varians* exposed to cadmium for 72 h. Data is presented as % of survival. The blue bars represents animals exposed to atmospheric pressure, the green bars represents animals pre-exposed to pressure before cadmium exposure, and the red bars the animals exposed to both pressure and cadmium simultaneously.

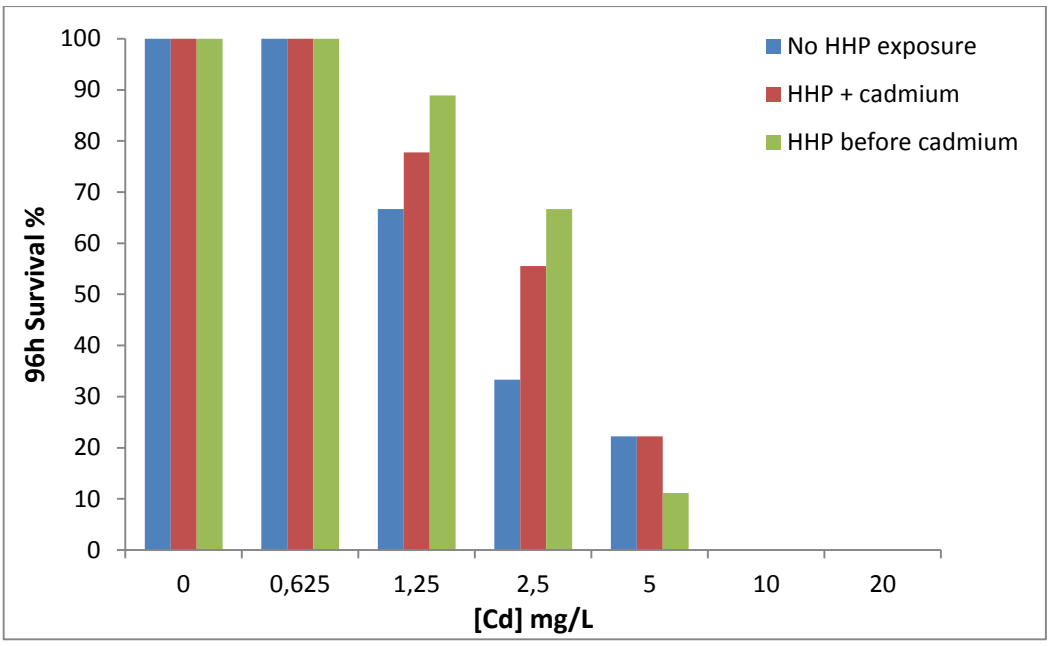

Figure 9 Total survival of *Palaemon varians* exposed to cadmium for 96 h. Data is presented as % of survival. The blue bars represents animals exposed to atmospheric pressure, the green bars represents animals pre-exposed to pressure before cadmium exposure, and the red bars the animals exposed to both pressure and cadmium simultaneously.

3.5 Discussion

The results of this work indicate that exposure to pressure does not caused any potentiation or decrease on the toxic effect of cadmium in *P. varians*. However, an exposure to HHP during the 96h was not possible to access, due the oxygen depletion in the vessels. Finding ways to increase the exposure times should be tested in future assays.

A synergism upon their combined exposure could be expected in this work, mainly for 2 reasons. First, high hydrostatic pressure cause the dissociation of ionic bounds, therefore more cadmium would be available in its ionic form, which is more toxic for marine organisms (Engel and Fowler 1979; Gross and Jaenicker 1994). However, the extent of the increase in the ionic cadmium is unknown. Although no work was performed with pressure, it is common that several natural stressors such as temperature

and oxygen levels that are out of a species natural range can cause an increase in the toxicity of a chemical (for review, see Holmstrup et al. 2010). However, the absence of response of *P. varians* to HHP in the previous work, can explain the equal response of *P. varians* to cadmium when exposed to different pressure regimes.

As other organisms, *P. varians* have shown increase sensitivity to cadmium with the increasing exposure time. While the seawater on open sea have a cadmium concentration of 5-20 ng/L, concentration up to 400 ng/L have been reported in certain coastal zones like in the Liverpool Bay, United Kingdom (Nordic Council of Ministers 2003; OSPAR 2002; UNEP 1985). Despite the lack of capacity of decapods to regulate internal cadmium levels (White and Rainbow 1982), the sensitivity of *P. varians* to an acute exposure is not relevant when the values are compared to the concentrations found in the environment.

Regarding the sensitivity of the species used in the present work, it can be considered in the same range or lower in sensitivity when compared to other species. Khan et al. (1988) calculated a 96 h LC50 of total metal concentration for cadmium of *Palaemon pugio* of 1.83 mg/L and Burton and Fisher (1990) calculated a 96 h LC50 for the same species of 1.3 mg/L. The 96 h LC50 for *Palaemon vulgaris* was calculated by Eisler was 0.42 mg/L (in EPA 2001). When compared with the values calculated for other species of the same genus, *P. varians* have shown a lower sensitivity to cadmium (LC50 to total concentration of 2.81 mg/L). It is important to notice that a higher salinity was used in this work when compared to the other studies mentioned above which can also justify these small differences. Both Howard and Hacker (1990) and Pierron et al. (2007) reported a positive relation between the increase on the toxicity of cadmium and the salinity decrease in the shrimps *Palaemon Pugio* and *Palaemon elegans* respectively. At higher salinities the amount of cadmium in the ionic form (Cd^{2+}) is lower and therefore less bioavailable (Pierron et al. 2007; Sunda et al. 1978).

While more research is needed to evaluate the effect of pressure on the sensitivity of different groups of organisms to metals, looking specially at sub-lethal and more ecological relevant parameters, this work provided evidences that exposure to high level pressure do not cause an increase in the sensitivity of *P. varians* to cadmium.

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PART 4 CONCLUDING REMARKS

The continuously growing of world's population is adding high levels of pressure to all ecosystems on Earth. As land resources become exhausted due to overexploitation, the interest on the deep-sea resources keeps growing. The rising of new technologies can today allow the exploitation of minerals which were unavailable a few decades ago. However, the management and protection of this previously undisturbed ecosystem require more knowledge to understand the effect of the very particular environmental conditions, the ecological services provided by the deep-sea and the ecology and vulnerability of the unique species which live there.

The main aim of the present study was to gain an insight knowledge on the pollution effects in the deep sea, and for that available tools were tested. So a shallow water marine species was used (*Palaemon varians*) based on its similarity in terms of phylogeny with deep-sea species, to understand the effects of HHP in their survival and selected biochemical biomarkers. These biomarkers are currently used tools in biomonitoring studies as a way of detect effects upon exposure of an organism to contaminants. In addition, a non-effective pressure was chosen in order to evaluate the joint effect with cadmium, as a model metal for environmental pollution.

For this work, an easy to operate and affordable high-pressure system was used. The simplicity of the system when compared to others, allows running series of tests at low costs. However, this system does not allow renewing the medium during the test duration. Because no aeration can be provided, the oxygen inside the test vessels quickly drops due the animal's respiration, causing them to be in hypoxia some hours. Therefore, the use of smaller species or younger animals, such as shrimp larvae can allow accessing the effects of longer exposures to HHP using this system. However, no mortality or changes in the biochemical biomarkers was observed in *P. varians*, showing that this shrimp can be used with this system while assessing effects of HHP during short term exposures.

At hydrostatic pressure lower than 20 MPa, the tested biomarkers did not show any response to HHP indicating possibly low to no effect under those exposures. At low

temperatures, the present work showed some changes of some biochemical biomarkers and interaction with HHP in terms of mortality. A long acclimation of the animals is a possible way to improve the use of these animals at low temperatures.

Exposure duration to pressure was also considered short termed and therefore looking at long term exposures would also be advisable.

Despite some technical limitations, the present study gave an important contribute for future research. While exposed to non-acute levels of HHP, *P. varians* have shown no sign of increased sensitivity to cadmium. Therefore, based on this work results, no potentiation of the toxic effect (mortality) from cadmium is expected when organisms are exposed to non-lethal levels of HHP for short periods of time. In the future, it is important to test longer exposures to metals under the effect of HHP.

While more research is needed to test the effect of several other metals and xenobiotics, the data presented in this work can be of use in the elaboration of hazard assessment studies due to the exploitation of deep-sea minerals.

In a nutshell:

1. The pressure system used successfully simulated the desired parameters and the conditions of the chambers did not have a significant effect in exposed animals.

2. *P. varians* did not survive pressures above 20 MPa, therefore cannot be used in assays which simulate conditions of depths higher than 2000 m.

3. Exposures to low temperatures cause some changes in *P. varians* biochemical biomarkers and further investigation is required.

4. Short-term exposure to HHP did not have any effect in the sensitivity of *P. varians* to cadmium.

PART 5 ANNEXES

ANNEX 1

Table 1 Oxygen and pH levels measured during the biomarkers assay using *Palaemon varians*. Oxygen was measured using a probe Oxi 330i/set WTW® and pH was measured using a probe pH 330/SET WTW®.

Treatment	8 h exposure (inside the hyperbaric chamber)		8 h recovery (optimal conditions)	
	Oxygen	pH	Oxygen	pH
Optimal conditions (aeration, light. 20 ºC)	95%	8	88%	7.9
	94%	8	89%	8
	95%	8	92%	8.1
	94%	8	91%	8
	77%	7.9	88%	8.1
	88%	7.9	89%	8.1
	87%	7.9	92%	8
	89%	7.9	92%	8
Atmospheric pressure (inside the hyperbaric chamber, 20 ºC)	46%	8.1	91%	8.1
	39%	8.2	88%	8
	12%	8.1	94%	8.1
	41%	8.2	92%	8
	54%	8	91%	8.1
	37%	7.9	89%	8.1
	51%	8	89%	8
	32%	7.8	88%	8
Atmospheric pressure (inside the hyperbaric chamber, 4 ºC)	70%	8.2	94%	8.1
	65%	8	93%	8.1
	66%	8	93%	8.2
	69%	8	88%	8
	60%	8	89%	8.1
	65%	7.9	90%	8
	59%	8	90%	8
	64%	8	89%	8
10 MPa, 20 ºC	43%	8.2	91%	8
	39%	8.2	91%	8.1
	15%	8.2	92%	8.1
	18%	8.2	89%	8
10 MPa, 4 ºC	58%	8.2	89%	8
	70%	8.1	91%	7.9
	65%	8.3	92%	8
	55%	8.2	91%	7.9
20 MPa, 20º C	41%	7.9	91%	8
	33%	7.8	90%	8
	27%	7.8	88%	7.9
	55%	7.9	93%	7.9

Date:	03/10/2015
Protocol developed by:	Gonçalo Domingues
Person responsible:	Susana Loureiro and Ricardo Calado
Protocol (new/adapted):	New
Language:	Portuguese
State:	Version 2; Annex 2

Grupo I&D Ecologia Aplicada e Ecotoxicologia- applEE- R&D Group Applied Ecology and Ecotoxicology
DEPARTAMENTO DE BIOLOGIA & CESAM, UNIVERSIDADE DE AVEIRO

TABELA DE CONTEÚDOS

Introdução

O seguinte protocolo destina-se à utilização do sistema de câmaras hiperbáricas para testes sobre pressão hidrostática com organismos aquáticos de pequenas dimensões.
O sistema está concebido para utilização de pressões até 500 Bar, não devendo este valor ser ultrapassado em caso algum.
O procedimento de pressurizar e despressurizar as câmaras exige alguma força física e deve ser efectuado sempre por duas pessoas.
O sistema deve ser utilizado cumprindo SEMPRE as regras de segurança neste protocolo enunciadas. As altas pressões com que se trabalha podem causar lesões graves e/ou permanentes quando não seguidas as normas de segurança.

Composição do sistema

O sistema é composto por:

- 5 câmaras hiperbáricas (CH)
- 1 bomba e respectiva mangueira de ligação
- Frascos para teste em plástico
    - 36 frascos 60 ml
    - 36 frascos de 125 ml
- Material de protecção
    - Botas de protecção
    - Luvas
    - Viseira
- Ferramentas para manutenção
    - 2 chaves de boca
    - 1 chave inglesa
- 1 spray WD-40
- 1 grampo para fixação da bomba a diferentes superfícies

Preparação das amostras

Os recipientes utilizados devem ser em plástico e apropriados para suportar altas pressões como aqueles indicados na figura 1.
NUNCA usar recipientes em vidro ou em material não deformável.

Figura 1 Frascos de plásticos para teste

Nos frascos deve ser colocado o organismo a testar e o meio de teste. Estes devem ser cheios até ao máximo possível e bem fechados.

- Reduzir ao máximo a presença de bolhas de ar no interior do recipiente. As bolhas de ar podem causar a implosão, aquando da pressurização.

Preparação do sistema de pressão

Utilizar material de protecção apropriado para manuseamento do sistema, principalmente durante a pressurização e manuseamento das CH sobre pressão.

- Luvas de protecção
    - Evitar cortes e aumentar a aderência das mãos ao manusear as CH
- Botas de biqueira de aço
- Viseiras de protecção
    - Durante a pressurização e despressurização e manuseamento das CH com pressão

1. Verificar o aperto das porcas e parafusos das CH antes de iniciar o procedimento, evitando assim fugas de água durante o processo de pressurização (fig. 2).

Figura 2 Verifica sempre o estado de aperto das porcas indicadas na figura pelas setas amarelas.

2. Abrir a CH e encher com água da torneira ou água de osmose, NUNCA com água salgada (fig. 3).

Figura 3 Enchimento da CH com água de osmose

3. Colocar os frascos na CH e fechar bem a mesma (fig. 4). Para fechar é necessário colocar a parte superior de forma correta na parte inferior.
    - Utilizar sempre a peça superior correspondente à inferior (as peças encontram-se marcadas com fita colorida correspondente).
    - Se a rosca entrar torta a peça vai enroscar um pouco mas depois bloquear. Um truque pode ser colocar a peça superior sobre a inferior e rodar lentamente no sentido oposto ao sentido de enroscar (no sentido contrário aos ponteiros do relógio) até ouvir um ligeiro

estalido, nesse ponto parar e começar a enroscar correctamente (no sentido horário).

Figura 4 Colocação dos frascos na CH e fecho da mesma

Manuseamento da bomba e pressurização

1. Prender a bomba à mesa com ajuda de um grampo e enroscar a mangueira e a alavanca como indicado na figura 5.
2. Fechar a válvula de despressurização indicado na figura com uma seta vermelha.
3. Encher o depósito indicado na figura com uma seta azul com água de osmose ou água da torneira.

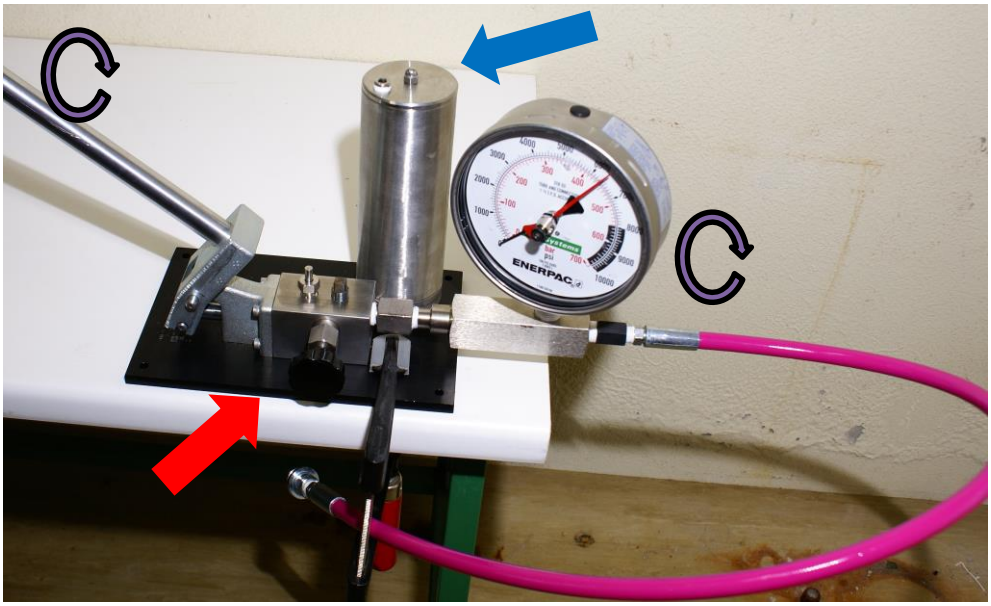

Figura 5 Bomba presa à mesa e pronta para a pressurização. A seta azul indica o depósito da água e a seta vermelha indica a válvula de despressurização.

4. Encaixar bem a mangueira na CH e enroscar usando a peça presente na mesma até apertar o encaixe como indicado na figura 6 e de seguida abrir a torneira (na figura a torneira encontra-se fechada, para abrir rodar no sentido indicado pela seta).

Figura 6 Encaixe da mangueira na CH. A seta vermelha indica a peça utilizada para enroscar a mangueira

5. Iniciar o bombeamento de água para o interior da CH utilizando a alavanca como indicado na figura 7. O volume de água no depósito deve ser verificado constantemente durante este processo, podendo ser necessário voltar a encher o mesmo. Manter sempre o depósito mais de meio e NUNCA bombear ar para dentro da CH.

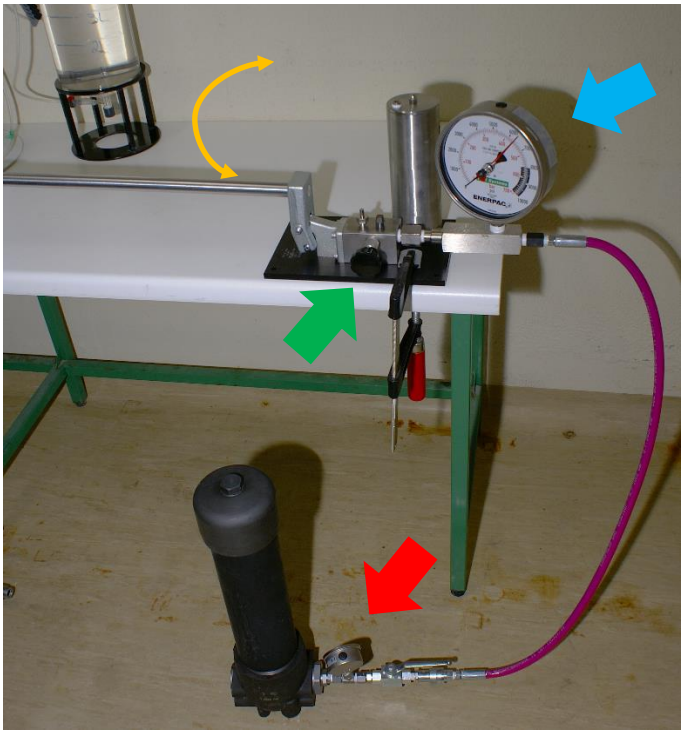

Figura 7 Sistema pronto para pressurizar. Seta verde indica a torneira de despressurização. A seta azul indica o manómetro que mostra a pressão na mangueira e a seta vermelha o manómetro que indica a pressão no interior da CH

6. Verificar constantemente o manómetro. Quando a pressão começar a subir, bombear mais lentamente (a pressão aumenta abruptamente nesta fase) até o manómetro da CH indicar a pressão pretendida. O manómetro indicado com uma seta azul indica a pressão na mangueira e o indicado com uma seta vermelha indica a pressão no interior da CH (estas podem diferir ligeiramente durante o processo de pressurização).

7. Quando atingida a pressão pretendida fechar a torneira da CH e <u>APENAS</u> de seguida despressurizar a mangueira usando a válvula de descompressão na figura indica com a seta verde. <u>NUNCA</u> retirar a mangueira da CH se o manómetro indicado pela seta azul não estiver a indicar 0 Bar de pressão.

8. A CH pode agora ser guardada onde pretendido. Durante a duração do teste verificar o manómetro diariamente, para controlar eventuais perdas de pressão.

Repetir o mesmo processo para as restantes CH se necessárias para o teste

Descompressão

1. Ligar a bomba à mangueira e de seguida abrir a torneira da CH. Isto vai causar a perda de uma parte da pressão contida na CH para a mangueira, levando a uma despressurização muito rápida. Para uma despressurização mais lenta, fechar a válvula de despressurização e pressurizar a mangueira antes de abrir a torneira da CH.
    - A mangueira pressuriza muito rápido, estar atento ao manómetro da bomba e NUNCA ultrapassar a pressão colocada previamente na CH. Após este processo abrir a torneira da CH.
2. LENTAMENTE abrir a válvula de descompressão, pode ser necessário remover água do depósito da bomba durante este processo se o mesmo encher.

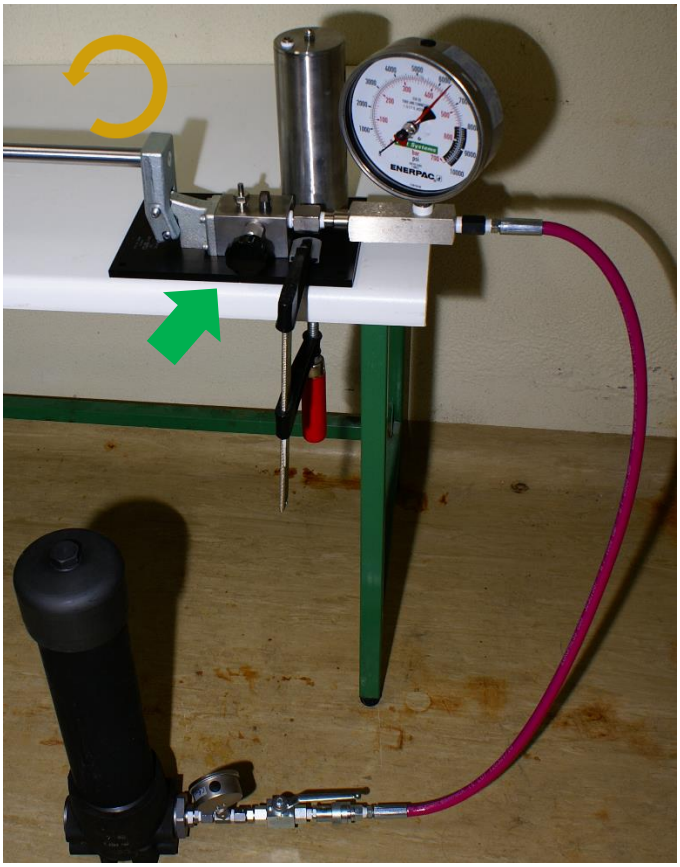

Figura 8 Sistema após despressurização. Mangueira pronta a ser retirada, repare que o manómetro indica pressão 0 na mangueira

3. Verificar no manómetro da CH a pressão no seu interior. Apenas para a despressurização quando o valor for 0.

4. Para abrir a câmara rodar a parte superior ou inferior mantendo a outra fixa. Pode ser necessário usar um torno para abrir as mesmas. Antes de abrir certificar SEMPRE que a pressão no interior da CH é 0. Mesmo com esta indicação alguma água vai sair com alguma pressão (insuficiente para provocar qualquer lesão), evitar ter perto de objectos que não devem ser molhados.

5. Retirar os frascos com as amostras e analisar.

Seguir o mesmo procedimento para as restantes CHs.

Finalizar

1. Secar as CH e a bomba o melhor possível e deixar alguns dias a secar a restante humidade.
2. Aplicar WD-40 ou similar nas CH, principalmente nas válvulas, porcas e parafusos.
3. **Arrumar em local seco**.