Universidade de Aveiro Departamento de Biologia
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ÂNGELA AUGUSTA SOARES DE ALMEIDA

PRESENCE OF CARBAMAZEPINE IN COASTAL SYSTEMS: EFFECTS ON BIVALVES

PRESENÇA DA CARBAMAZEPINA EM SISTEMAS COSTEIROS: EFEITOS EM BIVALVES

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Rosa de Fátima Lopes de Freitas (investigadora auxiliar do Departamento de Biologia e CESAM, da Universidade de Aveiro), da Doutora Vânia Maria Amaro Calisto (estagiária de pós-doutoramento do Departamento de Química e CESAM, da Universidade de Aveiro) e da Doutora Etelvina Maria Paula de Almeida Figueira (professora auxiliar do Departamento de Biologia e CESAM, da Universidade de Aveiro).

Dedico este trabalho aos meus pais por me proporcionarem o prazer que é VIVER

o júri

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palavras-chave

Toxicidade aguda e toxicidade crónica, biomarcadores, imunoensaio ELISA, drogas farmacêuticas, bivalves, *Venerupis decussata*, *Venerupis philippinarum*

resumo

A carbamazepina (CBZ), uma droga antiepilética, é uma das drogas farmacêuticas frequentemente detetadas em ecossistemas aquáticos, sendo utilizada como um marcador da poluição antropogénica.

Uma vez que a CBZ foi desenhada para exercer um efeito biológico, quando alcança o ambiente aquático existe uma elevada probabilidade para provocar efeitos tóxicos em organismos não-alvo.

Desta forma, no presente estudo foi avaliada a toxicidade aguda (96 h) e crónica (28 d) de concentrações ambientalmente relevantes de CBZ (0.00, 0.03, 0.30, 3.00, 9.00 µg/L) em amêijoas comestíveis *Venerupis decussata* (nativa) e *Venerupis philippinarum* (invasora) capturadas na Ria de Aveiro.

Os efeitos em ambas as espécies foram estudados utilizando uma bateria de biomarcadores principalmente relacionados com o estado de saúde e stress oxidativo nos organismos. Foi aplicada também uma técnica alternativa promissora, o imunoensaio ELISA para a quantificação direta de CBZ nos tecidos das amêijoas.

Os resultados obtidos para o teste agudo mostraram um aumento da concentração de CBZ nos tecidos das amêijoas ao longo do intervalo de exposição, tendo *V. decussata* acumulado mais CBZ (exceto para CBZ 9.00 µg/L) do que *V. philippinarum*. Após uma exposição de 28 d a *V. decussata* acumulou uma concentração superior de CBZ, exceto na concentração mais elevada, onde os níveis concentrados foram similares aos do teste agudo.

Apesar das amêijoas acumularem baixos níveis de CBZ comparando com a concentração de exposição, estes foram suficientes para comprometer o estado de saúde das espécies em estudo levando a uma condição de stress oxidativo. As duas espécies apresentaram uma resposta diferente à CBZ quando submetidas ao teste agudo. *V. philippinarum* aumentou os níveis de peroxidação lipídica na concentração mais alta de exposição, ao passo que *V. decussata* apresentou uma diminuição significativa deste parâmetro. A atividade da glutationa S-transferase foi estimulada no caso da *V. decussata* e diminuída para V. *philippinarum*. Não obstante, após a exposição a CBZ, em ambas as espécies ocorreu uma indução das atividades da glutationa reductase e superóxido dismutase. Os resultados indicaram que, provavelmente, *V. philippinarum* possui um sistema de defesa antioxidante deficiente quando comparada com *V. decussata*, sendo menos apta na neutralização das espécies reativas de oxigénio e portanto apresentou-se como a espécie mais sensível aos efeitos da CBZ.

Uma exposição de 28 d à CBZ resultou numa toxicidade superior na espécie *V. decussata* por comparação com o teste agudo. Tal foi principalmente refletido na reduzida atividade/conteúdo nos mecanismos envolvidos no sistema de defesa antioxidante, traduzindo-se numa baixa capacidade para combater o stress oxidativo provocado pela CBZ.

O quociente de risco determinado para a Ria de Aveiro foi superior a 1 indicando que se suspeita de um risco ecotoxicológico. A bioacumulação de CBZ pelas amêijoas torna evidente a possibilidade de ser transferida ao longo da cadeia alimentar, afetando em último caso o ser humano.

keywords

Acute toxicity and chronic toxicity, biomarkers, ELISA immunoassay, pharmaceutical drugs, bivalves, *Venerupis decussata*, *Venerupis philippinarum*

abstract

Carbamazepine (CBZ), an antiepileptic drug, is one of the most commonly detected pharmaceutical drugs in aquatic ecosystems, being used as a marker of anthropogenic pollution.

Since CBZ is designed to exert a biological effect, when it reaches aquatic environment high probability exists for toxic effects on non-target organisms.

In this way, the present study evaluated the acute (96 h) and chronic toxicity (28 d) of environmentally relevant concentrations of CBZ (0.00, 0.03, 0.30, 3.00, 9.00 µg/L) in the edible clams *Venerupis decussata* (a native species) and *Venerupis philippinarum* (an invasive species) collected from the Ria de Aveiro lagoon.

The effects on both species were assessed through the use of a battery of biomarkers mainly related with health status and oxidative stress in the organisms. Furthermore, it was applied a promising alternative technique, the immunoassay ELISA for the direct CBZ quantification in clams' tissues.

The results obtained for the acute test showed that CBZ levels in clams' tissues increased along the exposure concentration range, where *V. decussata* accumulated more CBZ (except for CBZ 9.00 µg/L) than *V. philippinarum*. After an exposure of 28 d *V. decussata* accumulated a higher concentration of CBZ with exception of the highest concentration of exposure, where the levels of CBZ accumulated were similar to the acute test.

Although the clams accumulated lower levels of CBZ than the concentration of exposure, these concentrations were enough to impair the health status of the species under study and induce oxidative stress. A different response to CBZ was observed for both species exposed to the acute test. *V. philippinarum* increased the lipid peroxidation levels at the highest CBZ concentration, whereas *V. decussata* presented a significant decrease in this parameter. Glutathione S-transferase activity was stimulated for *V. decussata* and decreased for *V. philippinarum.*

Nevertheless, after exposure to CBZ, for both species it was found an induction of glutathione reductase and superoxide dismutase. The results indicated that, probably, *V. philippinarum* have a deficient antioxidant defense system compared with *V. decussata,* being less capable to neutralize reactive oxygen species and thus appeared to be the most sensitive species to the CBZ effects.

A 28 d exposure to CBZ resulted in a higher toxicity in *V. decussata* compared with the same species exposed to the acute test. This was mainly reflected by a lower activity/content in the mechanisms involved in the antioxidant defense system and thus, a lower capability to lead with oxidative stress induced by CBZ.

The risk quotient determined for the Ria de Aveiro was higher than 1 indicating that an ecotoxicolgical risk is suspected. Furthermore, the bioaccumulation of CBZ in clams should be taken into consideration since it might be transferred along the food chain, ultimately affecting humans.

Contents

Abbreviations

BCF – bioconcentration factor

CAT – catalase

CBZ – carbamazepine

CI – condition index

CR – clearance rate

CYP3A4 – cytochrome P450 3A4

EC_{50} – median effective concentration

ELISA – enzyme-linked immunosorbent assay

GPx – glutathione peroxidase

GR – glutathione reductase

GSH – reduced glutathione

GSSG – oxidized glutathione

GST – glutathione S-transferase

LC_{50} – median lethal concentration

LPO – lipid peroxidation

LQ – quantification limit

MEC – measured environmental concentration

NOEC – no observed effect concentration

PCO – principal coordinates

PEC – predicted environmental concentration

PNEC – predicted no effect concentration

ROS – reactive oxygen species

RQ – risk quotient

SA – survival in air

SOD – superoxide dismutase

WWTP – wastewater treatment plant

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Introduction

1.1. Pharmaceuticals in the environment – emerging contaminants

The aquatic ecosystem has been target of increasing number of stressors associated with anthropogenic activities, namely metals, hydrocarbons, organic pollutants and amongst others, pharmaceuticals and their active compounds. Pharmaceuticals are a large and diverse group of bioactive chemicals used throughout the world, for the prevention, diagnosis and treatment of diseases (Fent et al., 2006), being applied in high amounts in human medicine but also in agriculture and aquaculture for animal use as veterinary drugs in livestock breeding (Boxall et al., 2004; Heberer et al., 2002).

Although the valuable help of these drugs, concerns have arisen on their use and consequent elimination by humans and animals. After intake, pharmaceuticals undergo metabolic processes in the organism, being excreted mainly in urine as a mixture of the unchanged parent compound, metabolites or conjugates (Heberer et al., 2002). Consequently pharmaceutical active compounds find their way through sewage systems to wastewater treatment plants (WWTPs). In WWTPs, elimination rates of many drugs are incomplete since metabolic stability and thus resistance to biodegradation is necessary for pharmacological action. In consequence, consumption and excretion of drugs and the discharge of WWTPs final effluents are considered the primary source of pharmaceutical drugs into the aquatic environment (Halling-Sorensen et al., 1998; Heberer et al., 2002). Other important pathways that contribute to the continuous introduction of pharmaceutical drugs into the environment are land application of the treated sludge and wastewater from WWTPs and incorrect household disposal of unused packages via trash or sewage (Kummerer, 2010).

In the last decade, researchers have detected a multitude of pharmaceutical residues in the aquatic environment (Nikolaou et al., 2007). The common analytical method for determining pharmaceutical drugs in environmental samples relies on liquid or gas chromatography coupled with single or tandem mass spectrometry. On the other hand, immunoassays have been used as an alternative to detect pollutants in water, being the enzyme-linked immunosorbent assay (ELISA) the most used.

Despite the valuable help of pharmaceutical drugs, their presence in the environment has been target of a growing concern due to the possibility of ecotoxicological risks to aquatic and terrestrial organisms, including humans (Huerta et al., 2012). It has been demonstrated that the low environmental concentrations at which pharmaceuticals drugs occur in the environment, in general, do not exert acute toxic effects on the organisms but, when these are submitted to a long term exposure sublethal effects can occur, impairing their health status and performance (Jones et

al., 2010). The ecosystem alteration due to the input of pharmaceuticals, in conjunction with other stressors, could also be related with the invasion by alien species that can have better chances of surviving when compared with native species.

Although the study of pharmaceutical drugs in the environment is a fairly new topic, a vast amount of literature has already been published and despite the effort made, there is still a necessity to conduct more chronic toxicity studies when assessing the impact of these contaminants in the environment (Fent et al., 2006). The potential impact on invertebrates is also scarce. In particular, bivalves are an example of benthic species that can be particularly exposed to pharmaceutical drugs and their impacts but few studies addressed the effects of drugs on these individuals. These long-lived and sessile organisms filter large quantities of water for feeding and respiration (McEneff et al., 2014) being therefore particularly susceptible to contaminants present in water column such as drugs (Gagné et al., 2006a). Besides their important role on ecosystem functioning, bivalves represent an important economic resource for many coastal populations in several countries, which highlights the importance to perform studies on pharmaceutical drugs ecotoxicity.

Despite the high amounts of medicine drugs released into the environment, concise regulations for ecological risk assessment are largely missing. Only in the last few years, regulatory agencies have issued detailed guidelines on how pharmaceuticals should be assessed for possible unwanted effects on the environment. The first requirement for ecotoxicity testing as a prerequisite for registration of veterinary pharmaceuticals was established in 1995 (Directive 92/18 EEC) (Fent et al., 2006). Later it was established that the application of human pharmaceuticals must be accompanied by an environmental risk assessment (Directives 2001/83/EU and 2004/27/EU), being necessary to the approval procedure for new substances (EMEA, 2005). However, there is still scarce toxicity data for the majority of pharmaceuticals in use, impeding their adequate risk assessment.

In this way, only after filling these gaps, more reliable environmental risk assessment about the presence of pharmaceutical drugs in the environment can be performed.

1.2. Occurrence, sources and fate of pharmaceuticals in the environment

About 3000 different medical substances are used in human medicine in European Union, for the prevention, diagnosis and treatment of diseases in humans (Fent et al., 2006). In addition, a large number of pharmaceuticals are used in veterinary medicine such as antibiotics and anti-inflammatory drugs.

Due to the vast amounts of pharmaceutical drugs used, which is expected to increase because more people are living longer and use more drugs as they age (Kummerer, 2010) and due to the development of more sensitive techniques for the detection of pharmaceutical drugs in the environment, a multitude of pharmaceutical drugs have been found, mainly in the aquatic environment.

Different routes are involved in the occurrence of pharmaceutical active compounds in the environment. However, in opposition to the majority of contaminants, the principal route of entrance on ecosystem is related with their proper use. After uptake by humans or animals, pharmaceuticals are excreted, mainly in urine and in less extent in sweat and feces, in their native form if they are not absorbed by the organism, or, they can be absorbed and suffer metabolization in order to facilitate their elimination, being excreted as metabolites (active or inactive) or conjugates (glucuronides and sulphates) (Daughton and Ternes, 1999; Heberer et al., 2002). Once excreted these compounds are conducted through sewage systems, reaching WWTPs.

In a conventional WWTP, the wastewater is submitted to primary, secondary and tertiary treatment. In primary wastewater treatment (a mechanical process) occurs the removal of large objects from the raw influent through the use of machinery and decantation. The secondary treatment is a biological treatment designed to degrade sewage through aerobic biological processes, including activated sludge and biological filters. The tertiary treatment is the final stage before treated wastewater is released into the environment, being used for nutrient removal (nitrogen, phosphorus) (Monteiro and Boxall, 2010). Pharmaceuticals drugs as acetaminophen, caffeine and salicylic acid were reported to be substantially removed during secondary treatment with activated sludge while others (e.g. gemfibrozil) are removed less efficiently. Moreover, very low removal was reported using this treatment for CBZ (Ternes, 1998). Sludges accumulated in the wastewater treatment processes are further treated for safe disposal, through aerobic or anaerobic digestion and composting. However, also in this case, some pharmaceutical drugs such as ibuprofen and iopromide presented a lower percentage of removal after anaerobic digestion of sludges (41 and 22%, respectively), with CBZ showing no elimination (Carballa et al., 2007).

In this way, the persistent drugs not removed during the passage through the treatments applied in WWTPs are released into the environment through treated effluents or sludge being transported and distributed in various compartments such as water bodies, soil and sediments. The biosolids (sludge) produced are also used in agriculture as soil amendment or disposed to landfill (Jelic et al., 2011). In this way, the use and excretion of pharmaceuticals and the discharge of WWTPs treated effluents are the major sources of pharmaceutical drugs into the environment,

resulting in the contamination of rivers, lakes, estuaries and less commonly, groundwater and treated drinking water, being detected in the range of ng/L to low μg/L (for a review please consult Monteiro and Boxall, 2010).

Other minority sources can contribute for the environment contamination with pharmaceutical active compounds. Although the correct procedure for the elimination of non-used pharmaceuticals is to return them to the pharmacy, the majority of people flush the unused drugs down the drain/toilet or dispose them in domestic refuse which will ultimately enter domestic waste landfill sites or, in less extent, sewage system (Jones et al., 2010). Bound and Voulvoulis (2005) interviewed the member of 400 households in UK and found that about half of the respondents did not finish their drugs and among those 63% discharged their unfinished drugs in household waste, 21.8% brought them to a pharmacist and 11.5% discharged the drugs into the drain or toilet. We should also take into consideration that many households don't have sewage systems so the wastewaters are direct released into the environment. Although the production and disposal of pharmaceuticals by manufacturers, wholesalers and retailers is subjected to strict control, Larsson et al. (2007) reported that the effluents from a WWTP serving about 90 bulk drug manufacturers in India contained by far the highest levels of pharmaceuticals reported in any effluent.

The numbers of reports on measurable concentrations of pharmaceutical drugs in environmental samples or reviews on pharmaceuticals found in the environment is growing (e.g. Carlsson et al., 2006; Daughton and Ternes, 1999; Fatta-Kassinos et al., 2011; Fent et al., 2006; Heberer et al., 2002; Monteiro and Boxall, 2010). Among the most frequently detected classes of pharmaceuticals detected in the environment are anti-inflammatory drugs and analgesics (e.g. ibuprofen, diclofenac), antibiotics (e.g. erythromycin), lipid regulators (e.g. benzafibrate), steroids and related hormones (e.g. 17-β-estradiol, estrone), β-blockers (e.g. metropolol), cancer therapeutics (e.g. cyclophosphamide) and neuroactive drugs (e.g. carbamazepine, fluoxetine) (Nikolaou et al., 2007). The majority of these studies rely on the determination of pharmaceutical drugs in environmental water samples. However, several studies investigated the occurrence and distribution of drugs in soil samples irrigated with reclaimed water or submitted to the application of biosolids from WWTPs (e.g. Kinney et al., 2006; Lapen et al., 2008), demonstrating that the use of these can result in the presence and accumulation of pharmaceutical residues in soil. The deposited pharmaceutical compounds may run off from soil into surface water after, for example, rainfall events, contributing even more to the contamination of aquatic environment.

The fate of pharmaceutical drugs in the environment includes sorption to soils and sediments, complexation with metals and organics compounds, chemical oxidation, photodegradation and biodegradation. However, the behavior and fate of pharmaceuticals and their metabolites in the aquatic environment is not well known. Please consult Fatta-Kassinos et al. (2011) and Monteiro and Boxall et al. (2010) for a review on these processes.

1.3. Environmental implications

Although the valuable help of pharmaceuticals in promoting organism's health, the presence of these compounds and their metabolites in the environment has been target of a growing concern due to the possibility of ecotoxicological risks to aquatic and terrestrial organisms, including humans.

Many reviews dedicated to the ecotoxicology and risk assessment of pharmaceuticals have been published (Carlsson et al., 2006; Fent et al., 2006; Halling-Sorensen et al., 1998; Li, 2014; Kummerer, 2010; 2009). It has been reported that the low concentrations at which pharmaceutical residues occur in the environment, in general are not enough to produce acute toxicity effects, but after a long term exposure they can lead to sublethal effects on the organisms, impairing its health status and performance.

But how pharmaceutical drugs can trigger a toxic effect to the organisms? They are lipophilic in order to be able to pass membranes; persistent in order to avoid the substance to be inactive before reaching their target and were designed to elicit specific effects in target organisms, modifying specific biochemical pathways (Halling-Sorensen et al., 1998). In this way, the accumulation of drugs by non-target organisms could result in a series of cascade reactions possibly leading to adverse effects. Since aquatic organisms are exposed to long-term continuous influx of wastewater effluents, contaminated with pharmaceutical residues, the majority of the studies regarding this field have focused on the effects of pharmaceutical drugs on aquatic biota, especially in vertebrates (e.g. fishes), under short term aqueous-exposure experiments (e.g. 24 to 96 h toxicity tests) with single substances. Although these studies provide important information on the toxic effects of drugs, they fail on the approximation to the reality, since these contaminants are persistent when released to the environment, and so, it is more likely that organisms are exposed for long periods of time, to a complex mixture of drugs, at low concentrations levels. Moreover, the effects of pharmaceutical drugs on benthic invertebrates have not been widely investigated despite their essential role in aquatic food chain and the potential for sediments to serve as repository for anthropogenic contaminants (Dussault et al., 2008). Bivalves are an example of benthic species that

are important members of aquatic ecosystem and markedly interact with water and sediment, being an excellent sentinel for the monitoring of organic micro-contaminants in environmental waters (Martínez-Bueno et al., 2013). In fact, they have been used to identify pollution trends in the marine environment due to the widespread distribution, sedentary lifestyle and ease of collection (Pruell et al., 1986). These long-lived organisms are constantly subjected to different types of environmental stressors, due to their sessile condition and filter feeding habits (Gagné et al., 2006b; McEneff et al., 2014). As they filter high quantities of water for feeding and respiration, any dissolved or suspended contaminants present in the water column may result in an ecotoxicological effect (McEneff et al., 2013). In consequence, these organisms can accumulate many organic contaminants to high concentrations and pharmaceutical drugs are not exception. The uptake of pharmaceutical residues in bivalves has been previously showed (Martínez-Bueno et al., 2013; Klosterhaus et al., 2013), being reported that a higher susceptibility to these contaminants exist if they have a high filtration activity and a low capability to metabolize them (Antunes et al., 2013). Furthermore, in bivalves, hemocytes circulate in an open vascular system favoring direct exposure to contaminants (Gagné et al., 2006b). As these organisms represent important economic resources for many coastal populations in several countries, their utilization as bioindicator species to assess environmental contaminants can provide important information about the physiological status of the animals, helping to diagnose the environmental quality of bivalves that are farmed/caught.

Besides toxicity to aquatic species, trace pharmaceutical concentrations have been previously detected in drinking water (Benotti et al., 2009) and in cooked seafood (McEneff et al., 2013). Thus, the presence of pharmaceutical drugs in water and seafood may, ultimately, act as a risk to humans.

1.4. Analytical methods for the analysis of pharmaceutical drugs in the environment

In order to infer about the fate of drugs in aquatic ecosystems, their quantitative determination is mandatory. Several review articles on the determination of pharmaceutical drugs and their metabolites in different kinds of environmental samples (usually in water and sediments) have been published in the last decade (Buchberger, 2011; 2007; Gros et al., 2006; Huerta et al., 2012; Fatta-Kassinos et al., 2011; Petrovic et al., 2005). The accurate quantification of pharmaceutical drugs in environmental samples can be an analytical challenge, due to the complexity of the matrix and their low levels of occurrence (Fatta-Kassinos et al., 2011). Several years ago, appropriate analytical techniques did not exist, but the presence of considerable expertise in pesticide residue analysis facilitated the fast progress in the development of analytical procedures for the analysis of pharmaceutical residues (Buchberger, 2007). Nowadays, the common procedures (reference techniques) for the quantification of drugs are gas and liquid chromatography (GC and LC, respectively) in combination with mass spectrometry (MS). Capillary electrophoresis (CE) has also been used for that purpose. The advances in these analytical techniques facilitated the detection of a multitude of pharmaceutical drugs in the environment, providing the opportunity to quantify them down to ng/L levels (Heberer et al., 2002; Fatta-Kassinos et al., 2011).

Gas chromatography is preferable for the analysis of non-polar and volatile compounds, but it can be applied for the analysis of low concentrations of pharmaceuticals by addition of a derivatization step. The advantages of GC include very high selectivity and resolution, good accuracy and precision and high sensitivity (Fatta-Kassinos et al., 2011). Liquid chromatography is the preferred technique for separation of polar organic pollutants, and has the advantage of shorter analysis time, necessary for monitoring studies. The main drawback of this technique for the analysis of pharmaceutical drugs in environmental samples is matrix effects which can reduce the sensitivity, linearity, accuracy and the precision of the method (Fatta-Kassinos et al., 2011). Capillary electrophoresis is less expensive than GC and LC, but less sensitive than these two techniques, with detection limits in the µg/L range. Therefore CE methods are more appropriate for analysis of wastewater samples rather than surface water samples.

The reference techniques have been applied for the quantification of CBZ in environmental samples (Contardo-Jara et al., 2011; Cueva-Mestanza et al., 2008; Ferrari et al., 2003; Huerta et al., 2013; Leclercq et al., 2009; Ramirez et al., 2007; Wang and Gardinali, 2012; Wille et al., 2011). In general these methods involve procedures of extraction, clean-up and concentration of the analyte

prior to analysis, and sometimes a derivatization step is required. Moreover, they are also time-consuming and expensive in terms of equipment and maintenance.

To overcome these problems and find cost-effective procedures, new alternatives have been developed and applied, where the immunoassays and biosensors are examples of these methods (Huerta et al., 2012). Immunoassays exploit the ability of specialized biological molecules, called antibodies, to selectively and reversibly bind organic molecules. The antibodies are large glycoproteins, composed by four polypeptide chains (two identical heavy chains and two identical light chains linked by one or more disulphide bridges) that belong to the family of immunoglobulins which have the capacity to bind specifically to an antigen (any substance able to trigger an immune response). A great part of the immunoassays have been developed and used for the analysis of pesticides in the aquatic systems (e.g. Lima et al., 2013), and only very few tests have been applied for pharmaceutical compounds (Bahlmann et al. 2012; 2011; 2009; Deng et al., 2003; Gagné et al., 2006c; Hintemann et al., 2006; Huo et al., 2007).

One of the most popular immunoassays formats is the enzyme-linked immunosorbent assay (ELISA) which is a heterogeneous assay with one of the components (the antigen or the antibody) bound to a solid phase (Law, 2005). This assay have been previously used for CBZ quantification in water samples (Bahlmann et al., 2012; 2011; 2009; Calisto et al., 2011a).

Bahlmann et al. (2009) applied ELISA for the determination of CBZ in wastewater and surface water samples in Germany. The authors found that the water samples could be analyzed without pre-concentration or sample clean-up, allowing the achievement of a detection limit of 0.024 μg/L with less than 1 mL of sample volume required. Moreover, concentrations up to 50 μg/L could be quantified without prior dilution. Calisto et al. (2011a) used the same assay to quantify CBZ in ground, surface and wastewaters from Aveiro region. The quantitation range determined varied between 0.03 and 10 μg/L. Cross-reactivity with CBZ metabolites was negligible, but for cetirizine, an antihistaminic pharmaceutical, cross-reactivity occurred in a significant level. However this could be overcome by selecting the optimal pH for analysis since the affinity of the monoclonal antibody towards cetirizine is highly dependent on the pH during the interaction step. The comparison of ELISA with LC–MS/MS showed that, for the same CBZ concentration, the results obtained by ELISA were overestimated being, in general, 2-29% higher than the LC-MS/MS results. Nevertheless, the authors reported that the results obtained using ELISA were highly satisfactory, referring the technique as being an inexpensive and simple alternative, well suited for the fast screening of samples.

Despite several articles have been published reporting the determination of pharmaceutical drugs on environmental samples such as water and sediments, little information on the detection of CBZ and its metabolites in real complex matrices such those that exist in aquatic organisms are available in literature (Chu and Metcalfe, 2007; Cueva-Mestanza et al., 2008; Ramirez et al., 2007), especially using the cost-effective immunoassays. Only one study developed by Gagné et al. (2006c) applied a competitive enzymatic immunoassay to quantify CBZ in the freshwater mussel (*Elliptio complanata*) soft tissue homogenate extracts. The importance of this assay on the study of bioavailability and accumulation of persistent xenobiotics in aquatic organisms was highlighted.

The reduced application of the immunoassays in this type of analysis could be partly explained by the high complexity of biological matrices which may be rich in components that could interfere with the analysis and the lack of suitable protocols to extract CBZ from these matrices. Moreover, usually, pharmaceutical drugs are present in biological matrices at trace levels (ng/L or lower), being required extensive and time consuming sample preparation techniques to solve these issues. For a review on the methods used for sample preparation please see Huerta et al. (2012).

In addition, considering the great complexity of biological matrices, combined with the large differences in physical and chemical properties (e.g. polarity, solubility, stability) between groups of pharmaceuticals, the main challenge for the development of an analytical method is to obtain efficient extractions for all the target compounds, which justifies why the majority of the analysis of pharmaceuticals in environmental matrices focus on single compound or a family of compounds (Huerta et al., 2012).

1.5. Risk assessment

Despite the high amount of drugs released to the environment, concise regulations for environmental risk assessment (ERA) are largely missing. Only in the last few years, regulatory agencies have issued detailed guidelines on how pharmaceutical drugs should be assessed for possible unwanted effects on the environment. The first requirement for ecotoxicity testing as a prerequisite for registration of pharmaceuticals was established in 1995 according to the European Union (EU) Directive 92/18 EEC and the corresponding “Note for Guidance” for veterinary pharmaceuticals (Fent et al., 2006). According to the directive 2001/83/EU modified by the directive 2004/27/EU, the application of human pharmaceuticals must be accompanied by an environmental risk assessment (EMEA, 2005), being necessary for the approval procedure of new substances. The U.S. Food and Drug Administration require environmental risk assessment to be performed for human and veterinary medicines on the effects on aquatic and terrestrial organisms before a

product can be marketed (Fatta-Kassinos et al., 2011). The applicants in the USA must provide a report when the expected introduction concentration of the active ingredient of the pharmaceutical drug in the aquatic environment is $\geq$1 μg/L (Fent et al., 2006). Despite these regulations, to date there is still scarce toxicity data for the majority of pharmaceuticals in use, impeding their adequate risk assessment. For pharmaceutical drugs risk assessment standard ecotoxicity tests are often used, focusing predominantly on short-term exposures and mortality as the endpoint (Ferrari et al., 2003; Halling-Sorensen et al., 1998). Further, the effects observed in these studies occur at much higher concentrations than those found in the environment. However, concerns about the possible environmental effects of the low and continuous input of pharmaceutical drugs in the environment have led to the development and implementation of chronic toxicity testing procedures using aquatic organisms. In this way, the chronic aquatic toxicity tests have been adopted in the risk assessment guidance document for human pharmaceuticals produced by the European Medicines Agency, in support of Directive 2001/83/EC (EMEA, 2005).

Environmental risk assessment procedures for pharmaceuticals based on principles already applied in ERAs for chemicals have been developed. This ERA is based on a step-wise tiered approach, starting from rough estimates and then to more elaborate methods if a potential risk cannot be excluded and where a risk quotient (RQ) is usually calculated (Carlsson et al., 2006; Ferrari et al., 2004). The first tier consists of deriving a crude predicted environmental concentration (PEC) or determining the MEC (the highest concentration measured in wastewater effluent during the sample period) in the aquatic compartment for the pharmaceutical or its metabolites (EC, 2003). If the PEC is above 0.01 μg/L an environmental analysis should be performed in a second tier, with the determination of the PNEC (predicted no effect concentration), the concentration below which an unacceptable effect will most likely not occur. The PNEC values are calculated by dividing the lowest short-term L(E)C50 (L, lethal; E, effect; C, concentration) or long-term NOEC (no observed effect concentration) by an appropriate assessment factor. The assessment factors are a reflection of the degree of uncertainty in extrapolation from laboratory toxicity test data for a limited number of species to the real environment, being smaller for long-term tests due to the best approximation to the natural environment that chronic tests can provide, in comparison with acute tests (EC, 2003). Then, the risk quotient between the MEC or PEC and the PNEC (RQ=MEC or PEC/PNEC) is calculated. If this ratio equals or exceeds 1, an ecological risk is suspected, and further considerations on a case-by-case basis are possibly needed in a third tier to refine the PEC and the PNEC (Martín et al., 2014).

1.6. The antiepileptic drug Carbamazepine

In the last decade, researchers have detected a multitude of pharmaceutical drugs in the aquatic environment which include carbamazepine (CBZ). CBZ (Figure 1.1), a dibenzazepine, is the most representative compound belonging to the class of anti-epileptic pharmaceuticals, used as the active substance in Tegretol. This drug has anti-epileptic and psychotropic properties so it has been applied in the treatment of psychomotor epilepsy, for relief of trigeminal and glossopharyngeal neuralgias and for a variety of mental disorders (Infarmed, 2013). CBZ exerts its effects by blocking sodium channels of excitatory neurons thus decreasing the neuronal activity on central nervous system (Ambrósio et al., 2002). The stabilization of neuron membranes through the decrease in glutamate was reported to justify its anti-epileptic effects (Houeto et al., 2012).

The current data about CBZ global consumption is not found in the literature. This is due to the difficulty in collect pharmaceutical consumption data since patients can obtain drugs in multiple ways that cannot be easily counted or because the same drug can be sold under different brand names, further obscuring the data. However, Zhang et al. (2008) estimated that 1 014 tonnes of CBZ are consumed worldwide, per year. In Portugal CBZ is among the 100 most prescribed medicines with a consumption of 297 437 packages per year (Infarmed, 2011).

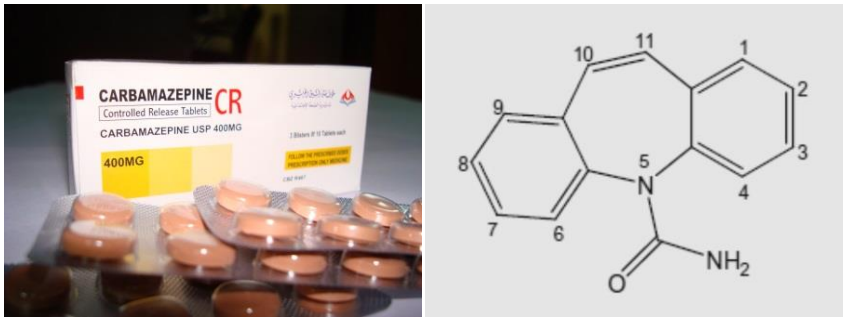

Figure 1.1. CBZ structure (Zhang et al., 2008).

1.6.1. Carbamazepine pharmacokinetics

The maximum daily dose of CBZ for adults is 1600-2000 mg (Infarmed, 2013). After administration, approximately 72% of CBZ is absorbed, while 28% is unchanged and eliminated through feces. Subsequently to its absorption, CBZ is metabolized by the liver and only about 1% of dosage leaves the body in an unaltered form. The metabolites and the unaltered form undergo enterohepatic cycling and finally are excreted mainly through the urine (Houeto et al., 2012; Zhang et al., 2008).

Many pharmaceuticals require biotransformation for elimination, being cytochrome P450 enzymes (CYP), namely the P450 3A4 the most important enzyme involved in drug metabolization (Anzenbacher and Anzenbacherová, 2001; Guengerich, 1999). The metabolization has the objective to form more polar and water-soluble derivatives which have reduced pharmacological activity compared with the parent compound and that are rapidly excreted (Fatta-Kassinos et al., 2011). So, in liver, CBZ is predominantly metabolized by CYP3A4 to 10,11-dihydro-10,11-epoxycarbamazepine (CBZ-epoxide), which is pharmacologically active (Figure 1.2). CBZ-epoxide is eliminated by hydroxylation of the aromatic ring to *trans*- 10,11- dihydro-10, 11 –dihydroxycarbamazepine (CBZ-diol) or by conjugation to form O-glucuronides (Martínez-Bueno et al., 2013; Houeto et al., 2012). Although CBZ-epoxide is pharmaceutically active just as CBZ, CBZ-diol is the main metabolite excreted in urine (about 30%). For CBZ-epoxide, the percentage of oral dosage that is excreted in urine is only 2% (Zhang et al., 2008).

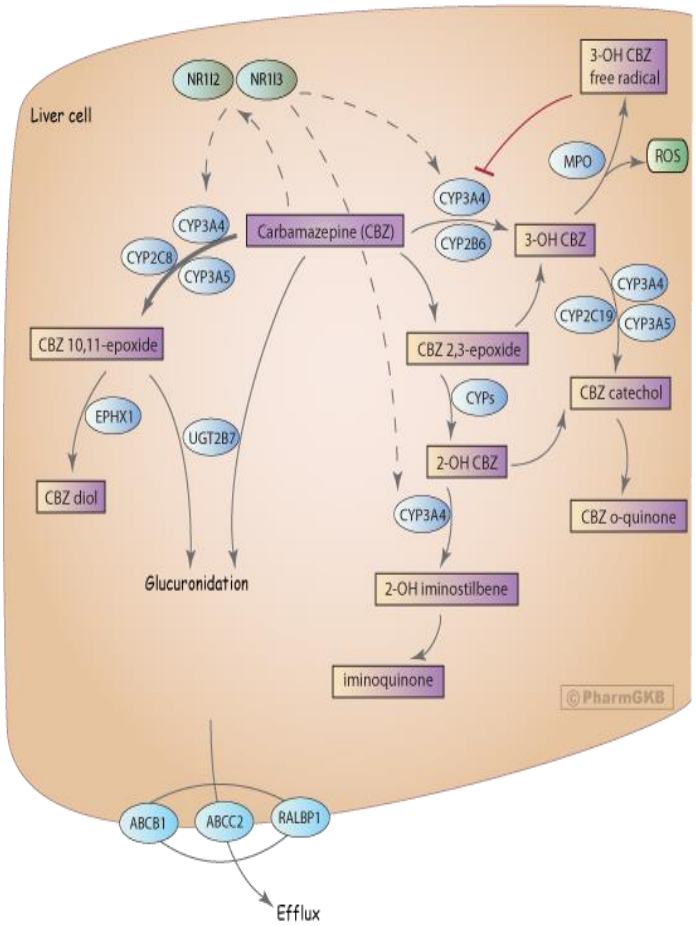

Figure 1.2. Metabolism of CBZ in liver cell (PharmGKB, 2011).

1.6.2. Carbamazepine in the aquatic environment – an anthropogenic marker

Carbamazepine enters aquatic bodies mainly through the discharge of WWTPs effluents in which its degradation rates, although depending on the treatment applied, are mostly below 10%. Ternes et al. (1998) investigated the removal of pharmaceutical drugs, including CBZ, during the passage through a German municipal sewage treatment plant. CBZ showed an elimination rate of 7% during this passage. A similar elimination rate (8%) of CBZ was determined by Heberer et al. (2002), revealing again that the drug was not removed during sewage purification on a sewage treatment plant in Germany. Other studies found similar elimination rates (Clara et al., 2004; Metcalfe et al., 2003), where the concentrations of CBZ in influent and effluent samples from WWTP were approximately equal. Clara et al. (2004) found that CBZ was not degraded or retained in an Austrian WWTP, independent of the solids retention time applied, neither during the soil passage and subsurface flow. However, these authors found high concentrations of CBZ in the effluent compared to the influent samples. Bahlmann et al. (2009) and Vieno et al. (2007) also reported an increase in CBZ concentration in WWTPs effluents attributing it to the cleavage of metabolite conjugates (glucuronides) by enzymatic processes in the treatment plant, possible due to the presence of glucuronidase activity in the activated sludge. Calisto et al. (2011a) found that no removal of CBZ occurred at different treatment stages in WWTPs from Aveiro region (Portugal), indicating that the applied treatments (primary decantation, biological treatment and secondary decantation) were not efficient in the removal of this pharmaceutical.

The low removal efficiency of CBZ can be explained by its properties. This drug presents a low distribution coefficient between water and secondary sludge indicating that CBZ is hardly attached onto sludge, remaining in the aqueous phase (Ternes et al., 2004). Andreozzi et al. (2003) demonstrated that CBZ exposed to sunlight can persist in aquatic environments for more than 100 d (in winter and higher latitudes). The same authors reported that the presence of dissolved humic acids in the water could result into an increase of CBZ half-life time, delaying its spontaneous photochemical degradation (Andreozzi et al., 2002). Calisto et al. (2011b) reported that CBZ is very slowly degraded by sunlight, requiring between 4.5 and 25 sunny summer days for its elimination.

In consequence, CBZ has been commonly detected in WWTPs influents and effluents, surface waters, groundwater and even in treated drinking water in concentrations from 0.03 to 6.3 μg/L (Table 1.1) (Bahlmann et al., 2012; 2009; Ferrari et al., 2003; Metcalfe et al., 2003; Sacher et al., 2001; Ternes et al., 1998). Another aspect to take into account about the presence of CBZ in aquatic environment is the occurrence of its metabolites. As referred before, CBZ-diol is the main metabolite excreted in urine, so it is probable to be found in water bodies. Miao et al. (2005)

detected CBZ and five metabolites in wastewater samples collected from four different stages of treatment in Canada. They found that 29% of the CBZ was removed from the aqueous phase during treatment in the WWTP, while the metabolites were not effectively removed. CBZ-diol was the predominant analyte, being its concentration in untreated and treated wastewater approximately three times higher than that of CBZ, and thus alerting for the necessity to conduct more studies on the environmental fate of CBZ metabolites and other degradation products. In this way, due to the high persistence of CBZ and thus, ubiquitous presence in aquatic environment it has been reported as an anthropogenic pollution marker, being qualified as a suitable marker for human influences in the aquatic environment (Clara et al., 2004).

Table 1.1. Measured environmental concentrations (MEC) of CBZ in different aquatic bodies.

MEC (µg/L)	Water type	Reference
6.3	WWTP effluent	Ternes (1998)
5.0	WWTP influent	Bahlmann et al. (2012)
4.5	WWTP effluent	
1.9	WWTP influent	Metcalfe et al. (2003)
2.3	WWTP effluent	
0.87	WWTP effluent	Ferrari et al. (2003)
0.7	WWTP effluent	Calisto et al. (2011a)
0.1	Surface water	Calisto et al. (2011a)
1.1	River waters	Ternes (1998)
3.2	Surface water	Bahlmann et al. (2009)
0.9	Groundwater wells	Sacher et al. (2001)

1.6.3. Ecotoxicological impact of carbamazepine in aquatic biota

Carbamazepine is widely present in water bodies so it is necessary to evaluate its ecotoxicological impact in aquatic environment and biota. Besides their continuous release to the ecosystem, exposing organisms during their entire life cycle, it was designed to exert a biological effect (even at low concentration) and in consequence to be resistant to biodegradation to exert

its pharmacological action. Thus, there is a high probability for this drug to interfere with non-target organisms, possible causing toxic effects. In fact, some authors have been classifying CBZ as "R52/53 Harmful to aquatic organisms and may cause long term adverse effects in the aquatic environment" (Tsiaka et al., 2013). However, the impact of CBZ on aquatic environment and its ecotoxicological data still remain largely unknown (Malarvizhi et al., 2012).

1.6.3.1. Assessment of CBZ toxicity

Aquatic organisms are particularly important targets for monitoring aquatic contamination, as they are exposed via wastewater residues over their whole life. Some studies have been investigating the effects of CBZ in organisms belonging to different groups, for example, algae (Tsiaka et al., 2013; Zhang et al., 2012), crustacean (Aguirre-Martínez et al., 2013b; Dussault et al. 2008; Kim et al., 2007), fish (Gagné et al., 2006a; Li et al., 2010a; 2010b; 2010c; Malarvizhi et al., 2012; Triesbskorn et al., 2007), insect (Dussault et al. 2008) and, of particular importance bivalves (Aguirre-Martínez et al., 2013a; Gagné et al., 2006b; 2004; Martin-Diaz et al., 2009b). The study of cells (in vitro tests) instead of organisms has also been reported (among others, Parolini et al., 2011).

Many investigations about the effects of CBZ on non-target organisms have focused on short-term toxicity, revealing that environmental concentrations do not cause acute toxic effects or that those concentrations causing effects, are in the mg/L range, which is much higher than found in surface or effluent waters. Kim et al. (2007) evaluated the acute toxicity of CBZ in the freshwater invertebrate *Daphnia magna* determining an acute median effective concentration (EC_{50}) of >100 and 76.3 mg/L after a 48 h and 96 h exposure, respectively. The authors reported that the ecotoxicological assessment of environmental concentrations of pharmaceutical drugs for long-term exposure is needed to more realistically characterize the ecological significance of drugs contamination in the environment. Zhang et al. (2012) found an EC_{50} of 72.97 and 239.84 mg/L for the algae *Scenesdesmus obliquus* and *Chlorella pyrenoidosa* after a 48 h exposure to CBZ. Moreover it was found a concentration dependent inhibitory effect for Clorophyll a synthethis. Malarvizhi et al. (2012) determined a median lethal concentration (LC_{50}) in the fish *Cyprinus carpio* of 59.7 mg/L after a 24 h exposure to CBZ. Dussault et al. (2008) evaluated the toxicity of CBZ in benthic invertebrates, the midge *Chironomus tentans* and the freshwater amphipod *Hyalella Azteca* in a 10 d water-borne exposure. CBZ was found to be toxic only at concentrations at least 100-fold higher than those typically observed in aquatic environments. The LC_{50} determined by these authors was 9.9 and 47.3 mg/L, for *H. Azteca* and *C. tentans*, respectively. Parolini et al. (2011) determined the

in vitro cytotoxicity of common drugs, including CBZ on different cell typologies from the zebra mussel *Dreissena polymorpha* determining an EC_{50} ranging from 5.09 to 6.78 mg/L.

Although acute toxicity testing provides answers for the assessment of potential risk of pharmaceuticals, some questions regarding its usefulness and validity as an appropriate predictor of potential environmental impacts have been raised and it has been reported that the information about acute toxicity alone is not enough to provide an adequate risk assessment, possibly underestimating the real impacts of drugs as CBZ in the environment (Oetken et al., 2005; Triebskorn et al., 2007; Zhang et al., 2012).

For example, Ferrari et al. (2003) studied the toxic effects of CBZ, clofibric acid and diclofenac on bacteria, algae, microcrustaceans and fish, estimating the acute and chronic toxicity on these species. They observed that CBZ had a relatively limited acute toxicity on the tested organisms. However, the chronic tests displayed higher toxicity than the acute assays. Concentrations that caused 50% effect were 77.7 μg/L on *Ceriodaphnia dubia* over 48 h. Regarding all acute endpoints tested, CBZ was found to have an intermediate toxicity comparing with diclofenac and clofibric acid. In the other hand, chronic toxicity test displayed higher toxicity, being the NOEC (no observed effect concentration) 25 μg/L on *C. dubia* over 7 d. We should note that this concentration (25 μg/L) is close to that found in environment. According to the chronic endpoints analyzed, CBZ was ranked as the highest-risk compound for aquatic environments when compared to diclofenac and clofibric acid. Other studies found a similar pattern, where the chronic tests presented a higher toxicity than the acute tests (e.g. Oetken et al., 2005; Zhang et al., 2012).

Some works with lower CBZ concentration and hence environmentally relevant levels demonstrated that this drug can exert a toxic effect after a long-term exposure, pointing out to the extreme importance of conducting chronic tests in the assessment of CBZ toxic effects. Most of these studies regarding the effects of CBZ in organisms are related to responses that can potentially lead to an oxidative stress condition due to the close relationship between environmental stress and the rate of cellular reactive oxygen and nitrogen species generation in these. These species can be produced as byproducts of cellular metabolism, and if they were not immediately trapped by antioxidant defenses, they could oxidize different cell components as proteins, DNA and lipids. For example, Martin-Diaz et al. (2009b) studied the effects of environmentally relevant concentrations of CBZ (0.1-10 μg/L) on the mediterranean mussel *Mytilus galloprovincialis* after a 7 d exposure. These authors found that the exposure to CBZ reduced the mussel health status and induced oxidative stress, interacting with targets for which it was designed and that are evolutionarily conserved.

Tsiaka et al. (2013) reported an increase in levels of cell death, superoxide anions and nitric oxide (in terms of nitrite) in the mussel *M. galloprovincialis* hemocytes in consequence of CBZ accumulation. The CBZ uptake in aquatic organisms has been shown (Contardo-Jara et al., 2011; Vernouillet et al., 2010). Some studies have been suggesting that CBZ - mediated oxidative stress could be a consequence of its accumulation by aquatic organisms as bivalves, interfering with the phagocytic activity through the disruption of lysosomal membrane with consequent release of hydrolytic enzymes and reactive oxygen species into the cytosol (Gagné et al., 2006b; Tsiaka et al., 2013). Moreover, during pharmaceuticals metabolization, reactive oxygen species can be formed leading to oxidative stress (Vernouillet et al., 2010).

Generally, after uptake, drugs undergo biotransformation (phase I and II) in order to be eliminated. Bivalves have some capacity to biotransform organic chemicals, being cytochrome P450 3A4 isoenzyme the most important drug-metabolizing enzyme (Gagné et al., 2006a). Some authors have been studying the activity of these enzymes in organisms exposed to CBZ (Aguirre-Martínez et al., 2013b; Gagné et al., 2006a; 2004; Quinn et al., 2004). The reactions catalyzed by cytochrome P450 are grouped according to the type of substrate, being benzylether resorufin (for enzymes CYP3A4 and 2B6) and dibenzylfluorescein (for enzymes CYP2C9, CYP3A4, and CYP3A5) valuable substrates in measuring the activity of these drug-metabolizing enzymes (Gagné et al., 2006; Martín-Diaz et al., 2009a). Martín-Diaz et al. (2009a) studied the activity of phase I (ethoxyresorufin-O-deethylase (EROD) and dibenzylfluorescein dealkylase (DBF)) and phase II (glutathione-S-transferase (GST)) detoxification enzymes after injection of CBZ in the mussel *Elliptio complanata*, finding that CBZ significantly induced DBF activity but no induction of EROD and GST activity was found.

In order to deal with oxidative damage, organisms have developed systems of antioxidant defense, involving the action of enzymes that prevent the onset of oxidative stress, namely catalase, glutathione peroxidase, glutathione reductase and superoxide dismutase. The analysis of these biomarkers has been reported in studies that evaluate the potential toxic effect of CBZ in different organisms. For example Chen et al. (2014) found a decrease in glutathione reductase activity in gills and digestive gland of the clam *Corbicula fluminea* when exposed during 30 d to environmentally relevant CBZ concentrations (0.5, 5 and 50 μg/L).

As reported before, if the control mechanisms involved in antioxidant defense are not effective in preventing the establishment of an oxidative stress condition, damage can be expected to occur at different cellular macromolecules, including lipids. Damage induced to lipid membranes produces malondialdehyde (formed by degradation of initial products from lipid membranes by the

free radical attack), which has been frequently assessed to observe the presence of lipid peroxidation. The induction of lipid peroxidation in aquatic organisms exposed to CBZ have also reported (Aguirre-Martínez et al., 2013b; Gagné et al., 2006a; 2006b; 2004; Li et al., 2010; Martín-Díaz et al., 2009b).

Another aspect to consider when evaluating the toxic impact of CBZ in aquatic organisms is the fact that it can be bioaccumulated and transferred along food chains, potentially harming other species. It is generally accepted that substances with octanol-water partition coefficient (log K_{ow}) values higher than or equal to 3 have potential to bioaccumulate in biological tissues. Thus, it is possible to suppose that CBZ, which has a log K_{ow} equal to 2.2, might not accumulate in organisms (Vernouillet et al., 2010). However, other factors must be taken into account when performing bioaccumulation studies as the different rates of metabolism in organisms, the accumulation behavior of the metabolites, the uptake and depuration kinetics (Huerta et al., 2012). In light of that, some investigators as Vernouillet et al. (2010) have been studying the effects of CBZ along the trophic chain, revealing the ability of CBZ to bioaccumulate in aquatic organisms through trophic chain transfer.

As referred previously, it is also important to study the ecotoxicity of CBZ degradation products. Donner et al. (2013) studied the toxic effects of CBZ and two of its ultraviolet transformation products, acridine and 9 (10H)-acridone, using bacteria, algae and crustaceans. The assay showed that the ecotoxicity increased with the CBZ degradation, with the mixture of degradation products being more toxic than the parent compound. The study highlighted the importance of considering transformation products and mixtures when assessing the environmental risk of CBZ.

1.7. Objectives

As reported previously, the ecotoxicological impact of pharmaceutical active compounds in aquatic organisms still remains largely unknown. This is the case of CBZ, an antiepileptic drug widely used. Although it has been shown that the concentrations at which CBZ occurs in the environment aren't enough to induce acute toxicity effects, some studies with environmental concentrations have been demonstrating that, after a prolonged exposure, this drug can trigger sublethal effects, impairing the organism health and performance. Nevertheless, there is a great gap concerning the chronic effects of CBZ in aquatic organisms, especially in benthic organisms as bivalves. This is of particular importance since CBZ is released continuously to the aquatic environment exposing the organism for long periods of time. As bivalves are sessile organisms and filter large quantities of water for feeding and respiration they can be particularly exposed to CBZ. Thus, it is of upmost importance to evaluate the toxic effects induced by CBZ on these populations, providing data for an adequate risk assessment. Moreover, the evaluation of contamination by CBZ in bivalves can also provide information relative to the quality of the organisms that are farmed/caught and the extent of urban contamination by CBZ.

In this way, the objectives of the present study included:

a) Evaluate the possible acute and chronic toxicity of CBZ by exposing the clams *Venerupis decussata* (native species) and *Venerupis philippinarum* (invasive species) to environmental concentrations;

b) Explore, through the use of a battery of biochemical and physiological parameters, how CBZ affects these species;

c) Evaluate the performance of the direct competitive immunoassay ELISA to directly quantify CBZ in clams tissues;

d) Assess CBZ ecotoxicological risk through the determination of risk quotient (RQ).

Chapter 2

Materials and Methods

2.1. Study area and test organisms

The Ria de Aveiro is a shallow lagoon (47 km^2 of maximum surface area) located in the Northwest Atlantic coast of Portugal (Figure 2.1). This lagoon, one of the most important estuarine systems of Portugal, has a maximum width and length of 10 and 45 km, respectively, and comprises a complex net of channels which cross both urban and rural areas and by wide intertidal areas (mudflats and salt marshes) (Jonkers et al., 2010; Sousa et al., 2010). It has three main channels radiating from the sea entrance, named Mira, São Jacinto and Ílhavo channels. The Ria de Aveiro is connected with the Atlantic Ocean through an artificial channel, local known as Barra de Aveiro (1.3 km long, 350 m wide and 20 m deep), and exchanges most part of its water by tidal input across this entrance (Dias et al., 2000). The water mass dynamics inside the system is controlled by tides and freshwater inflow from river runoff, mainly from river Vouga and Antuã (Dias et al., 2000). Due to the combined effects of the freshwater input and tidal penetration, the Ria de Aveiro exhibits a longitudinal gradient of salinity ranging from 0 in freshwaters (near river entrances), to about 36 at the bar entrance (Lopes et al., 2007).

This estuary has been heavily influenced by human activities. Besides the urban pressure derived from population settlement, the industrial and harbor structures have an important impact. In this way, the Ria de Aveiro has been submitted to anthropogenic contamination of different sources, namely by organochlorides (e.g. tributyltin), metals (e.g. mercury), endocrine-disrupting compounds (e.g. parabens) (Antunes and Gil, 2004; Galante-Oliveira et al., 2009; Jonkers et al., 2010; Sousa et al., 2010), and of the most relevance, pharmaceutical active compounds such as CBZ (Calisto et al., 2011a). CBZ was detected in this lagoon surface water as well as in wastewaters with concentrations ranging between 0.1 and 0.7 µg/L, but no contaminations of this drug was found in the Mira channel (Calisto et al., 2011a). Although the Ria de Aveiro is subjected to anthropogenic contamination, it is not contaminated across its entire extension existing channels with low or even no contamination, as the referred Mira Channel (Calisto et al., 2011a; Castro et al., 2006; Freitas et al., 2014). For this reason, this channel was chosen as the sampling site to this work, where the clams were collected, being considered non-polluted and used as a reference site.

As referred before, the presence of CBZ in aquatic systems is mainly attributed to the release of WWTPs effluents contaminated with the drug due to the low removal during the treatment process (< 10%). In Aveiro region, the effluents from municipalities bordering Ria de Aveiro are processed at three main wastewater: north, south and São Jacinto WWTP, being north and south WWTPs the main treatment plant facilities responsible for the treatment of wastewaters of 272 000 and 159 700 inhabitant equivalents, respectively (SIMRIA, 2013). In these WWTPs, wastewaters

are currently subjected to a pre-treatment, primary decantation, biological treatment and secondary decantation before effluent rejection. After treatment, the final effluent is redirected to a submarine outfall (São Jacinto outfall) and discharged into the Atlantic Ocean (3 km offshore, at approximately 16 m depth). In 2013, the effluent released by São Jacinto outfall corresponded to 33 639 million m^3 of treated effluent per year (SIMRIA, 2013). However, before 2006, the effluents of this WWTP were directly discharged into the Ria de Aveiro (Sousa et al., 2010), promoting the contamination of this ecosystem.

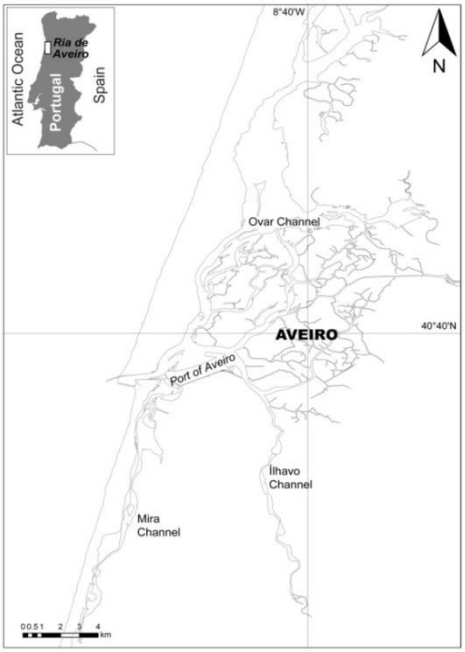

Figure 2.1. Representation of the Ria de Aveiro lagoon.

The Ria de Aveiro represents the habitat of innumerous species with high commercial interest besides their importance in the maintenance of the ecosystem integrity (Rodrigues et al., 2011). Bivalves are an example of these species where *V. decussata* and *V. philippinarum* are among the most exploitable bivalves commercialized for human consumption. In Portugal the farm/caught of bivalves is of prime relevance for the economy of the country, with 1230 tonnes of clams caught in 2013. In that year, the mollusk bivalves represented 38% of the total aquaculture, being the clams the most important species (INE, 2013).

V. decussata and *V. philippinarum* have been successfully employed throughout the world in ecotoxicological studies in order to assess organic and metal pollution under laboratory and field conditions, demonstrating to be suitable bioindicator species in marine environment quality assessment (e.g. Aguirre-Martínez et al., 2013a; Bebianno et al., 1993; Bebianno and Serafim, 2003; Dellali et al., 2001; Gomez-arisa et al., 1999; Martin-Diaz et al., 2007). *V. decussata* (Linnaeus, 1758), the European clam, can be found in Atlantic from the north of Africa to the south of Scandinavia, and in Mediterranean waters (Flassch and Leborgne, 1992; Usero et al., 1997). In Portugal, the main production areas of this species are the Ria de Aveiro and the Ria Formosa, being an endemic species of these ecosystems (Matias et al., 2013) and representing an important economic sector for aquaculture revenue. *V. philippinarum* (Adams & Reeve, 1850), the Manila clam, is an endemic species in the Indo-Pacific region but it was introduced in 1970 in North European Atlantic and Mediterranean coastal waters for commercial exploitation/cultivation due to its high growth rates and easy culture (Flassch and Leborgne, 1992; Delgado and Pérez-Camacho, 2007). This species is the most important shell species on the market and in 2011 was among the most cultivated species in the world, with a total of 3 681 436 tonnes (FAO, 2011). In Portugal, *V. philippinarum* was recently introduced in some aquatic systems (Tagus estuary and the Ria de Aveiro), being registered in the Tagus estuary in 2000 (ICES, 2011). In the Ria de Aveiro these two clam species coexist, sharing and competing for similar requirements. However, *V. philippinarum* has been rapidly spreading throughout this ecosystem, occupying low and high contaminated sites, possibly being responsible for the decrease in the abundance of the native species *V. decussata* (Antunes et al., 2013). This decline was already observed in other ecosystems as in Arcachon bay (Venice Lagoon) in which *V. philippinarum* was reported to be more resistant to environmental stress than the native species *V. decussata* leading to its replacement (Solidoro et al., 2000; Pravoni et al., 2006; Usero et al., 1997). Pravoni et al. (2006) reported that *V. philipinarum* shows a fast growth rate, extended breeding season, high number of offspring, which confer better chances when competing with other species. Thus, in the presence of an environment contaminated with pharmaceutical drugs, possibly *V. philippinarum* possess a higher ability to cope with this stress compared to *V. decussata* (Antunes et al., 2013) contributing to the decline of the native species. However, little information is available regarding the reasons behind this process.

In this way, to study the impact of CBZ in the Ria de Aveiro, the clams *V. decussata* and *V. philippinarum* were selected as bioindicators (Figure 2.2). The assessment of CBZ ecotoxicological risk using these species could provide important knowledge on the performance and health status of these clams which generate information on the extension of anthropogenic contamination in the Ria de Aveiro.

2.2. Experimental conditions – Toxicity tests

For the present study individuals (230 organisms for each species), collected by divers by hand in the Mira channel, were selected with similar size in order to minimize the effect of body weight on physiological and biochemical response and CBZ uptake. The mean length and width of both clams' species were, respectively, 4.5±0.5 cm and 3.5±0.4 cm (Figure 2.3). After sampling, organisms were transported to the laboratory where they were depurated during 48 h in seawater (salinity 25.0), under continuous aeration, at a temperature of 18±1^{o}C and a photoperiod of 12:12 h (light/dark). Moreover, in this work it was chosen a salinity of 25.0 instead of 28.0 as in previous works (Freitas et al., 2012) in order to minimize matrix effects caused by higher salt concentrations in CBZ quantification in clams. The salinity values are represented without units, as defined by UNESCO Practical Salinity Scale of 1978 (PSS78) which considers salinity as a conductivity ratio, and thus, dimensionless (NASA, 2010). The effects of salinity variation (0-42) on *V. philippinarum* during 7 d were studied, being conclude that the range between 21 and 28, where the salinity 25 is included, was optimal for this species (personal communication).

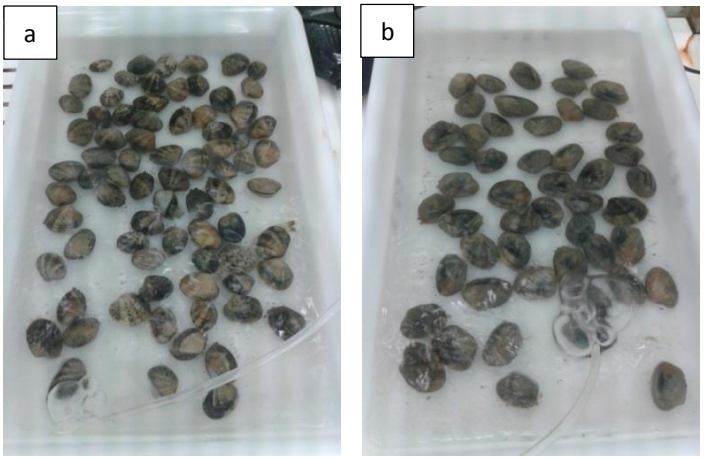

Figure 2.2. Test organisms used in the acute and chronic toxicity tests: (a) *V. philippinarum* and (b) *V. decussata*.

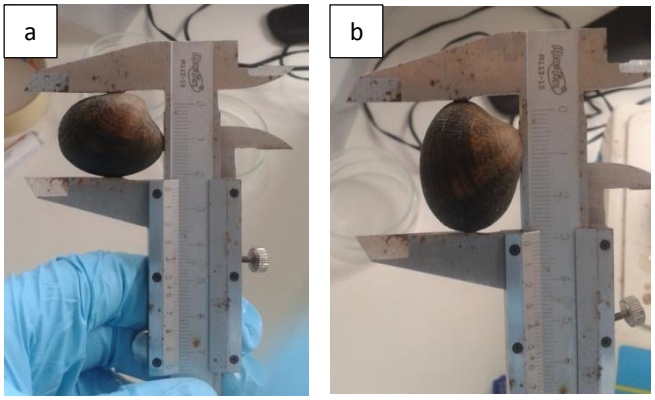

Figure 2.3. Measurement of the clams' width (a) and length (b) using a caliper.

Acute and chronic toxicity assays were performed in order to assess the impact of CBZ in the clams previously referred. In Table 2.1 are represented the experimental conditions of the toxicity assays performed. Acute and chronic exposures lasted, respectively, 96 h (performed in October) and 28 d (performed in February). Other studies evaluated the toxicity of CBZ in aquatic organisms using the same or similar exposure periods (Aguirre-Martínez et al., 2013b; Contardo-Jara et al., 2011). The acute and chronic tests were conducted by exposing, respectively, 95 and 40 organisms of each species (*V. decussata* and *V. philippinarum*), distributed by five concentrations of CBZ (control=0.00; 0.03; 0.30; 3.00 and 9.00 µg/L) with 19 replicates per concentration for the acute test and 8 replicates per concentration for the chronic test (one organism/replicate). The tested concentration range of CBZ was selected based on concentrations found in the environment, as referred before. For each replicate, organisms were placed in plastic containers filled with 300 mL of medium (artificial seawater, salinity 25), and submitted to the conditions of temperature, aeration and photoperiod reported previously. Figure 2.4 illustrates the experimental setup of the toxicity assays performed in this work. Daily, animals were checked for mortality. Organisms were considered dead when their shells gaped and failed to shut again after external stimulus. During the experiment, water was renewed every 48 h for the acute test and twice a week for the chronic test (after a 4 and 3 d cycle), and the concentrations of CBZ re-established. In the case of the chronic test, at each water renewal, the clams were fed with *Nannochloropsis gaditana* (1.99×10^6 cells/mL). At the end of the exposures, the surviving organisms were frozen at -20^{o}C for further analysis. With the aim to study the losses of CBZ during the experiment, a blank test, with no organisms, was performed for each condition of CBZ contamination (0.03; 0.30; 3.00 and 9.00 µg/L) and submitted to the same acute and chronic experimental conditions.

Table 2.1. Experimental conditions used to perform the acute and chronic toxicity tests.

		Toxicity tests	
Conditions		**Acute test**	**Chronic test**
Exposure duration		96 h	28 d
Test organisms		*V. decussata* *V. philippinarum*	
Nº of organisms used		95 for each species	40 for each species
CBZ concentrations		Control = 0.00 μg/L CBZ: 0.03, 0.30, 3.00, 9.00 μg/L	
Conditions	Salinity	25	
	Temperature	18 ± 1°C	
	Photoperiod	12 h light : 12 h dark	
	Water renewal	Every 48 h	Twice a week
	Food	-	*N. gaditana* (1.99×10^6 cells/mL)

Figure 2.4. Toxicity assays experimental setup.

2.3. Physiological parameters

The *condition index* (CI) can give meaningful information about the general physiological status of the animals, being often used to evaluate bivalve condition in environmental studies. A low value for this index indicates that a major biological effort has been expended, for example for the energy maintenance under poor environmental conditions or disease (Lucas and Beninger, 1985).

The deleterious effects of contaminants on *clearance rate* (CR) have been showed. At higher concentrations of contaminants, bivalves elicit avoidance behavior through shell closure to prevent tissue exposure. Under lower concentrations, ciliary beat is inhibited reducing food intake (Nicholson and Lam, 2005).

The *survival in air* (SA) test is considered as one of the simplest, feasible, reproducible and cost-effective for evaluating the negative effects of environmental stress in bivalves, having the potential of becoming a universally applicable stress parameter in marine and estuarine waters (Eertman et al., 1993).

Glycogen (GLYC) has been recognized as the principal energy storage in bivalves serving also as an energy reserve under unfavorable environmental conditions. Moreover, this parameter was reported as being a useful biomarker because changes in tissue concentrations are not as transient or as sensitive to non-toxicant stressors as many other metabolic responses (Lagadic et al., 1994).

The total protein content (PROT) has been showed to be altered when the organisms are under oxidative stress, due to the formation of carbonyl groups on proteins as a result of reactive oxygen species attack (Li et al., 2009). Moreover, an induction in enzymes used for antioxidant system defense can also result in an increase in total protein content.

In this way, the assessment of CI, CR, SA, GLYC and PROT provide a rapid initial screening about the bivalves' health status under different stress conditions, and for this reason they were analyzed presently. The parameters CI, CR and SA were applied only for the clams exposed to the acute test due to the reduced number of organisms that survived after the chronic exposure.

2.3.1. Condition index

The *Condition Index* (CI), measured with the objective of following the clams physiological status during the assay, was determined using clams non-exposed (control condition) and exposed to CBZ. For each condition eleven replicates were used. CI values were calculated following the equation:

$$CI\ (\%) = (\text{dry tissue weight}) / (\text{dry shell weight}) \times 100$$

where CI (%) corresponded to the percentage of the ratio between the dry weight of the soft tissues (g) and the dry weight of the shell (g) (Figure 2.5) (Matozzo et al., 2012a). Dry weight of soft tissues and shells were obtained at 60^{o}C, until reaching constant weight.

After exposure, for each tested concentration, the CI values were compared with the ones obtained for clams not contaminated (control).

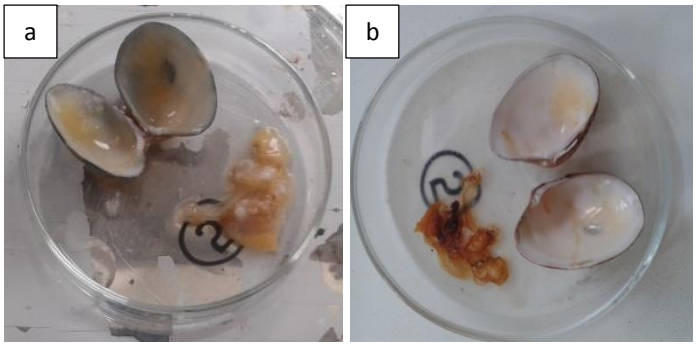

Figure 2.5. Clams' fresh tissue and shell (a). Clams' dry tissue and shell (b).

2.3.2. Clearance rate

The *clearance rate* (CR) was estimated adapting a previously developed method (Coughlan, 1969) based on the rate of absorption of a neutral red dye by animals (Masilamoni et al., 2002). This rate was determined to study how CBZ was affecting the clams' filtration capacity, by comparison with the control condition. For this assay it was prepared a stock solution of Congo Red (1.5 g/L) in deionized water. A known concentration of this solution (15 mg/L) was added to the CBZ exposed clams (n=3 per condition), after 96 h of exposure. In order to assess the initial concentration of the dye, a positive control, with no organisms, was prepared in deionized water by adding Congo Red (15 mg/L). After observing the valve aperture, the dye concentration in aliquots of 1 mL of the solution was measured spectrophotometrically at 498 nm (t_0). This

procedure was repeated in intervals of 1h during 3h. The clearance rate (L/h) was calculated using the equation (Coughlan 1969):

$$CR = (V/nt) \times \log (C_i/C_t)$$

where V is volume in the container (L), n is the number of organisms used, t is the time (h), C_i is the initial concentration of the dye, and C_t is the final concentration after time t.

2.3.3. Survival in air

To measure the capacity of animals to *survive in air* (SA), at the end of the exposure, 3 animals per condition were maintained in the plastic containers with filter paper that was soaked with artificial seawater to keep the humidity, at a constant temperature (18±1^{o}C) and a photoperiod of 12:12 h (light/dark). When the open valves did not close after mechanical stimulation the animals were considered dead. This procedure was repeated daily until the death of all organisms was recorded.

2.3.4. Glycogen and total protein content

For GLYC and PROT determination, frozen organisms (soft tissues) were mechanically homogenized, in a mill, with liquid nitrogen. For each organism, the homogenized tissues were distributed per aliquots of 0.5 g. The samples were sonicated for 15 s at 4^{o}C and centrifuged for 10 min at 10 000 *g* at 4^{o}C and the supernatant extracted used for the analysis.

Glycogen was quantified according to the sulphuric acid method, as described by Dubois et al. (1956). In the presence of sulfuric acid, glycosidic bonds between glucose units of glycogen are cleaved forming monomers. With the addition of phenol, furfural derivatives are formed resulting in a yellow color product that can be measured spectrophotometrically. Glycogen concentrations were determined against glucose standards (0-2 mg/mL). The extraction was done using 0.5 g of pulverized clams and sodium phosphate buffer (1:2, w/v), pH 7.0 (50 mM sodium dihydrogen phosphate monohydrate, 50 mM disodium hydrogen phosphate dihydrate, 1% (v/v) Triton X-100). To every sample (diluted 25x), 100 µL of 5% (v/v) phenol and 600 µL of sulfuric acid (H_2SO_4) 98% were added, shaken and incubated at room temperature for 30 min. Absorbance was measured at 492 nm. The concentration of GLYC was expressed in mg per g of fresh tissue.

Total protein content was determined according to the spectrophotometric method of Biuret (Robinson and Hogden, 1940), using bovine serum albumin (BSA) as standards (0-40 mg/mL). In this

method, cooper (II) ion forms violet-colored coordination complexes with peptide bonds in alkaline solutions. Supernatants were obtained using the same extraction buffer referred for glycogen, in the same conditions. Biuret reagent (600 µL) was added to the samples (50 µL). The mixture was shaken and let to incubate at 30ºC during 10 min. At the end of this time absorbance was read at 540 nm. Results were expressed in mg per g of fresh tissue.

2.4. Biochemical parameters

Bivalves have the capacity to induce biochemical defense and repair mechanisms in order to deal with environmental stressors (Almeida et al., 2007). In this sense, to assess the acute and chronic toxicity of CBZ on aquatic biota, several biochemical parameters have been applied (Aguirre-Martínez et al., 2013b; Martin-Díaz et al., 2009a; 2009b; Li et al., 2011; 2010). Many studies regarding the effects of CBZ in aquatic organisms are centered on responses that can potentially lead to an oxidative stress condition. This condition results from the imbalance between the generation and the neutralization of reactive oxygen species (ROS) by antioxidant mechanisms (Almeida et al., 2007). ROS as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) or hydroxyl radical ($OH^{\cdot}$) can be produced as byproducts of cellular metabolism of CBZ. The involvement of cytochrome P450 (mainly CYP3A4) and glutathione S-transferase (GST) in the biotransformation of CBZ in bivalves has been studied (Martin-Diaz et al., 2009a). Glutathione S-transferases are involved in the conjugation of xenobiotics with *glutathione* (GSH) in order to facilitate the excretion of chemicals after phase I biotransformation. Tsiaka et al. (2013) reported an increase in superoxide anions and nitric oxide (in terms of nitrite) in the mussel *M. galloprovincialis* hemocytes exposed to CBZ. The uptake of CBZ by organisms and its accumulation can also lead to lysosomal membrane impairment, resulting in the release of hydrolytic enzymes and ROS into the cytosol responsible for the induction of oxidative stress. Bivalve's defenses against ROS consists of low molecular weight free radical scavengers, such as GSH and antioxidant enzymatic defenses such *superoxide dismutase* (SOD) which reduces the superoxide anion into hydrogen peroxide; *catalase* (CAT) which reduces H_2O_2 produced by SOD to water and oxygen, and glutathione peroxidase (GPx) which reduces peroxides (H_2O_2 or hydroperoxides) by oxidizing two molecules of glutathione (Winston and Di Giulio, 1991). *Glutathione reductase* (GR) plays also an important role in cellular antioxidant defense protection, catalyzing the reduction of oxidized glutathione (GSSG) to GSH in a NADPH-dependent reaction (Almeida et al., 2007).

Variations in the levels/activities of these parameters have been proposed as useful biomarkers to assess susceptibility of organisms to oxidative stress (Soldatov et al., 2007). If ROS were not immediately trapped by antioxidant defenses they could oxidize different cell components as proteins, DNA and lipids. Lipid peroxidation was also reported in bivalves exposed to CBZ (Martin-Díaz et al., 2009b). Figure 2.6 presents the main biochemical responses involved in the response to a xenobiotic.

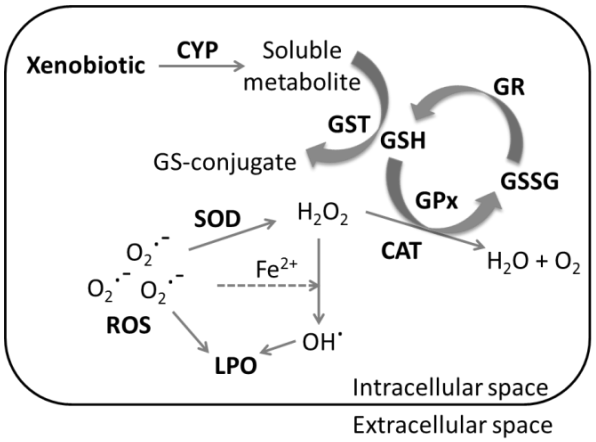

Figure 2.6. Biochemical parameters involved in a xenobiotic response. The abbreviations indicate: CAT, catalase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; GPx, glutathione peroxidase; GR, glutathione reductase; CYP, cytochrome P450 enzymes; LPO, lipid peroxidation; SOD, superoxide dismutase; ROS, reactive oxygen species.

In this way, in the present study it was evaluated the presence of oxidative stress through the occurrence of lipid peroxidation (LPO) in the clams' tissues. Moreover, GSH and its ratio with GSSG (oxidized glutathione) were analyzed as a marker of oxidative stress. The performance of antioxidant defense enzymes, namely SOD, CAT and GR, was evaluated in order to study the response of clams to CBZ. Finally, biotransformation enzymes such as GST and CYP3A4 were analyzed to confirm the presence of CBZ oxidative metabolism in the generation of ROS.

For these biochemical measurements, frozen organisms (soft tissues) were also homogenized with liquid nitrogen, and the homogenized tissues were distributed per aliquots of 0.5 g and the supernatants extracted using the same conditions as referred previously for GLYC and PROT. Supernatants were stored at -20^{o}C or used immediately to determine the biochemical parameters indicated before. These parameters were quantified for clams of both acute and chronic toxicity tests, with the exception of CYP3A4 which was quantified only for the acute test

due to the lack of surviving organisms from the chronic test. All the biochemical parameters were performed twice, using five replicates for each.

2.4.1. Indicators of oxidative stress

Lipid Peroxidation (LPO) was measured by the quantification of TBARS (ThioBarbituric Acid Reactive Substances), according to the protocol described by Ohkawa et al. (1979). The supernatants were extracted using 0.5 g of pulverized clams and 20% (v/v) trichloroacetic acid (TCA) (1:2, w/v) and then it was added 300 µL of thiobarbituric acid (TBA) (0.5% in 20% (v/v) TCA). Samples were incubated at 96^{o}C during 25 min. The reaction was stopped by transferring samples to ice. In this method, the byproducts of lipid peroxidation such as malondialdehyde (MDA) react with TBA, forming a colored product which can be read spectrophotometrically. The amount of MDA, was quantified at a wavelength of 535 nm (ε = 156 $mM^{-1}cm^{-1}$). Lipid peroxidation levels were expressed in nmol of MDA formed per g of fresh tissue.

Total glutathione content (GSH_t, expressed as SH equivalents, GSH+GSSG) was determined using the method described by Anderson (1985). This method employs the reaction of GSH present in the samples with Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid, DTNB), giving rise to a product that can be quantified spectrophotometrically. Glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH) are added to recycle the oxidized glutathione existent in the cells to GSH, enabling the quantification of total GSH with DTNB. Supernatants were extracted using 0.5 g of pulverized clams and 50 mM potassium phosphate buffer (1:2, w/v), pH 7.0 (50 mM dipotassium phosphate; 50 mM potassium dihydrogen phosphate; 1 mM ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1% (v/v) polyvinylpyrrolidone (PVP); 1 mM dithiothreitol (DTT)). Glutathione standards (0-500 µmol/L) and samples were incubated for 5 min at room temperature with 550 µL of potassium phosphate buffer, pH 7.0 (50 mM dipotassium phosphate; 50 mM potassium dihydrogen phosphate); 20 µL of 30 mM NADPH; 50 µL of 10 mM DTNB and 10 µL of 10 U/mL GR. Absorbance was measured at 412 nm. GSH_t was expressed as mmol per g of fresh tissue.

Reduced glutathione content (GSH) was determined according to Moron et al. (1979) with some modifications. Supernatants were extracted using 0.5 g of pulverized clams and 20% (v/v) TCA (1:2, w/v). Glutathione standards (0-500 µmol/L) were prepared in 20% (v/v) TCA. The samples and standards were neutralized with 2 M sodium hydroxide (NaOH). Then, 50 mM potassium phosphate

buffer (pH 7.0) and 50 µL of 10 mM DTNB (final volume of 620 µL) were added. Samples and standards were incubated during 5 min at room temperature and after this time, absorbance was measured at 412 nm. GSH was expressed as nmol per g of fresh tissue. Oxidized glutathione (GSSG) was calculated as being the difference between total and reduced glutathione and expressed as nmol per g of fresh tissue. The ratio GSH/GSSG was determined based on these data.

2.4.2. Antioxidant enzymes

Superoxide dismutase activity (SOD) was determined based on the method of Beauchamp and Fridovich (1971). In this assay, superoxide anions are generated from conversion, by xanthine oxidase, of xanthine and molecular oxygen to uric acid and hydrogen peroxide. The superoxide anion then converts a tetrazolium salt into a formazan dye. The addition of SOD to this reaction reduces superoxide anion levels, thereby lowering the rate of formazan dye formation. In this way, SOD activity is measured as the percent inhibition of the rate of formazan dye formation. For this method, supernatants were extracted using 0.5 g of pulverized clams and 50 mM potassium phosphate buffer (1:2, w/v), pH 7.0 (50 mM dipotassium phosphate; 50 mM potassium dihydrogen phosphate; 1 mM EDTA; 1% (v/v) Triton X-100; 1% (v/v) PVP; 1 mM DTT). Standards of SOD were prepared (0.25-60 U/mL). In a microplate, samples (25 µL) were incubated with 25 µL of 56.1 mU/mL xanthine oxidase and 250 µL of reaction buffer (50 mM Tris-HCl, pH 8.0; 0.1 mM diethylene triamine pentaacetic acid (DTPA); 0.1 mM hypoxanthine and 68.4 µM nitro blue tetrazolium (NBT)). Standards (25 µL) were incubated with 25 µL of xanthine oxidase, 25 µL of extraction buffer and 225 µL of reaction buffer. SOD activity was measured spectrophotometrically at 560 nm in a microplate reader. The enzymatic activity was expressed in U/g of fresh tissue, where U represents the quantity of the enzyme that catalyzes the conversion of 1 µmol of substrate per min.

Catalase activity (CAT) was quantified according to Lars et al. (1988). In this method, CAT activity was measured taking into account its peroxidatic function, with methanol as the hydrogen donor in the presence of hydrogen peroxide. The formaldehyde produced is determined with Purpald (4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole) as a chromogen. To obtain a colored compound, the product of the reaction between formaldehyde and Purpald is oxidized by potassium periodate. Supernatants were extracted using 0.5 g of pulverized clams and 50 mM potassium phosphate buffer (1:2, w/v), pH 7.0 (also used for SOD determination). Standards of formaldehyde (0-150 µM) were prepared. In a microplate, samples and standards (25 µL) were incubated with 125 µL of 50 mM potassium phosphate buffer (pH 7.0), 37.5 µL of methanol and 25

µL of 35.28 mM hydrogen peroxide. After, the microplate was incubated at room temperature during 20 min in a shaker. To stop this reaction it was added 37.5 µL of 10 M potassium hydroxide and 37.5 µL of 34.2 mM of Purpald. Again, the microplate was incubated during 10 min at room temperature in a shaker. At the end of this time, it was incubated with 12.5 µL of 65.2 mM potassium periodate, at room temperature for a period of 5 min in a shaker. Then, the absorbance was read at 540 nm in a microplate reader. Catalase activity was expressed in U/g of fresh tissue, where U, in this case, represents the quantity of enzyme that catalyzes the conversion of 1 nmol of substrate per min.

The activity of *glutathione reductase* (GR) was determined according to Carlberg and Mannervick (1985). Supernatants analyzed were obtained using 0.5 g of pulverized clams and sodium phosphate buffer (1:2, w/v), pH 7.0 (50 mM sodium dihydrogen phosphate monohydrate, 50 mM disodium hydrogen phosphate dihydrate, 1% (v/v) Triton X-100). In a microplate it was added 100 µL of 200 mM potassium phosphate buffer pH 7.0 (200 mM dipotassium phosphate; 200 mM potassium dihydrogen phosphate; 2 mM EDTA), 30 µL of 10 mM GSSG, 90 µL of deionized water and 50 µL of samples (dilution 1:5). The reaction was initiated with the addition of 30 µL of 1 mM NADPH. The potassium phosphate buffer and deionized water were equilibrated first at 25^{o}C. Glutathione reductase activity was measured spectrophotometrically at 340 nm (ε = 4.665 $mM^{-1}cm^{-1}$) in a microplate reader. Absorbance values were read at intervals of 10 s during 5 min. GR activity was expressed in U/g of fresh weight (U = µmol/min).

2.4.3. Biotransformation enzymes

The activity of *glutathione S-transferase* (GST) was determined following an adaptation of the method described by Habig et al. (1974). This enzyme catalyzes the conjugation reaction of GSH with electrophilic substrates such as 1-chloro-2,4-dinitrobenzene (CDNB), which recognizes all GST isoenzymes in tissues (Vernouillet et al., 2010). Supernatants analyzed were obtained using 0.5 g of pulverized clams and sodium phosphate buffer (1:2, w/v), pH 7.0 (50 mM sodium dihydrogen phosphate monohydrate, 50 mM disodium hydrogen phosphate dihydrate, 1% (v/v) Triton X-100). In a microplate samples (100 µL of supernatant) were incubated with 200 µL of a reaction solution consisting of 60 mM CDNB, 10 mM GSH and 0.1 M potassium phosphate buffer, pH 6.5 (0.1 M dipotassium phosphate, 0.1 M potassium dihydrogen phosphate). GST activity was measured spectrophotometrically at 340 nm (ε = 9.6 $mM^{-1}cm^{-1}$) in a microplate reader after the addition of

the reaction solution. Absorbance values were read at intervals of 10 s during 5 min. The enzymatic activity was expressed in U/g of fresh tissue (U = µmol/min).

The activity of *cytochrome P450 3A4* (CYP3A4) was determined in supernatants extracted using isolated clam's hepatopancreas and sodium phosphate buffer (1:2, w/v), pH 7.4 (0.1 M sodium dihydrogen phosphate monohydrate; 0.15 M potassium chloride (KCl); 1mM EDTA; 1 mM DTT). Standards of resorufin (0.01-0.08 µmol/L) and specific substrate resorufin benzyl ether (BzRes) were used (Quinn et al., 2004). The reaction mixture consisted of samples (dilution 1:3) or standards, 0.5 µmol/L BzRes and 5 mmol/L NADPH. The signal of resorufin formation was read after 32 sec of incubation with NADPH by fluorometry (excitation wavelength 530 nm, emission wavelength 585 nm). Enzyme activity was expressed in nmol/min/g of fresh tissue.

2.5. Carbamazepine quantification by ELISA

Immunoassays have been used as a promising alternative to the reference techniques (gas and liquid chromatography coupled with single or tandem mass spectrometry) to quantify CBZ in environmental matrices. The direct competitive immunoassay ELISA (Enzyme-Linked Immunosorbent Assay) has been used to detect CBZ in water (Bahlmann et al., 2009; Calisto et al., 2011a), however, the quantification of CBZ in biological matrices is less regarded. To the best of my knowledge, only one work, performed by Gagné et al. (2006c) applied a competitive enzymatic immunoassay to quantify CBZ in the soft tissue homogenate extracts of the freshwater mussel (*E. complanata*). In this way, for this work I intended to study the performance of the immunoassay ELISA to quantify directly, CBZ in clams' tissues of the acute and chronic tests. The direct competitive ELISA is based on a direct competition of the antigen (the analyte) and tracer (an analyte analogue linked to an enzyme) for antibody binding sites that is immobilized in a 96-well microtiter plate. The removal of the remaining free reagents in solution (not bound to the antibody) and the addition of a substrate results in development of color (catalyzed by the enzymatic tracer), which can be measured spectrophotometrically. The optical density is proportional to the amount of tracer bound and inversely proportional to the amount of antigen present in samples/standards (Law, 2005).

2.5.1. Immunoassay procedure and calibration curve

Carbamazepine was quantified in the clams' tissues, blanks and water samples as will be described in the following sections through the application of a direct competitive ELISA, following the procedure developed by Bahlmann et al. (2009) and optimized by Calisto et al. (2011a). This assay is based on a direct competition of the antigen (CBZ) and tracer (CBZ analogue linked to the enzyme horseradish peroxidase) for the primary antibody (monoclonal antibody against CBZ) binding sites that is linked to the secondary antibody immobilized in a 96-well microtiter plate. After a period of incubation, the plate is washed to remove the free reagents in solution and the substrate is added. This substrate will be used by the enzyme linked to the CBZ analogue (tracer), resulting in the development of colored product. The signal produced is proportional to the amount of tracer bound to the antibody. However if a higher content of CBZ, present in samples/standards, bind to the antibody, the opposite will be seen, with the color produced being inversely proportional to the amount of CBZ. As less tracer bind the substrate, a lower optical signal will be developed.

In this way, high-binding microtiter plates were coated with polyclonal antibody against mouse IgG (1 mg/L, 200 µL per well) diluted in phosphate buffered saline (PBS) (10 mM sodium dihydrogen phosphate, 70 mM sodium hydrogen phosphate, 145 mM sodium chloride, pH 7.6). The plates were covered with Parafilm® to prevent evaporation and incubated overnight (approximately 16 to 18 h) on a Titramax 100 plate shaker at 900 rpm. After overnight incubation, the plates were washed three times with PBS containing 0.05% (v/v) Tween™ 20 (PBS-T) using an automatic 8-channel plate washer. Monoclonal antibody against CBZ also diluted in PBS (7.61×10^{-5} mg/mL, 200 µL per well) was added and incubated for 1 h. Then a three-cycle washing step of the plate was performed again. After, 50 µL of tracer solution (147 pmol/L in sample buffer) and 150 µL of CBZ standard solutions and samples were added per well and incubated for 30 min. Sample buffer consisted of 1 M glycine, 3 M sodium chloride and 2% (w/v) EDTA, pH 9.5. After another three-cycle washing step (PBS-T), 200 µL of substrate solution was added per well and incubated for 30 min. Substrate solution consisted of 540 µL TMB-based solution (41 mM 3,3',5,5'-tetramethylbenzidine (TMB), 8 mM tetrabutylammonium borohydride (TBABH) prepared in dimethylacetamide (DMA) under nitrogen atmosphere) in 21.5 mL of substrate buffer (220 mM citric acid, 0.66 mM sorbic acid potassium salt and 3 mM hydrogen peroxide). The TMB solution was freshly prepared for each run. The enzyme reaction was stopped by the addition of 1M sulfuric acid (100 µL per well) and the optical density was read on a microplate spectrophotometer at 450 nm and referenced to 650 nm. Data was analyzed using SoftMax® Pro Software (version 5.3,

Molecular Devices). All samples and standards were determined in triplicate on each plate. The standards' mean values were fitted to a four-parametric logistic equation (4PL) (Findlay and Dillard, 2007).

For each plate a calibration curve was performed using eight calibrators, with concentrations between 0 and 100 µg/L for a better convergence of the curve fitting. These were prepared, in ultrapure water, by diluting a 10 mg/L stock solution of CBZ (also prepared in ultrapure water).

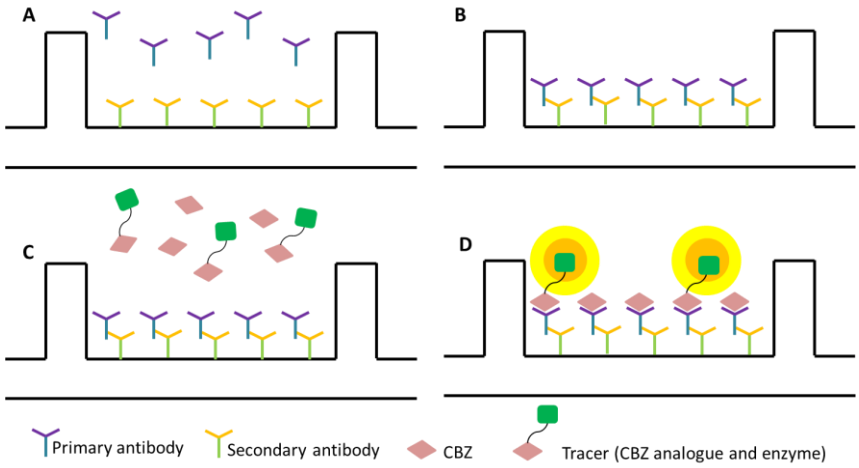

Figure 2.7. Principle of the direct competitive ELISA used in this work. A) Immobilization of the secondary antibody on the microtiter plate symbolized as a well; B) Bound of the primary antibody (monoclonal antibody against CBZ) onto secondary antibody; C) Samples containing CBZ and tracer (CBZ analogue linked to the enzyme) are added to the microplate and directly compete for the antibody binding sites (D). The addition of substrate in the presence of the enzyme labeled tracer results in an enzyme catalyzed colorimetric reaction (represented by the orange and yellow circles).

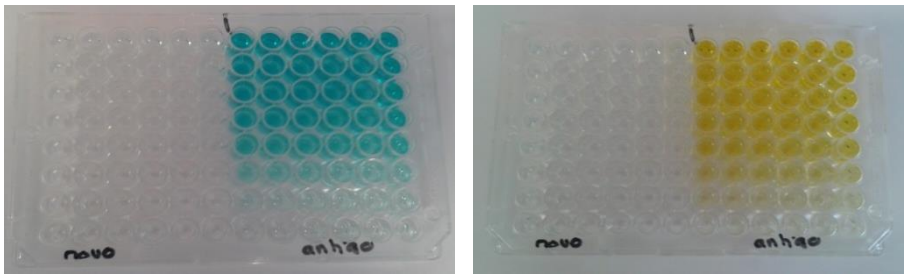

Figure 2.8. ELISA assay. Microtiter plate with reaction developed. Only standards were used and incubated following an increasing order of CBZ concentrations (from top to bottom). After the addition of sulfuric acid to stop the reaction the color changed from blue to yellow. Note that the absence of color in half of the plate is because it was not used.

2.5.2. Quality control

To test the confidence on the results obtained for the CBZ quantification in clams through ELISA, a quality control was performed. For this, supernatants were obtained from non-contaminated clams (collected in the same channel as the clams used in the toxicity tests) by extraction with deionized water (dilution 1:2). The samples were enriched with a known concentration of carbamazepine: 0.10, 0.30, 1.00 and 6.00 µg/L. Also, in order to investigate if sample filtration influenced the CBZ determination by ELISA, the spiked samples were analyzed without supernatant filtration, and with filtration before and after the enrichment with CBZ. These samples were analyzed by ELISA as referred in the section 2.5.1.

2.5.3. Toxicity tests clams tissue – sample preparation

Carbamazepine quantification by ELISA was performed in clams (*V. decussata and V. philippinarum*) non-exposed (control condition) and exposed to CBZ (conditions: 0.03, 0.30, 3.00 and 9.00 µg/L of CBZ) from the acute and chronic toxicity tests. For this quantification, supernatants were obtained by extraction of clams' tissues with deionized water (dilution 1:2). Supernatants were analyzed as they were, with no other treatment. For CBZ concentration of 9.00 µg/L supernatants were diluted (1:2) in ultrapure water.

2.5.4. Blanks and determination of CBZ concentration in water from chronic toxicity test

To investigate the losses of CBZ during the exposure (namely photodegradation or adsorption onto the test vessels), CBZ was quantified in water samples from blanks (conditions 0.03, 0.30, 3.00 and 9.00 µg/L of CBZ), collected at the beginning and at the end of each assay.

The concentration of CBZ in water where clams were exposed was also determined in order to assess the variation of CBZ along the exposure duration. This was done only for the chronic test due to the longer period between water renewals (twice a week). In the acute test because the water was renewed after 48 h and the concentrations re-established, the CBZ was not quantified. A dilution in ultrapure water (1:2) was performed for samples contaminated with CBZ concentration of 9.00 µg/L.

2.6. Data analysis

In order to evaluate the CBZ accumulation by clams, the bioconcentration factor (BCF) was calculated, for each exposure condition, dividing the concentration of CBZ present in clams' tissues by the spiked CBZ concentration (Gobas and Morrison, 2000). Bioconcentration studies investigate the analyte uptake through water under controlled laboratory conditions, being important to assess chemical behavior, environment impact and potential health risks to aquatic organisms. Bioconcentration by aquatic organisms have been reported for pharmaceutical drugs including CBZ (Contardo-Jara et al., 2011; Wang and Gardinali, 2012; Schwaiger, 2004). The current state of research regarding pharmaceuticals bioconcentration in aquatic ecosystem was reviewed by Zenker et al. (2014).

To determine the CBZ ecotoxicological risk, the risk quotient (RQ) was calculated dividing the MEC (measured environmental concentration), the highest CBZ concentration measured in wastewater treatment plants effluents from the Ria de Aveiro (Calisto et al., 2011a), by the predicted no effect concentration (PNEC) (Martin et al., 2014). For RQ determination it was used two different PNEC values: i) the PNEC value of 0.00032 µg/L was determined by Aguirre-Martínez et al. (2013a), by dividing the lowest effective concentration (EC_{50}), the concentration of CBZ that reduced the lysosomal membrane stability in *V. philippinarum,* by an assessment factor of 1000 as indicated in EU Technical Guidance Document (EC, 2003); the PNEC value of 0.42 µg/L calculated by Ferrari et al. (2003) was obtained by an extrapolation using a log-normal distribution of chronic NOEC (no observed effect concentration) values for a set of species (algae, rotifers, crustaceans and fish) after being exposed to CBZ. Moreover, for this determination it was assumed an estimate of hazardous concentration for 5% of species (HC5), which means that 95% of the species in the environment are protected. The PNEC values were calculated dividing HC5 by an assessment factor of 5, as indicated in EC (2003).

Data obtained from physiological and biochemical analyses and CBZ quantification obtained in the acute and chronic toxicity tests was submitted to hypothesis testing using permutation multivariate analysis of variance with the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008). A one-way hierarchical design, with CBZ exposure concentration as the main fixed factor, was followed in this analysis. The pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance. When the main test revealed statistical significant differences ($p \leq 0.05$), pairwise comparisons were performed. The t-statistic in the pair-wise comparisons was evaluated in terms of significance. Values lower than 0.05 were considered as significantly different.

The null hypotheses tested were: a) for each exposure concentration, no significant differences exist between species; b) for each species, no significant differences exist among exposure concentrations. Significant differences ($p \leq 0.05$) among exposure concentrations, for each species, were represented with letters. For each exposure concentration, significant differences comparing the two species were represented with an asterisk. For each clam species, the matrix gathering, the physiological and biochemical responses per exposure concentration were used to calculate the Euclidean distance similarity matrix. This matrix was simplified through the calculation of the distance among centroids matrix based on the 5 exposure conditions, which was then submitted to ordination analysis, performed by Principal Coordinates (PCO). Pearson correlation vectors (r> 0.75) of physiological and biochemical descriptors were provided as supplementary variables being superimposed on the top of the PCO graph.

Chapter 3

Results

3.1. ELISA performance on carbamazepine quantification

3.1.1. Quality control

The results obtained for the quality control (Table 3.1) indicated that in the aqueous extracts (supernatants) from non-contaminated clams by extraction with deionized water and spiked with CBZ, all the concentrations spiked could be quantified through ELISA, revealing that no matrix interferences was occurring. Moreover, the results of the filtration test, realized to observe if the filtration of the aqueous extracts could interfere with the concentration of CBZ determined, showed that the best recoveries were obtained without aqueous extract filtration, reaching similar CBZ concentrations to those of spikes, with average recoveries between 91 and 118%. The filtration step seemed to interfere negatively in the quality of the results, because for some concentrations the recovery was higher than 120%, the upper limit of an acceptable result.

Table 3.1. CBZ concentration (µg/L) determined by ELISA, for the quality control test. Values are the mean (STDEV) of fifteen replicate. Significant differences ($p \leq 0.05$) between exposure concentrations, for each condition (without and with aqueous supernatant (aqueous extract) filtration), are presented with letters (a-d).

Without supernatant filtration		
Spiked concentration (µg/L)	ELISA response (µg/L)	Recoveries (%)
0.10	0.11 (0.03)[a]	110
0.30	0.29 (0.07)[b]	98
1.00	1.2 (0.3)[c]	118
6.00	5.5 (0.6)[d]	91
With supernatant filtration before spike		
0.10	0.20 (0.06)[a]	202
0.30	0.45 (0.1)[b]	150
1.00	1.3 (0.1)[c]	132
6.00	3.9 (0.2)[d]	66
With supernatant filtration after spike		
0.10	0.20 (0.02)[a]	195
0.30	0.35 (0.08)[b]	116
1.00	1.1 (0.4)[c]	110
6.00	4.5 (0.1)[d]	75

3.2. Acute toxicity test

3.2.1. Carbamazepine in clams' soft tissues

The results for CBZ quantification in clams' tissues (Table 3.2) revealed that both species responded similarly to CBZ exposure, showing significant differences among concentrations along the exposure gradient. Except for CBZ 9.00 μg/L, along the CBZ exposure, *V. decussata* presented significantly higher levels of CBZ in tissues than *V. philippinarum*.

Table 3.2 also presents the bioconcentration factor (BCF) determined for each species. These results showed that, for both species, BCF increased along the concentration range and *V. decussata* presented higher values than *V. philippinarum.* Moreover, the results obtained for control and CBZ 0.03 μg/L were below the lower limit of the quantitation range (<LQ) determined by Calisto et al. (2011a) for this ELISA assay (0.024-10 μg/L). In this case, the range defines the lowest and the highest CBZ concentration which can be determined with a maximum allowable relative error of 30%.

Table 3.2. CBZ concentration (μg/g FW), determined by ELISA, and bioconcentration factor (BCF) in *V. decussata* and *V. philippinarum* when exposed to increasing concentrations of CBZ in the acute toxicity test (96 h). Values are the mean (STDEV) of fifteen replicates. Significant differences ($p \leq 0.05$) between exposure concentrations, for each species, are presented with letters (a-c). Significant differences ($p \leq 0.05$) between species, for each exposure concentration, are presented with an asterisk (*). LQ: quantification limit

Exposure concentration (μg/L)	**CBZ in clams (μg/g FW)**		**BCF**	
	V. decussata	*V. philippinarum*	*V. decussata*	*V. philippinarum*
0.00	<LQ	<LQ	-	-
0.03	<LQ	<LQ	-	-
0.30	0.00008 (0.00001)^{a*}	0.000034 (0.000004)^{a}	0.26 (0.02)^{a*}	0.11 (0.01)^{a}
3.00	0.0014 (0.0008)^{b*}	0.0006 (0.0001)^{b}	0.5 (0.2)^{b*}	0.20 (0.04)^{b}
9.00	0.010 (0.004)^{c}	0.0109 (0.0009)^{c}	1.1 (0.4)^{c}	1.2 (0.1)^{c}

3.2.2. Blanks

Aqueous standards of CBZ in seawater with concentrations between 0.03 and 9.00 μg/L, exposed to the same conditions as acute test but with no organisms showed that CBZ was not adsorbed onto the inner surface of containers neither degradation of CBZ was observed during the 96 h exposure (Table 3.3). As the concentration of the standard of 0.03 μg/L is near the lower value of the quantitation range (0.024 μg/L) it was not possible quantify it with good precision (<LQ).

Table 3.3. CBZ concentration (μg/L), determined by ELISA, in blanks obtained at the beginning (day 0) and at the end (day 4) of the assay. Values are the mean (STDEV) of fifteen replicates. Significant differences ($p \leq 0.05$) between quantified CBZ by ELISA for exposure concentrations (spiked) are presented with letters (a-c). Significant differences ($p \leq 0.05$) between exposure days for each spiked concentration are presented with an asterisk (*). LQ: quantification limit

Spiked concentration (μg/L)	CBZ in blanks (μg/L) – Day 0	CBZ in blanks (μg/L) – Day 4
0.00	< LQ	< LQ
0.03	< LQ	< LQ
0.30	0.162 (0.002)[a *]	0.211 (0.009)[a]
3.00	3.1 (0.5)[b]	3.2 (0.9)[b]
9.00	9 (1)[c]	10 (1)[c]

3.2.3. Physiological responses

3.2.3.1. Mortality

After exposure, none of the treatments induced mortality, so the subsequent effects were considered sublethal.

3.2.3.2. Condition index

The results obtained for the condition index (CI) are presented in Figure 3.1A revealing that *V. philippinarum* presented significantly lower levels of CI than *V. decussata* in control, 0.30 and 3.00 μg/L CBZ concentrations. Furthermore, although not significant, for both species, the tendency was for a decrease in CI levels along the exposure range, especially at the higher CBZ concentrations. This pattern was more noticeable for *V. decussata* than for *V. philippinarum.*

3.2.3.3. Clearance rate

Along the exposure range, the clearance rate (CR) results (Figure 3.1B) indicated a similar trend for the two species. *V. philippinarum* presented significantly lower CR at 0.30 and 3.00 μg/L CBZ concentrations comparing to the remaining conditions. *V. decussata* decreased significantly CR at the highest concentration (9.00 μg/L CBZ), also in comparison with the remaining conditions.

3.2.3.4. Survival in air

Although not clear, the results for survival in air (SA) showed a similar response comparing *V. decussata* with *V. philippinarum* (Figure 3.1C). Despite the absence of significant differences, for *V. philippinarum*, there was a tendency for a decrease in SA comparing the control condition and the higher CBZ concentrations (3.00 and 9.00 μg/L). For *V. decussata* this decrease was more evident only at CBZ 9.00 μg/L.

3.2.3.5. Glycogen and total protein content

Alterations in glycogen content (GLYC) are present in Figure 3.1D. At each exposure condition, *V. decussata* presented significantly higher glycogen levels than *V. philippinarum*. However, the variation pattern of GLYC along the CBZ gradient was similar for both species, showing a decrease from the control condition at the lowest concentration (CBZ 0.03 μg/L), followed by a progressive increase as CBZ concentration increased. For *V. decussata*, the reduction in GLYC

content was significant for 0.03 µg/L of CBZ when compared with the remaining conditions. In the case of *V. philippinarum* GLYC content decreased in individuals exposed to all concentrations when compared to the control.

The total protein content (PROT) (Figure 3.1E) obtained for both species indicated that, in general, *V. decussata* presented higher PROT values than *V. philippinarum*. For *V. philippinarum* the PROT content decreased significantly at the highest exposure concentration, comparing with the remaining conditions. In *V. decussata,* PROT significantly increased at concentrations of 0.30, 3.00 and 9.00 µg/L, compared to the control.

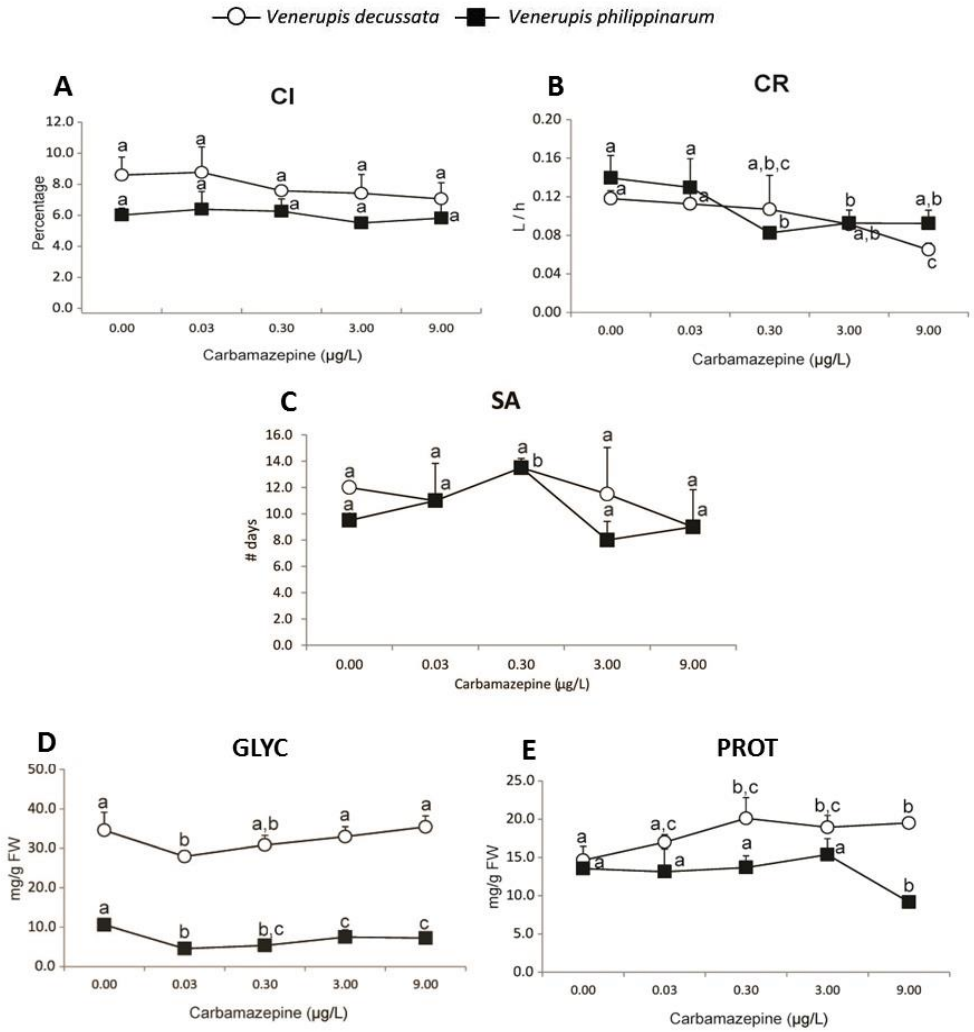

Figure 3.1. Physiological parameters (CI, condition index; CR, clearance rate; SA, survival in air; GLYC, glycogen content; PROT, total protein content) in *V. decussata* and *V. philippinarum* when exposed to increasing concentrations of CBZ in the acute toxicity test (96 h). Values are the mean (STDEV) of eleven (CI), three (CR) or ten (GLYC and PROT) replicates. Significant differences ($p \leq 0.05$) among exposure concentrations, for each species, are presented with letters (a-c).

3.2.4. Biochemical parameters

3.2.4.1. Indicators of oxidative stress

The results for lipid peroxidation (LPO) are represented in Figure 3.2A. Only in the control condition and in the CBZ concentration of 9.00 µg/L LPO levels differed between species, with *V. philippinarum* presenting significantly higher levels than *V. decussata.* For both species, significant differences were found between the control and the higher exposure concentration (9.00 µg/L), where a significant increase was observed for *V. philippinarum,* while for *V. decussata* a significant decrease was noticed.

The results obtained for the total glutathione content (GSH_t, expressed as SH equivalents, GSH+GSSG) are showed in Figure 32B. *V. philippinarum* presented significantly higher GSH_t content than *V. decussata,* when exposed to all CBZ concentrations (except for the control). In both species, the tendency was for an increase in GSH_t content with the increase in CBZ concentration. Although only slightly, *V. decussata* significantly increased GSH_t comparing the control condition and CBZ concentrations of 0.30 and 3.00 µg/L. In *V. philippinarum* the GSH_t was significantly increased along the increasing exposure gradient, although at 9.00 µg/L GSH_t tended to decrease.

Regarding the reduced glutathione (GSH) content results (Figure 3.2C), *V. decussata* decreased significantly this defense comparing the higher CBZ concentrations (0.30, 3.00 and 9.00 µg/L) with the control condition. The response for *V. philippinarum* was the opposite, showing that GSH increased significantly along the concentrations tested. Except for CBZ 3.00 µg/L, *V. philippinarum* showed significantly lower GSH levels than *V. decussata*.

The GSH/GSSG ratio for *V. decussata* decreased when comparing all CBZ conditions tested with the control (Figure 3.2D). Relatively to *V. philippinarum* the tendency was for an increase in GSH/GSSG along CBZ exposure, being significantly higher for CBZ 3.00 and 9.00 µg/L. Further, *V. decussata* presented significantly higher GSH/GSSH values comparing with *V. philippinarum* at all conditions tested.

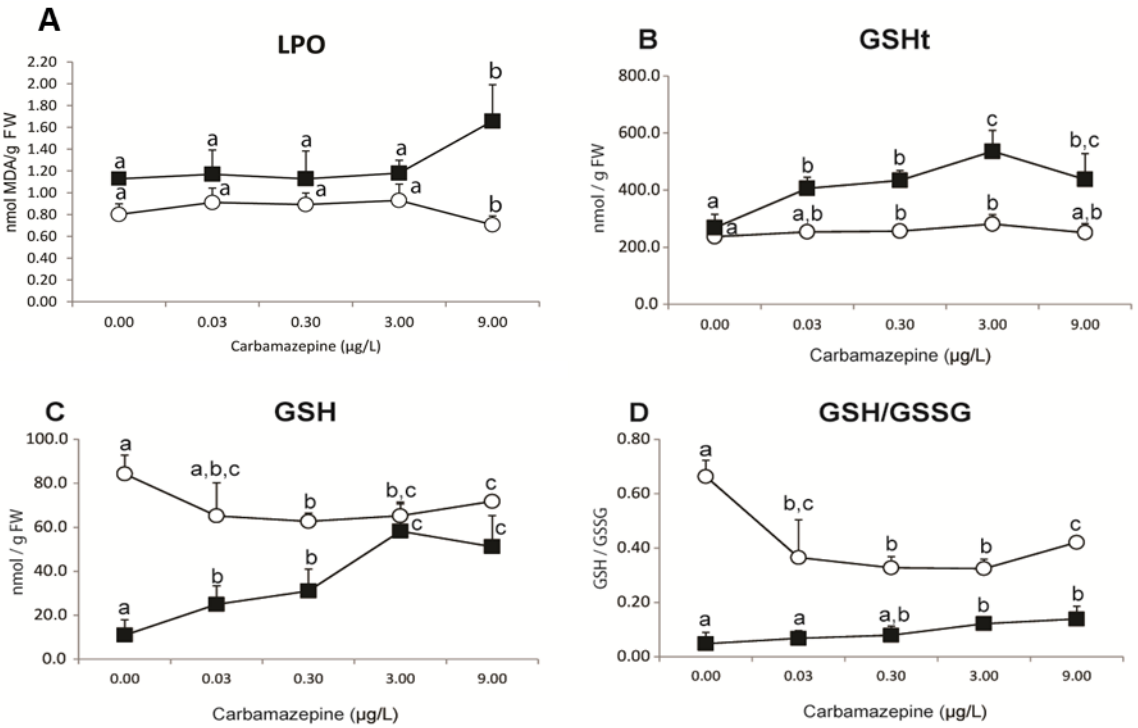

Figure 3.2. Indicators of oxidative stress (LPO, lipid peroxidation; GSH_t, total glutathione; GSH, reduced glutathione; GSH/GSSG, ratio between reduced and oxidized glutathione) in *V. decussata* and *V. philippinarum,* when exposed to increasing concentrations of CBZ in the acute toxicity test (96 h). Values are the mean (STDEV) of ten replicates. Significant differences ($p \leq 0.05$) among exposure concentrations, for each species, are presented with letters (a-c).

3.2.4.2. Antioxidant enzymes

Superoxide dismutase (SOD) activity (Figure 3.3A) was significantly increased for *V. decussata* in CBZ concentrations of 0.30, 3.00 and 9.00 µg/L, and although significantly higher than the control, at the highest CBZ concentration the SOD activity significantly decreased in comparison to the former concentrations (0.30 and 3.00 µg/L). On the other hand, *V. philippinarum* increased significantly SOD activity only at the highest CBZ concentration. At all exposure concentrations, *V. decussata* presented significantly higher SOD activity than *V. philippinarum*.

The activity of catalase (CAT) is presented in Figure 3.3B. *V. decussata* exhibited significantly higher CAT activity than *V. philippinarum* in the control condition and at the two lowest CBZ concentrations (0.03 and 0.30 µg/L). For *V. decussata*, CAT activity significantly decreased in all CBZ exposures, comparing to the control. In the case of *V. philippinarum*, the activity of this enzyme significantly decreased at 0.03 µg/L, increasing in the remaining concentrations.

The analysis of glutathione reductase (GR) activity (Figure 3.3C) demonstrated that *V. decussata* increased significantly the activity of this enzyme for CBZ concentrations of 0.30, 3.00 and 9.00 µg/L, in relation to the remaining conditions. On the other hand, *V. philippinarum* increased the GR activity only at the highest CBZ concentration. With the exception of the control condition, *V. decussata* showed significantly higher GR activity than *V. philippinarum.*

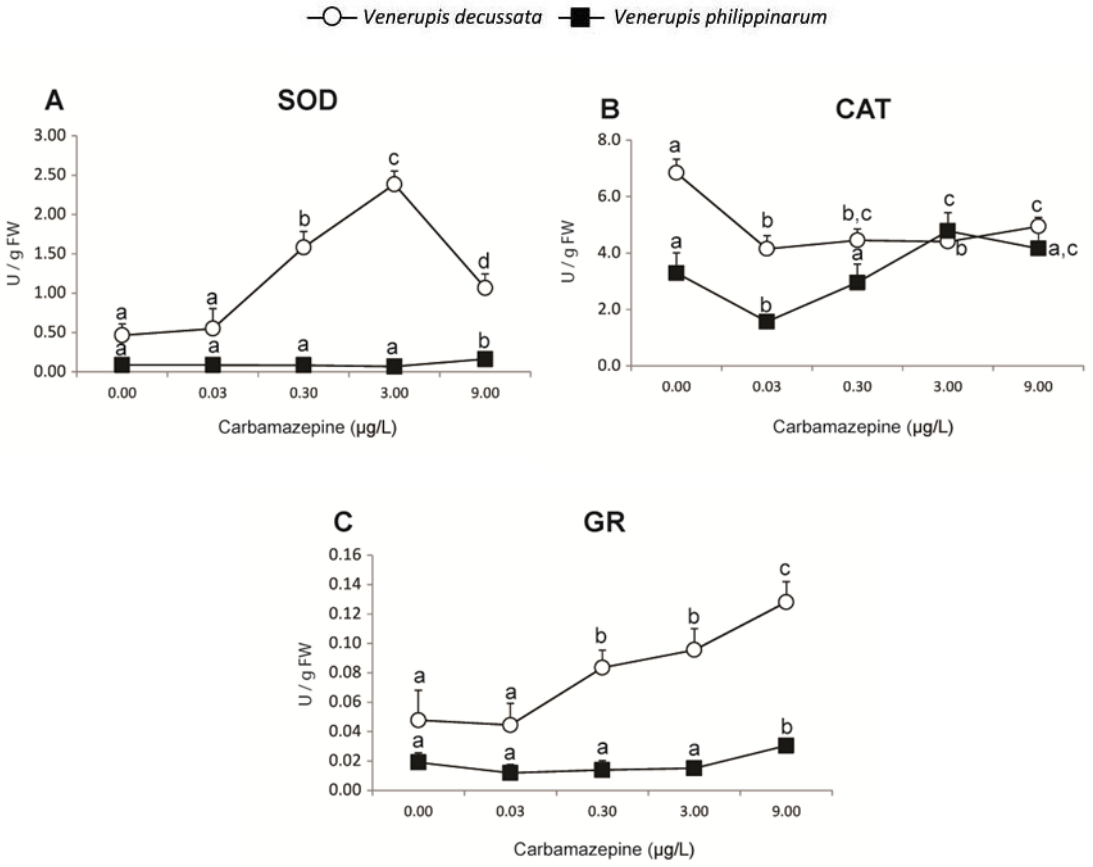

Figure 3.3. Antioxidant enzymes (SOD, superoxide dismutase; CAT, catalase; GR, glutathione reductase) in *V. decussata* and *V. philippinarum,* when exposed to increasing concentrations of CBZ in the acute toxicity test (96 h). Values are the mean (STDEV) of ten replicates. Significant differences ($p \leq 0.05$) among exposure concentrations, for each species, are presented with letters (a-d).

3.2.4.3. Biotransformation enzymes

The activity of glutathione-S-transferase (GST) is represented in Figure 3.4A. Along the exposure gradient *V. decussata* showed significantly higher GST activity than *V. philippinarum*. *V. decussata* increased significantly the GST activity for CBZ concentrations of 0.03 and 3.00 µg/L compared to the control but the activity decreased at 9.00 µg/L. On the other hand, *V. philippinarum* presented a significant decrease in GST activity for CBZ concentrations of 0.03, 3.00 and 9.00 µg/L when comparing to the control condition.

The analysis of CYP3A4 activity (Figure 3.4B) in *V. decussata* revealed a significantly increase in the activity of this enzyme comparing all the concentrations tested with the control. *V. philippinarum* tend to maintain the activity of CYP3A4 along CBZ conditions. Comparing the two species at each exposure concentration, at the control condition and 0.03 µg/L CBZ, *V. philippinarum* presented a significantly higher enzymatic activity than *V. decussata.*

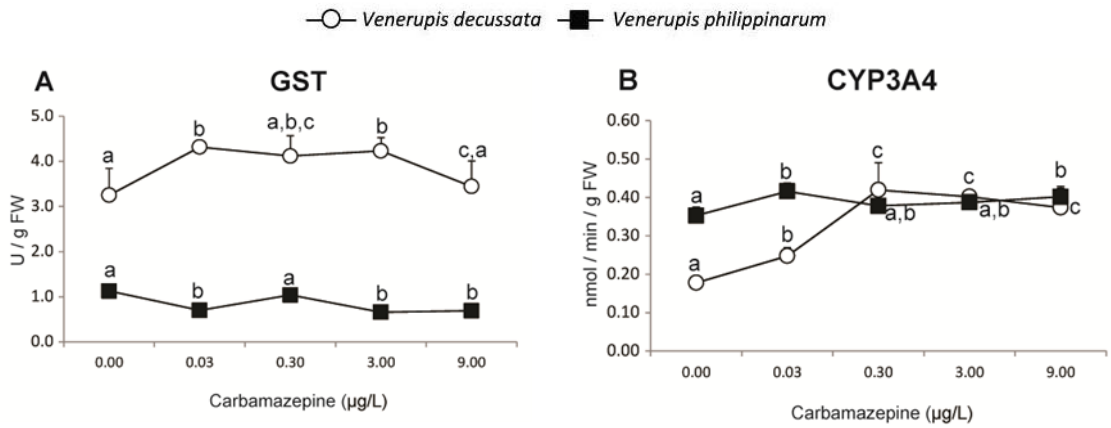

Figure 3.4. Biotransformation enzymes (GST, glutathione S-transferase; CYP3A4, cytochrome P450 3A4) in *V. decussata* and *V. philippinarum,* when exposed to increasing concentrations of CBZ in the acute toxicity test (96 h). Values are the mean (STDEV) of ten replicates. Significant differences ($p \leq 0.05$) among exposure concentrations, for each species, are presented with letters (a-c).

3.2.5. Multivariate analysis

Figure 3.5 shows the centroids PCO ordination graph resulting from applying a multivariate analysis to the physiological and biochemical parameters. The PCO axis 1 explained 65% of the total variation of data, clearly separating the two species based on their physiological and biochemical alterations due to CBZ exposure. The PCO axis 2 only explained 17.5% of the total data variation. In this axis, it was possible to observe that, among conditions, higher differences were noticed for *V.*

decussata than for *V. philippinarum*, which is shown by the lower variation along axis 2, among *V. philippinarum* conditions. The clams exposed to the higher CBZ concentrations (0.30, 3.00 and 9.00 μg/L) for *V. decussata* were characterized by the increase in SOD and GR activity and PROT. Moreover, the PCO indicated that a high correlation (r> 0.75) exists between GST levels and *V. decussata* (especially at higher concentrations). For *V. philippinarum* the clams exposed to the same conditions, a high correlation was observed with LPO and GSH_t levels.

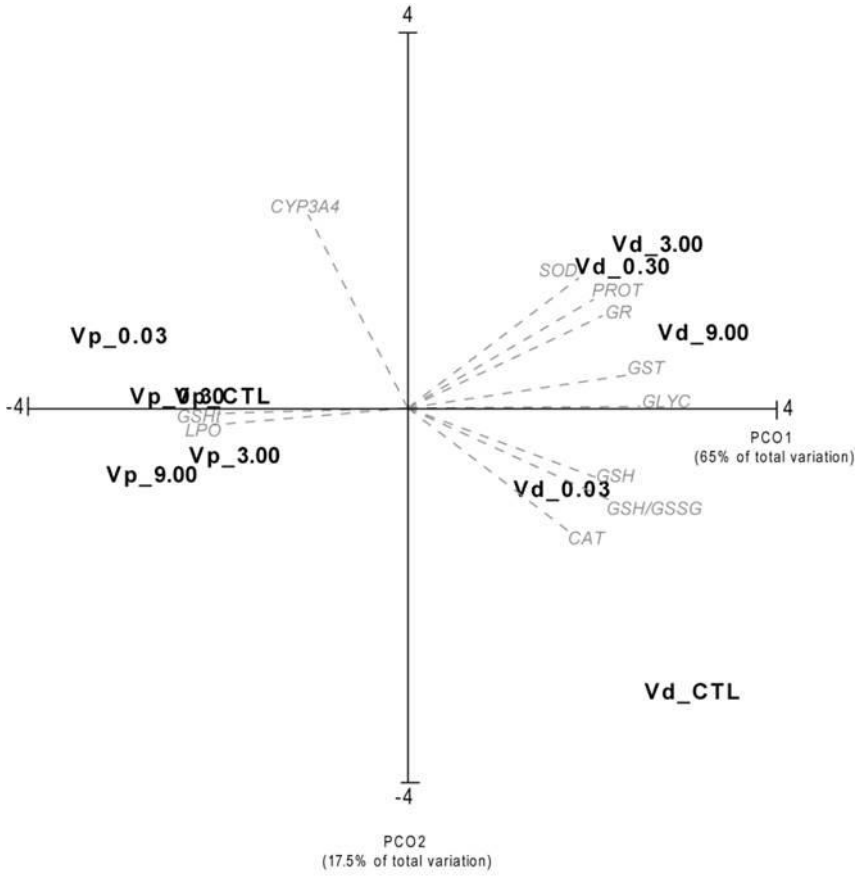

Figure 3.5. Physiological and biochemical responses of *V. decussata* and *V. philippinarum* exposed to increasing CBZ concentrations in the acute toxicity test (96 h), plotted on axes 1 and 2 of a Principal Coordinates (PCO) graph. Physiological and biochemical responses are superimposed on the PCO (r > 0.75). The control (CTL) and concentrations of exposure (0.03, 0.30, 3.00, 9.00 μg/L) are indicated for each species (Vp, *V. philippinarum*; Vd, *V. decussata*). The biomarkers presented are: CAT, catalase; GLYC, glycogen content; GSH, reduced glutathione; GSH/GSSG, ratio between reduced and oxidized glutathione; GSHt, total glutathione; LPO, lipid peroxidation; GST, glutathione S-transferase; PROT, total protein content; SOD, superoxide dismutase; GR, glutathione reductase.

3.3. Chronic toxicity test

3.3.1. Mortality

As referred previously, the clams were considered dead when, after external stimulus their shells gaped and failed to shut again. During the chronic exposure, clams from both species died, even under control condition. *V. philippinarum* reached 50% of mortality on the control condition (results not shown) leading to the necessity to abort the test for this species. Although a reason for this high percentage of death in the control condition was not find, it was assumed that clams came already in a stressful condition from the environment. Another hypothesis raised is that the clams used in the two toxicity tests came from the environment with a different physiological status due to the period of time in which they were collected, and the initial period in the laboratory, before the toxicity tests, maybe was not enough for their adequate depuration/acclimatization. *V. decussata* also presented a high mortality rate in the control condition (37.5 %) (Figure 3.6). However, despite this high mortality rate, the experiment was carried out (28 d) and it was possible to observe a higher mortality values for clams exposed to higher CBZ concentrations (3.00 and 9.00 µg/L) when compared with clams exposed to the lower CBZ concentrations (0.03 and 0.30 µg/L).

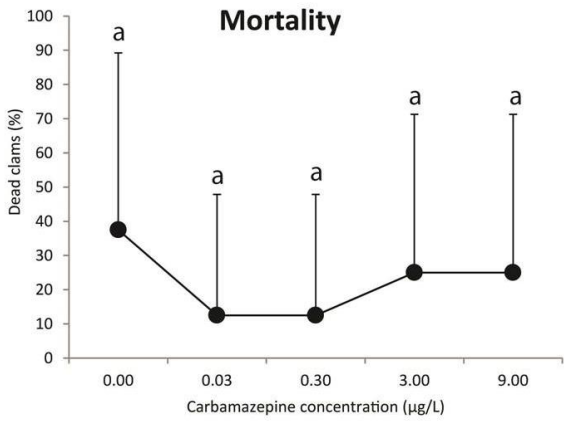

Figure 3.6. Percentage of mortality in *V. decussata* exposed to increasing concentration of CBZ after the chronic toxicity test (28 d).

3.3.2. Why it was decided to proceed with the chronic test analysis?

Although both toxicity tests have not run as expected, with an abnormal mortality in animals under the control condition (not contaminated with CBZ), it was decided to perform the physiological and biochemical parameters and the quantification of CBZ in the clams of the species

V. decussata. Besides the lack of time to perform a new assay, the results showed differences between the control and the exposure to CBZ indicating that the clams were responding to the presence of CBZ in water. However, the results must be interpreted carefully due to the low confidence in the assay. In this way, the present chronic test will serve as a preliminary study to evaluate the toxic effects of CBZ on *V. decussata* when exposed for 28 d to this drug, and thus being more representative of the conditions that occur in the environment. This is of particular importance since the majority of works studying the toxic effects of CBZ are based on acute short term toxicity tests which, by themselves, are not adequate to make a good ecotoxicological risk assessment of this drug.

The results obtained for the chronic toxicity test using *V. decussata* as test organism will be compared with those obtained in the acute test for the same species.

3.3.3. Carbamazepine in clams' soft tissues

The results for CBZ quantification in clams' tissues (Table 3.4) revealed that, for both assays, the clams responded similarly to CBZ exposure, increasing the concentration of CBZ in their tissues along the exposure gradient. *V. decussata* exposed to the chronic test showed a higher increase of CBZ in their tissues compared with *V. decussata* exposed to the acute test, mainly at CBZ 0.30 and 3.00 µg/L. At these concentrations, respectively, the increase in accumulated CBZ was approximately 6 and 3 fold higher in the chronic test than in the acute test. However, at the highest spiked CBZ concentration (9.00 µg/L), the clams in the two toxicity tests accumulated similar levels of CBZ.

Table 3.4 also presents the bioconcentration factor (BCF) determined for each toxicity test. The results showed that, for *V. decussata* exposed acutely to CBZ, BCF increased along the concentration range. A different pattern was observed for *V. decussata* exposed chronically to CBZ, where BCF decreased comparing CBZ 0.30 with 3.00 µg/L and maintained at CBZ 9.00 µg/L. Again, both toxicity tests presented a similar BCF at the highest CBZ concentration, but at concentrations of 0.30 and 3.00 µg/L the BCF was approximately 6 and 2 fold higher in the clams from chronic test comparing with the clams from the acute toxicity test. Moreover, the results obtained for control and CBZ 0.03 µg/L were below the lower limit of the quantitation range determined by Calisto et al. (2011a) for this ELISA assay (0.024-10 µg/L), so it couldn't be determined with confidence (< LQ).

Table 3.4. CBZ concentration (µg/g FW), determined by ELISA, and bioconcentration factor (BCF) in *V. decussata* submitted to the acute (96 h) and chronic (28 d) CBZ exposures. Values are the mean (STDEV) of fifteen replicates. Significant differences ($p \leq 0.05$) between exposure concentrations, for each species, are presented with letters (a-c). Significant differences ($p \leq 0.05$) between species, for each exposure concentration, are presented with an asterisk (*). LQ: quantification limit

Exposure concentration (µg/L)	**CBZ in *V. decussata* (µg/g FW)**		**BCF**	
	Acute test	*Chronic test*	*Acute test*	*Chronic test*
0.00	<LQ	<LQ	-	-
0.03	<LQ	<LQ	-	-
0.30	0.00008 (0.00001)^{a*}	0.00047 (0.00005)^{a}	0.26 (0.02)^{a*}	1.6 (0.2)^{a}
3.00	0.0014 (0.0008)^{b*}	0.0036 (0.0003)^{b}	0.5 (0.2)^{b*}	1.2 (0.1)^{b}
9.00	0.010 (0.004)^{c}	0.011 (0.001)^{c}	1.1 (0.4)^{c}	1.2 (0.1)^{b}

3.3.4. Blanks

The results of the quantification of CBZ in blanks, aqueous standards of CBZ in seawater with concentrations between 0.03 and 9.00 µg/L, exposed to the same conditions as chronic test but with no organisms showed that CBZ was not adsorbed on the inner surface of containers neither degradation of CBZ was observed during the period of exposure (Table 3.5). As the concentration of the standard of 0.03 µg/L was near the lower value of the quantitation range (0.024 µg/L) it was not possible to quantify it with good precision (<LQ).

Table 3.5. CBZ concentration (µg/L), determined by ELISA, in blanks obtained at the beginning (day 0) and at the end (day 28) of the chronic assay. Values are the mean (STDEV) of fifteen replicates. Significant differences ($p \leq 0.05$) between quantified CBZ by ELISA for exposure concentrations (spiked) are presented with letters (a-c). Significant differences ($p \leq 0.05$) between exposure days for each spiked concentration are presented with an asterisk (*). LQ: quantification limit

Spiked concentration (µg/L)	CBZ in blanks (µg/L) – Day 0	CBZ in blanks (µg/L) – Day 28
0.00	< LQ	< LQ
0.03	< LQ	< LQ
0.30	0.25 (0.02) [a]	0.293 (0.007) [a]
3.00	2.9 (0.4) [b]	3.02 (0.08) [b]
9.00	10.6 (0.8) [c]	9 (3) [c]

3.3.5. Carbamazepine concentration in water samples where the clams were exposed

The results of the quantification of CBZ in the seawater solutions spiked with CBZ used to expose clams to the drug showed concentrations similar to those of the spikes indicating that the accumulation of CBZ by clams was too low, and thus did not change in an appreciable extent the concentration in the surrounding water (Table 3.6). This was evident by the absence of significant differences between exposure concentrations among exposure periods (cf. Table 3.6). Moreover, the CBZ quantified in control and 0.03 µg/L were below the lower limit of the quantitation range determined by Calisto et al. (2011a) making not possible to quantify it with good precision (<LQ).

Table 3.6. CBZ concentration (µg/L), determined by ELISA, in water where the clams were exposed to the chronic assay at the beginning (day 0), after 4 and 3 days of exposure (water renewal cycle) and at the end of the assay (day 28). Values are the mean (STDEV) of fifteen replicates. Significant differences ($p \leq 0.05$) between CBZ quantified by ELISA for exposure concentrations (spiked) are presented with letters (a-c). Significant differences ($p \leq 0.05$) between exposure periods for each spiked concentration are presented with an asterisk (*). LQ: quantification limit

Spiked concentration (µg/L)	CBZ in water (µg/L) – Day 0	CBZ in water (µg/L) – 4 days water renewal cycle	CBZ in water (µg/L) – 3 days water renewal cycle	CBZ in water (µg/L) – Day 28
0.00	< LQ	< LQ	< LQ	< LQ
0.03	< LQ	< LQ	< LQ	< LQ
0.30	0.23 (0.02) [a]	0.23 (0.01) [a]	0.22 (0.02) [a]	0.22 (0.05) [a]
3.00	4 (1) [b]	2.8 (0.7) [b]	2.8 (0.6) [b]	3 (1) [b]
9.00	8 (1) [c]	8 (1) [c]	9 (2) [c]	10.2 (0.8) [c]

3.3.6. Physiological parameters

3.3.6.1. Glycogen and total protein content

Alterations in glycogen (GLYC) content are present in Figure 3.7A. The results obtained showed that *V. decussata* submitted to the acute test presented higher GLYC content at all conditions tested than when the individuals were exposed to the chronic test. In clams exposed to the chronic test, for all the CBZ concentrations tested, except 0.30 µg/L, the results showed a significantly increase in GLYC content compared with the control. For *V. decussata* exposed to acute test the results showed that GLYC content decreased from the control to the lowest concentration (CBZ 0.03 µg/L), and then a progressive increase as CBZ concentration increased was observed.

When exposed to the acute and chronic toxicity tests, individuals increased the total protein content (PROT) at the lowest CBZ concentration (0.03 µg/L) when compared to the control (Figure 3.7B). However, a different response was observed, at the remaining concentrations, for clams exposed to both test conditions. In *V. decussata* exposed to the acute test the PROT content significantly increased at 0.30, 3.00 and 9.00 µg/L compared with the lower concentrations. For *V. decussata* submitted to the chronic test the PROT content significantly decreased at concentrations of 0.30, 3.00 and 9.00 µg/L when compared with the remaining conditions.

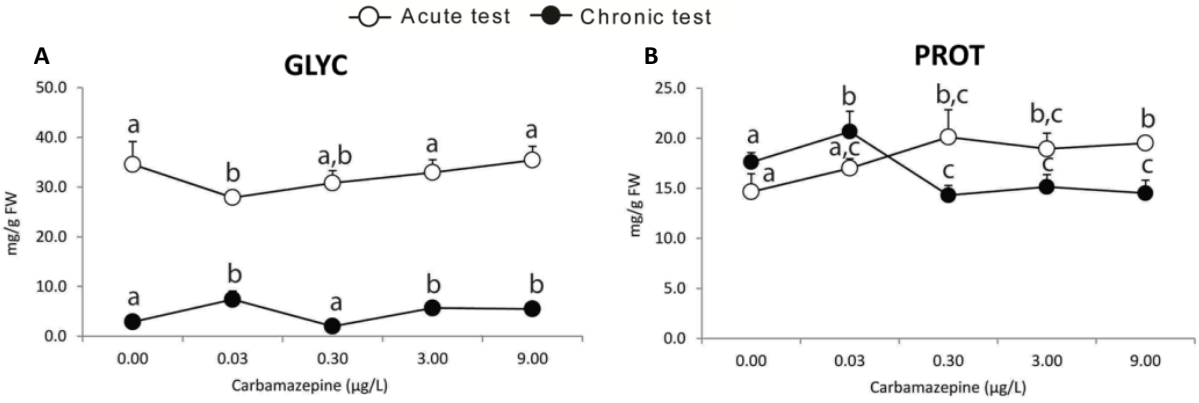

Figure 3.7. Physiological parameters (GLYC, glycogen content; PROT, total protein content) in *V. decussata* when exposed to increasing concentrations of CBZ in the chronic (28 d) and acute (96 h) toxicity tests. Values are the mean (STDEV) of ten replicates. Significant differences ($p \leq 0.05$) among exposure concentrations, for each toxicity test, are presented with letters (a-c).

3.3.7. Biochemical parameters

3.3.7.1. Indicators of oxidative stress

In order to verify the presence of oxidative imbalance induced by CBZ, lipid peroxidation (LPO) levels were measured (Figure 3.8A). *V. decussata* exposed to the chronic test showed higher LPO levels than the same species submitted to the acute test at all conditions tested. After 28 d of exposure, oxidative damage significantly increased at 9.00 μg/L compared with the remaining conditions. On the other hand, the results for the acute test showed an opposite response, were the oxidative damage was significantly lower at the highest CBZ concentration compared to control and the lower CBZ concentrations.

The results obtained for the total glutathione content (GSH_t) are shown in Figure 3.8B. In general, comparing the toxicity assays, *V. decussata* exposed chronically to CBZ showed lower GSH_t levels compared with *V. decussata* exposed to the acute test, especially at the control and 0.03 μg/L. GSH_t variation was more pronounced for *V. decussata* exposed to the chronic test in comparison with the acute test and it was observed a significantly increase along the increasing exposure gradient. The same tendency was observed for *V. decussata* submitted to the acute test, although less pronounced.

Regarding the reduced glutathione (GSH) content results (Figure 3.8C), *V. decussata* demonstrated a different response under both toxicity tests. In the chronic test the clams increased significantly GSH content comparing the CBZ concentrations of 0.03, 3.00 and 9.00 μg/L with the control. On the other hand, *V. decussata* exposed to the acute test decreased significantly the GSH content comparing the higher CBZ concentrations (0.30, 3.00, 9.00 μg/L) with the control condition.

In Figure 3.8D are represented the results for GSH/GSSG ratio. For both toxicity tests, *V. decussata* present the same response being possible to observe a decrease in GSH/GSSG ratio along the exposure range. *V. decussata* exposed to the chronic test showed a significantly higher GSH/GSSG ratio at the control and 0.03 μg/L concentrations when compared with the same species exposed to the same concentrations in the acute test.

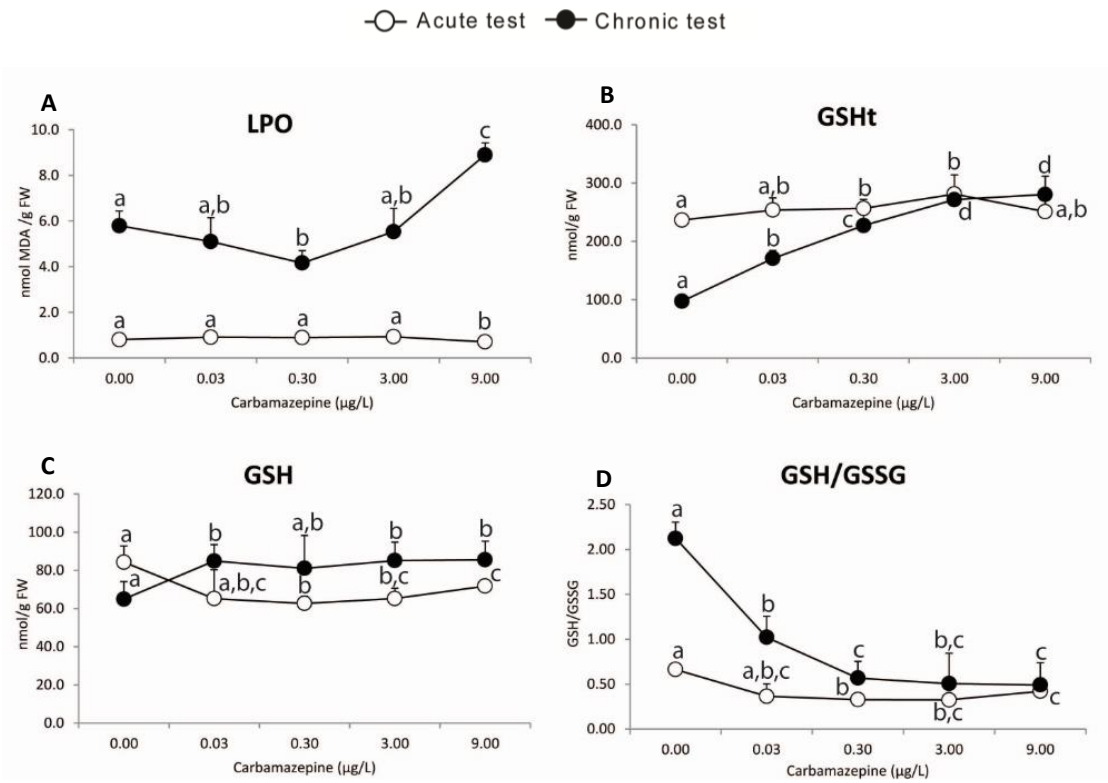

Figure 3.8. Indicators of oxidative stress (LPO, lipid peroxidation; GSH_t, total glutathione; GSH, reduced glutathione; GSH/GSSG, ratio between reduced and oxidized glutathione) in *V. decussata* when exposed to increasing concentrations of CBZ in the chronic (28 d) and acute (96 h) toxicity tests. Values are the mean (STDEV) of ten replicates. Significant differences ($p \leq 0.05$) among exposure concentrations, for each toxicity test, are presented with letters (a-d).

3.3.7.2. Antioxidant enzymes

Superoxide dismutase (SOD) activity (Figure 3.9A) showed that *V. decussata* exposed do the acute test presented a higher activity at all concentrations tested with the exception of 9.00 µg/L, in comparison with *V. decussata* exposed to the acute test. SOD activity was significantly increased for *V. decussata* exposed to the chronic test only at the highest CBZ concentration (9.00 µg/L). In the same species exposed to the acute test an increase in SOD activity was found in CBZ 0.30, 3.00 and 9.00 µg/L compared with the lower concentrations. Moreover, *V. decussata* exposed to the acute test showed significantly higher enzymatic activity than the clams exposed to the acute test in all conditions tested with the exception of 9.00 µg/L.

The activity of catalase (CAT) is presented in Figure 3.9B, showing that *V. decussata* exposed to the acute test presented a higher CAT activity than the same species submitted to the chronic

test at all concentrations tested. However, along the increasing exposure concentration, *V. decussata* exhibited the same pattern for both toxicity tests, where a decrease in CAT activity in all concentrations tested compared with the control was observed. Nevertheless, for *V. decussata* submitted to the chronic test, the variation in CAT activity between the control and the exposure concentrations were lower than that observed in the acute test.

Relatively to glutathione reductase (GR) activity (Figure 3.9C), the clams exposed to the acute test presented higher GR activity than the clams exposed to the chronic test at all CBZ concentrations tested (exception of the control). In *V. decussata* exposed do the chronic test, a general tendency for maintenance in GR activity was observed at the control, 0.03 and 0.30 µg/L, but at higher CBZ concentrations, GR activity decreased significantly when compared to the control. For *V. decussata* exposed to the acute test, an opposite response was observed, occurring an increase in the enzymatic activity at the higher CBZ concentrations (0.30, 3.00 and 9.00 µg/L) in relation with the control and 0.03 µg/L.

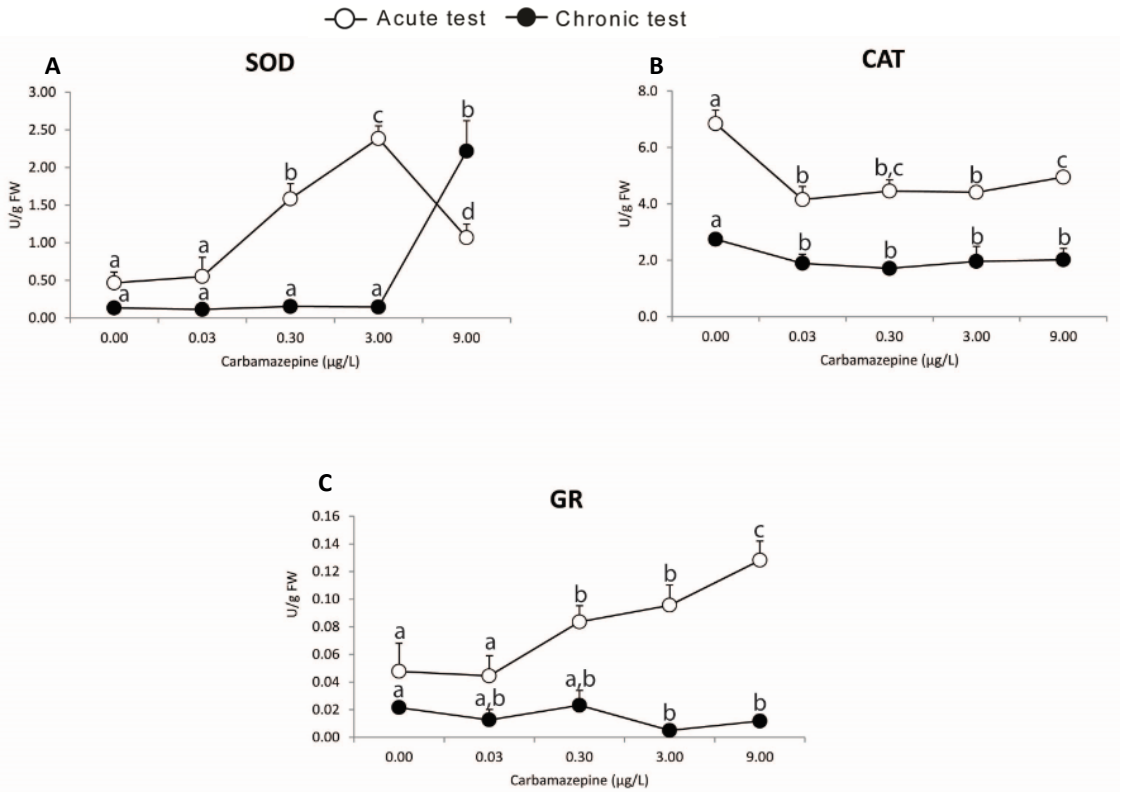

Figure 3.9. Antioxidant enzymes (SOD, superoxide dismutase; CAT, catalase; GR, glutathione reductase) in *V. decussata* when exposed to increasing concentrations of CBZ in the chronic (28 d) and acute (96 h) toxicity tests. Values are the mean (STDEV) of ten replicates. Significant differences ($p \leq 0.05$) among exposure concentrations, for each toxicity test, are presented with letters (a-d).

3.3.7.3. Biotransformation enzymes

The activity of glutathione-S-transferase (GST) is represented in Figure 3.10, showing a higher activity for *V. decussata* exposed to the acute test, compared with the same species exposed to the chronic test at all conditions tested. Although *V. decussata* submitted to the chronic test showed a significantly increase in GST activity at CBZ 0.03 µg/L compared with the control, for the remaining conditions the enzymatic activity decreased, being similar or lower than the control. In individuals submitted to the acute test, the tendency was for an increase in GST activity after exposure to CBZ, especially when comparing the control with 0.03, 0.30 and 3.00 µg/L.

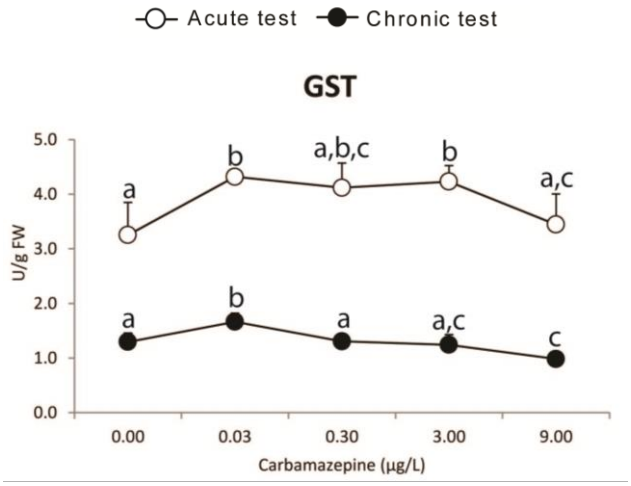

Figure 3.10. Biotransformation enzymes (GST, glutathione S-transferase) in *V. decussata* when exposed to increasing concentrations of CBZ in the chronic (28 d) and acute (96 h) toxicity tests. Values are the mean (STDEV) of ten replicates. Significant differences ($p \leq 0.05$) among exposure concentrations, for each toxicity test, are presented with letters (a-c).

3.3.8. Multivariate analysis

In the Figure 3.10 are represented the centroids PCO ordination graph resulting from applying a multivariate analysis to the physiological and biochemical data obtained from chronic and acute test. The PCO axis 1 explained 57.9% of the total variation of data, clearly separating the two toxicity assays, based on their physiological and biochemical alterations due to CBZ exposure. The PCO axis 2 explained 20.6% of the total data variation. However, through this axis it was observed a higher separation of the responses according to the concentration tested, where higher differences were noticed for *V. decussata* exposed 28 d to CBZ comparing to the same species in a

4 d exposure to CBZ. A higher correlation (r> 0.75) was observed between the LPO and GSH/GSSG levels and the clams exposed to the chronic test, where the conditions were well separated.

For acute test, the PCO indicated a high correlation (r> 0.75) between this assay and GLYC, GST, GR and CAT parameters. Concerning GSH_t the PCO analysis showed an equally correlation with both tests. This occurred because, the species presented similar levels of GSH_t at higher CBZ concentrations.

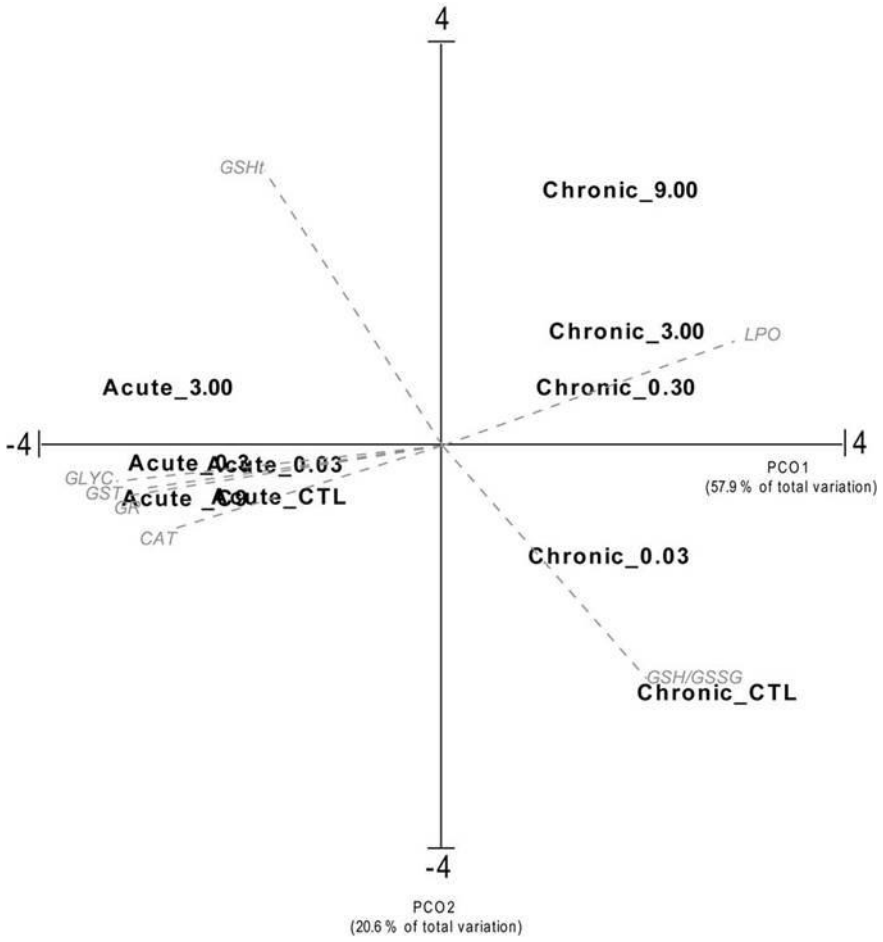

Figure 3.11. Physiological and biochemical responses of *V. decussata* exposed to increasing CBZ concentrations in the acute (Acute) and chronic (Chronic) toxicity tests, plotted on axes 1 and 2 of a Principal Coordinates (PCO) graph. Physiological and biochemical responses are superimposed on the PCO (r > 0.75). The control (CTL) and concentrations of exposure (0.03, 0.30, 3.00, 9.00 µg/L) are indicated for each toxicity test. The biomarkers presented are: CAT, catalase; GLYC, glycogen content; GSHt, total glutathione, GSH/GSSG, ratio between reduced and oxidized glutathione; LPO, lipid peroxidation; GST, glutathione S-transferase; GR, glutathione reductase.

3.4. Carbamazepine risk assessment: risk quotient determination

In order to determine CBZ risk assessment in the Ria de Aveiro, two risk quotients (RQ) were obtained using data from literature. These RQ values were calculated dividing the MEC (measured environmental concentration) value, the highest concentration of CBZ in WWTP effluent from Aveiro region (0.60 µg/L) by PNEC (predicted no effect concentration) values obtained by the application of two approaches (see section 2.6-Data analysis). Using a PNEC of 0.00032 µg/L based on the Aguirre-Martínez et al. (2013) work, the RQ value obtained was 1875; using the PNEC of 0.42 µg/L obtained by Ferrari et al. (2003), the RQ determined was 1.43. When the RQ is higher than 1, an ecotoxicological risk is suspected.

Chapter 4

Discussion

4.1. Contextualization

Carbamazepine is poorly removed by WWTPs. For this reason, it is one of the most commonly detected pharmaceutical drugs in water bodies, being used as a marker of anthropogenic pollution (Clara et al., 2004). Since CBZ is designed to exert a biological effect, when it reaches aquatic environment there is a high probability of exerting toxic effects on non-target organisms.

In most of the studies assessing the CBZ toxicity in the aquatic environment, the organisms are exposed to high concentrations, where EC_{50} concentrations are in the mg/L range (Ferrari et al., 2003), which are not representative of those occurring in the environment. Only a few studies have been performed with environmentally relevant concentrations showing that aquatic organisms can be impaired by CBZ (Aguirre-Martínez et al., 2013b; Chen et al., 2014; Martin-Diaz et al., 2009; Li et al., 2010b; 2010c; 2009). Among other effects, the involvement of antioxidant defense system as a response to CBZ effects has been reported, for example, in bivalves (Martin-Diaz et al., 2009b; Contardo-Jara et al., 2011), fish (Li et al., 2010a; 2010b; 2010c; 2009) and crabs (Aguirre-Martínez et al., 2013b). CBZ biotransformation and the possible interaction with targets that are also evolutionarily conserved in bivalves were also documented (Martin-Diaz et al., 2009b; Quinn et al., 2004). Martin-Diaz et al. (2009b) investigated whether CBZ affected the cyclic adenosine monophosphate (cAMP) - dependent pathway in the mussel *M. galloprovincialis,* finding that the drug decreased cAMP contents and protein kinase A (PKA) activities in all tissues of the mussel, thus acting on specific biochemical pathways for which it was designed and that are evolutionarily conserved.

4.2. ELISA performance on the quantification of carbamazepine

The reference techniques used to quantify pharmaceutical residues in environmental matrices, including CBZ have the inconvenient of being time-consuming, expensive, require costly instrumentation and dedicated personnel (Bahlmann et al., 2009; Buchberger, 2011). Alternative techniques, such as immunoassays (namely ELISA) have been applied to this field (Huerta et al., 2012), proven to be valuable tools for the sensitive detection of pollutants in water since they require little sample preparation, display high sensitivity and may be less expensive in comparison with the conventional methods. Deng et al. (2003) applied ELISA for the determination of diclofenac in tap, surface and wastewater samples. Estradiol and ethinylestradiol, estrogenic compounds, were quantified in surface and wastewater samples through ELISA by Hintemann et al. (2006). CBZ, the drug under study, was also quantified in water bodies by ELISA as referred previously (Bahlmann

et al., 2012; 2011; 2009; Calisto et al., 2011a). However, the application of immunoassays in environmental analysis is still scarce, since the majority of immunoanalytical test kits for pharmaceuticals are optimized for biological samples as blood and urine or food (Buchberger, 2011). Moreover, there is a need to apply these methods to real complex matrices such those that exist in living organisms (Chu and Metcalfe, 2007; Cueva-Mestanza et al., 2008; Ramirez et al., 2007), especially using immunoassays techniques due to the advantages that these techniques provide over reference methods. To the best of my knowledge, only one study developed by Gagné et al. (2006c) applied a competitive enzymatic immunoassay to quantify CBZ in the freshwater mussel (*E. complanata*) soft tissue homogenate extracts. In this way, in the present study the direct competitive ELISA was applied to quantify, directly, CBZ in clams' tissues.

In the work under study, to investigate the clams' homogenates matrix effects on the quantification of CBZ, a quality control was performed. Good recoveries were obtained (between 91 and 118%) demonstrating that CBZ could be quantified directly in clams homogenates, with no matrix interferences and with no sample pre-treatment, turning this assay suitable for rapid, large and economic environmental screening. The requirement of a filtration step for the CBZ quantification was tested due to the high complexity of the samples, revealing to be unnecessary. Only for homogenates exposed to 9.00 µg/L a dilution step was required, in order to determine the concentration within the quantitation range, and thus with good precision. This is a valuable advantage comparing with the reference techniques for quantifying CBZ in complex matrices such as biological tissues which require pre-concentration techniques. For example, in the study performed by Contardo-Jara et al. (2011), mussel (*D. polymorpha*) tissues samples were subjected to a microwave-assisted micellar extraction combined with solid-phase extraction to concentrate CBZ. The same procedure was applied by Cueva-Mestanza et al. (2008) to extract a group of pharmaceuticals including CBZ from mussel (*M. galloprovincialis*) samples. In this way, the application of ELISA to determine CBZ concentration in clams' tissues in the present work allowed to save time, to reduce the amount of materials (reagents and equipment) used and to reduce losses of the compound which could occur during the sample pre-treatment or the sample degradation.

4.3. Acute toxicity test

4.3.1. Carbamazepine uptake by clams

The results for CBZ quantification showed that both species increased CBZ in their tissues with the increase of concentration in water. Moreover, the blank test ensured that no CBZ losses (by photodegradation or adsorption on the test vessels) occurred during the acute test. The capacity of organisms to increase their internal pharmaceutical drugs concentration relative to their environment has been studied (e.g. Gomez et al., 2012; Schwaiger et al., 2004; Zhang et al., 2010). Relatively to CBZ, only few studies are published reporting its accumulation in aquatic species. Contardo-Jara et al. (2011) determined the bioconcentration factor (BCF) by measuring the tissue concentration of CBZ in the mussel *D. polymorpha* after a 1, 4 and 7 d of exposure to the concentration range 0.236-236 μg/L. Although, the authors showed an increase in CBZ accumulated in mussels with the increase of exposure concentration, the determined BCF was highest in animals exposed to the lowest CBZ concentration. After 4 d of exposure, the BCF increased to 60, corresponding to a concentration of 0.0142±0.0021 μg/g dry weight (dw), demonstrating the risk of CBZ bioaccumulation in wildlife populations. Although in the results of the present work it was observed an increase in CBZ tissue concentration with the increase in concentration of exposure, the BCF increased along the exposure range, reaching a value of approximately 1 in clams exposed to the highest CBZ concentration (9.00 μg/L).

Vernouillet et al. (2010) studied the bioaccumulation of CBZ (150 mg/L) through an experimental food chain composed by the green alga, *P. subcapitata*, the crustacean, *T. platyurus*, and the cnidarian, *H. attenuata*. A BCF of 2.2 was found to *P. subcapitata*; *T. platyurus* (fed with the algae) accumulated 12.6 times more CBZ than the algae and only traces of CBZ were found in *H. attenuata* (fed with the crustacean). The authors justified that the low concentration of CBZ determined in the cnidarian *H. attenuata* could be the result of a low uptake of the drug or due to a high detoxification activity in this organism. Moreover, although the high concentration of CBZ used in the study performed by Vernouillet et al. (2010), it was reported the ability of CBZ to bioaccumulate in aquatic organisms through food contamination.

4.3.2. Relationship between the carbamazepine exposure and the physiological parameters

The condition index (CI) was measured to observe if clams' weight was influenced by CBZ exposure. The accumulation of CBZ in clams' tissues was accompanied, although not significantly in both species, by a decrease in CI. The results for clearance rate (CR), a measure of the clams filter activity, showed a decrease at the higher CBZ concentrations for both species. The reduction in filter feeding activity may be the result of stressed clams attempting to limit their exposure to CBZ contamination by keeping their shells closed. Gosling (2003) reported that bivalves could immediately close their valves as a strategy of protection when exposed to a stressful condition. By doing so, their metabolism would tend to slow down, being reflected as a reduction in CI. These results are in accordance with those obtained by Farcy et al. (2011) after exposing the mussel *E. complanata* to a tertiary-treated municipal effluent, during 2 weeks. The survival in air (SA) test has been used to measure how long bivalves survive when removed from the contaminant environment (Eertman et al., 1993). However, although in the present study the SA assay didn't provide a good response, a slightly tendency for a decrease with the increase in CBZ exposure seemed to occur. Probably, the concentration of exposure to CBZ was not enough to accelerate their death under the absence of water. Although the removal from water for several days is unlikely to occur in the natural environment, this method has been used to test the fitness of bivalves in a number of studies, showing that organisms exposed to pollution died in air much earlier than the unexposed since the first spend a great part of their energy budget in detoxification processes and maintenance of homeostasis (Pampanin et al., 2012; Thomas et al., 1999; Wang and Widdows, 1992). Pampanin et al. (2012) showed a reduced survivability in air accompanied by a decrease in CI in mussels collected and transplanted to the historic center of Venice lagoon, contaminated by a variety of sources, including the discharge of untreated sewage directly into the canals.

The results obtained in the present work showed that although the glycogen content (GLYC) tend to decrease in *V. philippinarum* after exposure to CBZ, this decrease was not related with the CBZ dose, since the GLYC levels were in general maintained or slightly increased comparing the concentrations tested. For *V. decussata*, a decrease in this reserve was only noticed at the lowest CBZ concentration and for higher concentrations a progressive increase occurred, although the values were not statistically different from the control. Thus, it was assumed that, probably the range of CBZ concentrations tested was not enough to alter GLYC content in both species and so, the variation observed is a species characteristic and not a response to the contaminant. Kim et al.

(2001) reported that the shell closure and concomitant reduced oxygen consumption rate could result in a less energy expenditure for respiration and feeding activity. However, the reduction in feeding activity (through the shell closure) is also related with a lower production or higher expenditure of GLYC. So, it was expected a decrease in GLYC under a stressful condition and that was not observed in the present study. Other authors reported a different response, observing a reduction in GLYC in organisms exposed to stressful environmental conditions, due to its mobilization to combat the stress (e.g. Duquesne et al., 2004).

When exposed to CBZ, the total protein content (PROT) in *V. philippinarum* presented an opposite pattern comparing to *V. decussata.* The increase observed for *V. decussata* after the exposure to the highest CBZ concentrations may be an attempt of this species to induce proteins to be used in the defense against CBZ. For *V. philippinarum*, the PROT content decreased at the highest CBZ concentration possibly indicating that this species was under oxidative stress. Li et al. (2010c) revealed an induction of protein carbonylation in the rainbow trout (*Oncorhynchus mykiss*) exposed to CBZ (0.2 and 2 mg/L) after 21 d, as a result of reactive oxygen species (ROS) attack on proteins, under an oxidative environment. The formation of carbonyl groups triggered conformational changes on proteins, decreasing their catalytic activity and resulting in a higher susceptibility to protease action. Thus, CBZ-exposure could induce cellular protein metabolism disruption, as observed for *V. philippinarum*.

4.3.3. Relationship between carbamazepine exposure and biochemical parameters

Oxidative and reductive processes are involved in the biochemical properties of biologically active xenobiotics, including carbamazepine, as showed by Martin-Diaz et al. (2009b) when assessed the effects of environmental concentrations of this drug in the mussel *Mytilus galloprovincialis*. The accumulation of CBZ within lysosomes (involved in phagocytic activity) has been reported to trigger lysosomal membrane impairment. Because intralysosomal environment is a site of oxyradical production, the release of hydrolytic enzymes and reactive oxygen species into the cytosol could induce oxidative effects (Tsiaka et al., 2013). Moreover, ROS can also be produced during the oxidative metabolism of CBZ. The oxidation of polyunsaturated fatty acids, as those found in cellular membranes, is an important consequence of oxidative stress, resulting in lipid peroxidation (LPO). For both species under study, significant differences were found between the control and the highest CBZ exposure concentration (9.00 µg/L), where a significant increase was observed for *V. philippinarum* and a significant decrease for *V. decussata.* These results suggest that *V. philippinarum* was under oxidative impairment while *V. decussata* seemed to be more effective

in the combat to oxidative stress, presenting lower levels of LPO. Aguirre-Martínez et al. (2013a) reported a significant reduction in lysosomal membrane stability of *V. philippinarum* exposed to CBZ (0-50 μg/L) during 35 d, justifying the lysosomal damage as a result of lysosome ability to concentrate CBZ. In the study performed by Tsiaka et al. (2013) an increase in LPO was observed in the mussel *M. galloprovincialis* haemocytes exposed to CBZ (0.01-10 μg/L) for 1 h. This study also demonstrated the pro-oxidant behavior of CBZ, with an enhancement of free radicals such as superoxide anion and nitrite. As referred by these authors this behavior could result in the formation of peroxynitrite which has strong oxidizing effects on proteins, being highly toxic for the cells. Gagné et al. (2006a) found an induction in LPO after an exposure of rainbow hepatocytes to CBZ during 48 h (CBZ 0-0.236 g/L). LPO was also induced in all tissues of the crab *Carcinus maenas* exposed to increasing concentrations of CBZ (CBZ 0-50 μg/L, 28 d) (Aguirre-Martínez et al., 2013b).

The present study showed that, in *V. decussata*, the decrease in GSH after exposure to CBZ concentrations was accompanied by a slight increase in GSH_t and by a decrease in GSH/GSSG. Thus, it was possible to infer that, the reduction in GSH, responsible for the decrease in GSH/GSSG, was not due to a decrease in its production but due to its use as a substrate by other defense enzymes such as GST. The results obtained in this work further demonstrated that, in *V. philippinarum*, GSH increased after exposure to CBZ, followed by an increase in GSH_t and GSH/GSSG. In this case, the increase in GSH indicated an effort of cells to produce this non-enzymatic defense to participate in antioxidant reactions which could consequently increase the GSH_t levels. However, the variation of GSH was too low comparing with the amount of GSSG in the cells, being possible to attribute the increase in GSH_t to an increase in GSSG. Comparing the two species, the GSSG content (data not shown), at the same exposure concentration was approximately 2 fold higher in *V. philippinarum* comparing with *V. decussata,* revealing a higher oxidant environment in the former species.

These results are supported by those obtained for glutathione reductase (GR). This enzyme is important for the GSH turnover, catalyzing the reduction of GSSG (Li et al., 2010b). Comparing the two species, a higher GR activity was found in *V. decussata* revealing that a higher GSH turnover exists even though it is being used. For *V. philippinarum*, although the clams were trying to reduce GSSG, reflected as a higher GR activity at CBZ 9.00 μg/L, the enzymatic activity was lower when comparing with *V. decussata*. Thus, it was assumed that these defenses did not lower properly the cell's oxidative environment. Li et al. (2010c; 2009) found a decrease in GSH in rainbow trout brain after exposure for 42 d to 0.2 and 2 mg/L of CBZ. However, in these studies, the depletion in GSH was attributed to the decreased GR activity, suggesting that GSH was not rapidly recovered after the CBZ stress. Li et al. (2010a) found that in fish spermatozoa exposed to CBZ concentrations

ranging from 0.2 to 20 mg/L for 2 h, glutathione reductase activity was significantly inhibited only at the highest concentration.

The increase observed in SOD activity at higher CBZ concentrations in *V. decussata*, revealed the important role of this enzyme to neutralize ROS generation, and thus, lowering its effects as LPO. On the other hand, for *V. philippinarum* a slight increase in SOD activity was found only in clams exposed to 9.00 μg/L, meaning that a higher concentration of superoxide anions exist at this concentration. However, for this species SOD activity was lower than in *V. decussata* and again, despite the increase in GSH and GR activity this defense was not enough to combat LPO triggered by ROS in *V. philippinarum*. Li et al. (2011) found an increase in SOD activity in the intestine, muscle and gills of the fish *O. mykiss* exposed to CBZ (19.9 mg/L, the LC50) during 96 h. These authors assumed that CBZ could have different modes of action, depending on the tissues used for the analysis and the type of antioxidant enzyme studied. Other studies found a variable SOD response for *V. philippinarum*, comparing with other pharmaceutical drugs. SOD activity decreased markedly in *V. philippinarum* exposed to ibuprofen (100 and 1000 μg/L) comparing the contaminated animals with the control after a 5 d, suggesting that the contaminant reduced the antioxidant defenses of this clams (Milan et al., 2013). According to Matozzo et al. (2012b), the pattern of SOD activity for *V. philippinarum* exposed to triclosan (7 d exposure) varied with the type of tissue analyzed, where a significant increase was observed in gills at exposure concentrations of 600 and 900 ng/L comparing with the control, and where a significant decrease was observed at the same concentrations in digestive gland. Other organic contaminants (e.g. polycyclic aromatic hydrocarbons) have been found to induce SOD and CAT activity in *V. decussata* (among others, Bebianno and Barreira, 2009).

Further results revealed that the CAT activity for *V. decussata* decreased when comparing the control to all CBZ concentrations tested, indicating that the hydrogen peroxide produced by SOD was possibly being converted not by CAT but by other enzyme with the same role, as glutathione peroxidase (GPx). For *V. philippinarum*, although there was a tendency for an increase in CAT activity at the higher CBZ concentrations, these results seem to indicate that the fluctuations observed are species characteristics and not a response to the presence of CBZ. Other studies showed a different trend in the CAT activity. In the study performed by Li et al. (2011), which exposed the fish *O. mykiss* to CBZ (19.9 mg/L, 96h), an increase in the activity of CAT was found in intestine, muscle and gills. Martin-Diaz et al. (2009b) reported no significant alteration on CAT activity in the mussel *M. galloprovincialis* exposed to CBZ (10 μg/L, 7 d).

In the present study, exposure to CBZ induced the activity of CYP3A4, an enzyme predominantly involved in CBZ metabolism in humans, in the clam *V. decussata*, at all CBZ concentrations tested, suggesting induction in this cytochrome family. However, the activity of CYP3A4 for *V. philippinarum,* although not increasing along the CBZ concentration range, was similar to the control or slightly increased for some concentrations (0.03 and 9.00 μg/L). The results obtained here are in line with those obtained by Gagné et al. (2006a) which found an induction of benzylether resorufin (CYP3A4 substrate) dealkylase activity in trout hepatocytes exposed for 48 h to CBZ. Martín-Diaz at al. (2009b) and Aguirre-Martínez (2013b) found a significantly increase in dibenzylfluorescein (another CYP3A3 substrate) dealkylase activity in aquatic organisms exposed to CBZ. In the study performed by Aguirre-Martínez et al. (2013b), the activity of dibenzylfluorescein in hepatopancreas was 5.0 and 10.4 times higher than in controls, respectively, at the concentrations of 10 and 50 μg/L (28 d exposure). In the present work the activity of CYP3A4 was found to be 2.1 and 1.1 times higher for 9.00 μg/L than in controls for *V. decussata* and *V. philippinarum*, respectively. Although the CYP3A4 activities determined in this work were lower than those reported by the previous authors, these values are referred to an acute exposure. So, it is expected a pronounced effect after the exposure to a chronic test, as obtained by Aguirre-Martínez et al. (2013b).

In the present work, *V. decussata* increased the GST activity with the increase in CBZ exposure concentration. This could be caused by the formation of thiol metabolites resulting from CBZ oxidation, indicating that CBZ is being conjugated for elimination purposes. CBZ is a carboxylated iminostilbene (R-N-CO-NH_2) where the carbonyl (C=O) moiety might react with GSH (Vernouillet et al., 2010). The formation of GS-conjugates could explain the reduction in GSH and consequently in GSH/GSSG. An induction in GST activity was also found in the studies previously reported by Aguirre-Martínez et al. (2013b) and Martín-Díaz et al. (2009b). Contardo-Jara et al. (2011) observed an increase in GST activity after a 4 d exposure to the β- blocker receptor metoprolol in the bivalve *D. polymorpha.* The increase in GST activity for *V. decussata* can also be explained as the enhanced requirement for biotransformation waste products resulting from the potential cellular damage due to the CBZ exposure. GST isoenzymes are also capable of inactivating lipoperoxidation products, such as lipid hydroperoxides (Sturve et al., 2008) by the use of GSH as a reducing agent that is oxidized to GSSG when peroxides are reduced. Thus, for *V. decussata*, the increase in GST activity after exposure to CBZ may also indicate an involvement of GST isoenzymes in the reduction of LPO, as observed at the highest CBZ concentration (9.00 μg/L) with the consequent reduction in GSH verified also for this species. An increase in piGST mRNA expression,

a GST isoenzyme involved in the inactivation of LPO products, was reported in bivalves exposed to other pharmaceutical drugs. For example, Contardo-Jara et al. (2010) found an increase in piGST mRNA expression in the mussel *D. polymorpha* exposed to the β-blocker metoprolol (0.534-534 µg/L, 7 d). However, in the present work, for *V. philippinarum,* it was possible to observe a general decrease in GST activity along CBZ concentrations, showing that probably, for this species, lower levels of GSH were available to participate in the conjugation reactions due to a lower production of this defense and thus, the referred enzyme might not be involved in the CBZ phase II biotransformation. Moreover, the participation of GST in the elimination of lipoperoxidation products seemed to be compromised due to the low levels of GSH production observed for *V. philippinarum*, justifying the increase in LPO observed at the higher CBZ concentration (CBZ 9.00 µg/L). Gonzalez-Rey and Bebianno (2012) reported that the GST, CAT and GR inhibition favored a higher formation of LPO in the mussel *M. galloprovincialis* exposed to ibuprofen (250 ng/L, 2 weeks of exposure). At days 0, 3, 7 and 15 mussels were sampled from control and exposure conditions and submitted to the biomarker analysis. The increase in LPO, observed after 3 and 7 d of exposure comparing with the control was justified mainly through the reduced GR activity, which decreased the GSH, limiting the participation of GST in biotransformation of CBZ and its LPO products. An increase in GST activity was also reported in bivalves exposed to other organic compounds (Canesi et al., 2007; Zhang et al., 2014).

Other studies have been demonstrating the different response of *V. decussata* and *V. philippinarum* when exposed to the same pharmaceutical. For example, in the study performed by Antunes et al. (2013), it was evaluated the effects of an acute acetaminophen exposure on *V. decussata* and *V. philippinarum* in terms of biochemical responses. These authors found that both species responded distinctly to the occurrence of acetaminophen. *V. decussata* was more responsive to acetaminophen than *V. philippinarum* since it presented more alterations in terms of LPO, GST and GR activity. *V. philippinarum* only responded to acetaminophen in terms of GST activity. So the authors suggested the higher response of *V. decussata* in terms of the detoxification/antioxidant defense mechanisms as being a disadvantage since it reflected that this species was being affected by oxidative stress. On the other hand, the lack of response to acetaminophen of *V. philippinarum* indicated that the drug did not induce oxidative damage in this species, reflecting that *V. philippinarum* possess a higher ability to cope with chemical aggression. The results obtained in the referred study are not in agreement with those found in the present work, in which, *V. decussata* seemed to be more capable to cope with CBZ due to the higher antioxidant defense mechanisms, while *V. philippinarum* was not so efficient combating the stress

induced by CBZ, since it presented a lower response of these defenses. In this way, and in opposition to other studies that showed that *V. philippinarum* was more resistant to environmental stress than the native species *V. decussata*, leading to its replacement (Solidoro et al., 2000; Pravoni et al., 2006; Usero et al., 1997), in the present study, *V. philippinarum*, the invasive species, was more sensitive to CBZ than *V. decussata*, the native species.

4.4. Chronic toxicity

4.4.1. Carbamazepine uptake by clams

Measuring the CBZ concentrations in whole body homogenates clearly evidenced its immediate uptake by *V. decussata*. The results for CBZ quantification showed that, in both toxicity tests, *V. decussata* increased CBZ in their tissues with the increase of CBZ concentration in water. However, the CBZ accumulated by clams were low and didn't change significantly the concentration of exposure in water. As demonstrated here, the blank test ensured that no CBZ losses occurred during the assay. However, a different trend was found between the two toxicity tests when comparing the BCF results. For *V. decussata* exposed to the acute test, BCF increased with the increase in the exposure concentration. However, when *V. decussata* was submitted to the chronic assay, although it was observed an increase in CBZ accumulated in clams' tissues with the increase in the spiked concentration, the determined BCF was highest in the clams exposed to 0.30 μg/L, the lowest concentration determined with confidence by ELISA. Contardo-Jara et al. (2011) found a similar trend when determined the BCF values in the mussel *D. polymorpha* after exposure to CBZ (0.236-236 μg/L) for 1, 4 and 7 d. The highest value of BCF was obtained at the lowest CBZ concentration. The authors reported that although BCF were lower than the predicted, an ongoing uptake was likely to occur due to the increase of CBZ until the end of exposure (BCF 0.236 μg/L CBZ = 17, 60 and 90 after 1, 4 and 7 day, respectively). So, it was possible to expect an increase in BCF value after a longer exposure to CBZ. In the present study, BCF was determined in *V. decussata* after 28 d of exposure to CBZ, being approximately 1.6 at CBZ 0.30 μg/L (the lowest CBZ concentration determined). However, the BCF calculated for *V. decussata* after a 4 d exposure to CBZ reached a maximum value of approximately 1 at the highest CBZ concentration (9.00 μg/L). It seemed that at lower CBZ concentrations, the clams had a higher filter activity, not considering the presence of CBZ in water as a threat. Moreover, as the accumulated CBZ at the highest concentration of exposure was similar for both assays it was suggested that a threshold exists for the CBZ uptake, above which no more CBZ is accumulated by the clams because it will exert a toxic effect. In this case, it seemed that the threshold concentration was between 3.00 and 9.00 μg/L of CBZ, concentrations that occur in the environment. Contardo-Jara et al. (2010) observed a similar trend when exposed the mussel *D. polymorpha* to the β-blocker metoprolol (0.534-534 μg/L) during 1, 4 and 7 d. Again, a higher BCF was found at the lowest concentration, where after just 4 d, the mussels accumulated 20 times higher concentration of metoprolol compared with the exposure

medium (0.534 µg/L). At higher concentrations, the animals accumulated the substance to a less extent, on average 6 to 8 times within one week. The authors suggested that the decrease of BCF with the increase in exposure concentration could be the result of an active uptake of metoprolol by mussels, occurring its regulation due to a competition for binding sites at the carrier, and thus resulting in an inhibition of influx. At lower concentrations no inhibition would take place, justifying the highest accumulated concentrations. In this way, for the present work it was thought that a certain type of carrier is possible involved in the uptake of CBZ by cells, limiting the concentration accumulated at higher concentrations as observed in the acute test at 9.00 µg/L and in the chronic test at 3.00 and 9.00 µg/L. At lower concentrations (0.03 µg/L for the chronic test and 0.03 and 0.30 µg/L in for acute test) no regulation in the uptake of CBZ seemed to occur. However, more understanding about the CBZ uptake needs to be established in future studies to confirm these suggestions.

4.4.3. Relationship between the carbamazepine exposure and the physiological parameters

The accumulation of CBZ in clams' tissues was accompanied by a general increase in GLYC content and by a decrease in PROT content after the exposure of clams chronically to CBZ. Carbohydrates as GLYC are assumed to constitute the most important energy reserve in bivalves. Because of their water solubility they are immediately available for utilization. The general increase in GLYC content obtained in the chronic test seemed to be related with the acute test, where a slightly tendency for an increase in its content occurred at the higher CBZ concentrations. However, just like in the acute test these variations were not attributed to the presence of CBZ. Although significant differences were observed for the GLYC content it was supposed that CBZ was not enough to produce these effects. Moreover, the clams exposed chronically to CBZ were fed with algae, so they could use this food supply to obtain energy for their needs.

Relatively to PROT content, for *V. decussata* exposed to the acute test, the observed increase was previously justified as an attempt to induce proteins to be used in the defense against CBZ. However, after a long period of exposure to CBZ, an opposite response was observed at the highest concentrations. Although in the control and at 0.03 µg/L a higher protein content occurred for *V. decussata* exposed to the chronic test, which also may reveal a higher response of the clams to combat the stress induced by CBZ, its levels decreased at the higher CBZ concentrations. So, it was assumed that an initial attempt of cells to induce proteins might be involved in the response to CBZ oxidative stress, but at higher CBZ concentrations the clams failed in this induction possibly due to

the toxic effects caused by the drug. Moreover, the decrease in PROT content may be the result of reactive oxygen species attack on proteins, as reported by Li et al. (2009). These authors studied the effects of CBZ (1.0 µg/L, 0.2 mg/L and 2.0 mg/L) on fish (*O. mykiss*) after a 7, 21 and 42 d of exposure. They found that a prolonged exposure to CBZ resulted in an excess of ROS formation leading to oxidative damage to lipids and proteins. In particular, the authors observed a significant induction in the protein carbonyl levels after a 21 d exposure to CBZ (0.2 and 2.0 mg/L). As reported previously the formation of carbonyl groups results of the attack of ROS on proteins.

4.4.4. Relationship between carbamazepine exposure and biochemical parameters

As referred before, the uptake of CBZ could result in its accumulation by lysosomes, leading to lysosomal membrane impairment. As lysosomes are organelles involved in phagocytic activity and consequently in the production of ROS, its membrane damage could result in the release of hydrolytic enzymes into the cytosol inducing oxidative effects (Tsiaka et al., 2013). In this way, in the present work, LPO levels were measured in order to verify the presence of oxidative damage induced by CBZ. Comparing the two assays, in the chronic exposure to CBZ, *V. decussata* presented higher LPO levels when comparing to all the concentrations tested with the acute test. Possibly the clams were already in a stressful condition, or, due to the different period of their capture and thus, seasonal variations, the clams came with a different physiological state. However, after 28 d of exposure, oxidative damage significantly increased at CBZ 9.00 µg/L compared to the remaining conditions. On the other hand, the results for the acute test showed an opposite response, were the oxidative damage was significantly lower at the highest CBZ concentration compared to the remaining conditions. In this way, the results seemed to indicate that, after a long-term exposure to CBZ, the ROS formed were not eliminated, exerting its toxic effects, as it was seen by the increased LPO levels at the highest CBZ concentration. However it is possible to observe a general decrease of LPO levels at the lower CBZ concentrations in the clams submitted to the chronic test, possibly indicating an attempting of cells to neutralize ROS effects, although without success. These results clearly show a higher oxidative damage in clams exposed chronically to CBZ in opposition to the clams exposed to the acute test. LPO was also induced in all tissues of the crab *C. maenas* exposed to increasing concentrations of CBZ (CBZ 0-50 µg/L, 28 d) (Aguirre-Martínez et al., 2013b).

Reduced glutathione is considered to be one of the most important scavengers of ROS, and its ratio with GSSG may be used as a marker of oxidative stress. An exposure of 28 d to CBZ resulted in an increase in GSH_t along the exposure range, being followed by an increase in GSH and a decrease in GSH/GSSG. Although, the results of the present work showed a similar trend for GSH_t

after exposing *V. decussata* in the two toxicity tests, the variation in GSH_t was more pronounced for *V. decussata* exposed to the chronic test than in clams exposed to the acute test, which only slightly increased GSH_t. GSH content showed to be, in general, higher in clams exposed to the chronic toxicity assay. This is possibly related with other factors than CBZ, indicating again that the clams already came in a stressful condition and that the higher GSH content was the response involved in the combat to the environmental stressors. However, differences were noticed between the exposure concentrations for clams exposed to chronic assay, being observed an increase in GSH comparing the clams exposed to CBZ with the clams in the control. This increase in GSH was possibly an attempt of organisms to use it as a cofactor by other defense enzymes such as GST or GPx, but as clams were already in a stressful condition, GSH variation was small. So, the increase in GSH_t and the consequent decrease in GSH/GSSG ratio were attributed mainly to the increase in GSSG, showing a higher oxidative environment in cells. The highest GSH/GSSG in the control condition for the clams submitted to chronic test was due to the highest content in GSH, reported previously. However, the increase in GSSG superimpose the GSH content, and thus reduced amounts of GSH were available to participate in detoxification reactions, and clams became less capable to handle with oxidative stress. The results obtained for GR activity in *V. decussata* exposed to the chronic test seemed to support those obtained for GSH, GSH_t and GSH/GSSG ratio. At lower CBZ concentrations (0.03 and 0.30 µg/L), the activity of GR was similar to the control, possibly indicating that at these concentrations the enzyme was still operating without being influenced by CBZ. However, at higher CBZ concentrations (3.00 and 9.00 µg/L), a decrease comparing with the remaining conditions was observed for GR activity. So it was supposed that at higher CBZ exposures, GR was inhibited by the presence of CBZ. In this way, a lower production of GSH occurred, resulting in the accumulation of GSSG, and thus justifying the decrease in GSH/GSSG after 28 d exposure to CBZ. Although a decrease in GSH content was not observed in clams exposed chronically to CBZ, *V. decussata* didn't increase this content between exposure concentrations, perhaps indicating that the organisms were responding at their maximum capacity and were not able to increase GSH when submitted to increasing concentrations of CBZ. The same response was not observed in clams exposed to the acute test, where the decrease in GSH content was justified as being the result of its utilization by GST.

Generally, exposure to ROS generating contaminants results in an imbalance between low induction of antioxidant enzymes and high ROS production causing oxidative stress (Almeida et al., 2007). The reactive oxygen species can also attack the enzymes involved in the antioxidant system defense through the formation of carbonyl groups on these, thereby causing, for example, the

decrease in catalytic activity (Li et al., 2010c). To counteract the damaging effects of ROS, SOD exerts its activity catalyzing the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen. In both toxicity tests, the SOD activity revealed a significant increase in clams after exposure to CBZ. For *V. decussata* exposed to the chronic test, SOD activity increased only at the highest CBZ concentration (9.00 μg/L), while in *V. decussata* submitted to the acute test, the enzymatic activity was increased at 0.30, 3.00 and 9.00 μg/L. This may indicate that, after a 4 d exposure, to CBZ the clams responded more effectively to CBZ than after a 28 d exposure. As in chronic test, SOD was only activated at the highest CBZ concentration, the superoxide anions produced due to the accumulation of CBZ could not be eliminated by this enzyme and thus, produced higher LPO levels than for the acute test. The delayed activation verified in SOD could be due to a diminished state of alert, in such way that clams were only capable to "sense" the higher CBZ concentrations. Chen et al. (2014) found a decrease in SOD activity in gills and digestive glands of the freshwater clam, *C. fluminea*, when exposed to CBZ (0.5-50 μg/L), indicating an enhanced production of ROS. Li et al. (2010b) reported a significant increase in SOD activity in liver of the fish *O. mykiss* exposed to 2 mg/L of CBZ during 21 d.

Catalase is an antioxidant enzyme responsible for the reduction of hydrogen peroxide resulting from the action of SOD into molecular oxygen and water. The results obtained revealed for both toxicity tests that *V. decussata* decreased CAT activity when comparing the control with all CBZ concentrations tested. As referred before, the decreased enzymatic activity may suggest that hydrogen peroxide produced by SOD is possibly being converted not by CAT but by other enzyme with the same role, as GPx. However, *V. decussata* exposed chronically to CBZ showed lower levels of CAT activity comparing with *V. decussata* exposed to acute test. Thus, in consequence of lower enzymatic activity in clams exposed to CBZ during 28 d, a lower content in hydrogen peroxide is being enzymatically decomposed. As a result it can be converted to the hydroxyl radical, inducing the LPO observed in clams exposed chronically to CBZ (Yoon et al., 2010). In the case of the acute test, CAT activity seemed to be enough to degrade hydrogen peroxide. In this perspective, in the clams submitted to the acute test, the formed ROS could be neutralized by the joint action of SOD and CAT, which with the increase in antioxidant molecules (e.g. GSH) could have decreased the LPO at the highest concentration of CBZ. In the study performed by Chen et al. (2014), referred previously, the increase in CAT activity was the consequence of the hydrogen peroxide formation resulting from an increase in superoxide anions production and its conversion by SOD. Gonzalez-Rey and Bebianno (2012) showed an inhibition tendency in CAT activity overtime in the mussel *M. galloprovincialis* exposed to the non-steroidal anti-inflammatory drug ibuprofen (250 ng/L) for 15

d. However, these authors suggested that the response observed was possibly due to an overwhelming excess of hydrogen peroxide originated by SOD. The same tendency was reported by Li et al. (2009) which found an inhibition in CAT activity for the fish gill (*O. mykiss*) after the exposure to CBZ (1 µg/L-2 mg/L) during 7, 21 and 42 d. The inhibition was stronger with prolonged exposure duration which was reported as being due to the flux of superoxide radicals, resulting in hydrogen peroxide in the cells.

Second phase detoxification enzymes (such as GSTs) catalyze the synthetic conjugation reactions of the xenobiotic parent compounds and their metabolites, in order to facilitate the excretion of chemicals after phase I detoxification. In the present work, the clams exposed to CBZ during 28 d showed a slight increase in GST activity at the lower CBZ concentration (0.03 µg/L) and then a progressive decrease was observed, where the enzymatic activity was similar or lower to the control. These results reveal an initial attempt to neutralize CBZ, forming GS-conjugates but at higher CBZ concentrations this enzyme seemed to be impaired by CBZ, not exerting it effects. Moreover, as GSH was not being produced (since GR activity was decreased at higher CBZ concentrations), possibly GST didn't use this substrate for the conjugation with CBZ, neither for the inactivation of LPO products. A different trend was observed in the clams exposed to the acute test, where GST was increased after exposure to CBZ. In this case, GST was possibly operating in the formation of thiol metabolites resulting from CBZ oxidation and in the utilization of GSH to inactivate lipoperoxidation products thus, promoting the decrease in LPO levels at the highest CBZ concentration (9.00 µg/L). The results obtained for GST activity in the clams exposed to CBZ during 28 d are not in line with those obtained for Aguirre-Martínez et al. (2013b) which found a significantly increase in GST activity in all tissues of the crab *C. maenas* exposed to 10 and 50 µg/L of CBZ during 28 d. Martin-Diaz et al. (2009b) also found an induction in GST activity in digestive glands and mantle/gonads after exposing the mussel *M. galloprovincialis* to CBZ 0.1 and 10 µg/L during 7 d. In addition, it has been demonstrated that pharmaceuticals from a municipal effluent plume produced a significant increase in GST activity in tissues of the mussel *E. complanata* (Gagné et al., 2004).

In this way, the decreased response of GST, SOD, CAT and GR activity and the higher content in $GSH_{t,}$ mainly attributed to GSSG at the higher CBZ concentrations explained the higher LPO levels found at CBZ 9.00 µg/L. The higher production of ROS due to the higher accumulation of CBZ at the lower CBZ concentrations could lead to the impairment of antioxidant defense mechanisms.

4.5. Carbamazepine risk assessment

To better assess CBZ ecotoxicological risk in the Ria de Aveiro, two risk quotient (RQ) were determined using data from literature. One, using the values of PNEC reported for a set of aquatic species (RQ = 1.43) and another using a PNEC obtained only for the clam *V. philippinarum* (RQ= 1875). Although very different, both values are above 1 which means that an ecotoxicological risk is suspected, requiring a better ecotoxicological assessment of the presence of CBZ in the referred lagoon. The differences between the values are due to the species used for the PNEC determination. The PNEC using a set of species is less specific than using only clams and thus, the RQ is lower. However, this PNEC is more representative of what happens in aquatic environments since not only clams are exposed to CBZ. The RQ values obtained for the Ria de Aveiro are within the range of values obtained for other CBZ contaminated aquatic environments (Aguirre-Martínez et al. 2013a; Ferrari et al., 2003).

The requirement for a detailed risk assessment is a priority to ensure there are no major risks to environment and human health (McEneff et al., 2014). The presence of pharmaceutical drugs in caged mussels suggests a possibility for pharmaceutical uptake in wild and farmed species with potential exposure to humans via ingestion (McEneff et al., 2013). The presence of CBZ in drinking water and in cooked seafood has been reported (Benotti et al., 2009; McEneff et al., 2013). Benotti et al. (2009) screened drinking water that served more than 28 million people (source water, finished drinking water and distribution system (tap) water) from 19 water utilities and found that CBZ was among the 11 frequently detected compounds, with a median concentration less than 10 ng/L. McEneff et al. (2013) detected the presence of pharmaceutical residues in cooked and uncooked blue mussels (Mytilus spp.), including CBZ. CBZ was the pharmaceutical drug that underwent the least change in concentration after cooking (by steaming), with an increase of only 12%. According to these authors, the potential risk for pharmaceutical exposure to humans through the food chain exists but, this risk cannot be evaluated at present due to the lack of pharmaceutical exposure risk assessments carried out on aquatic organisms and humans (McEneff et al., 2013).

Chapter 5

Conclusions and Future Works

5.1. Conclusions

The occurrence of pharmaceutical drugs in the environment, especially in the aquatic ecosystem raised concerns about the possible ecotoxicological impacts on non-target species. Although in the last years a great number of studies have been published on this field, there is still a long way to go through. Despite the effort that is being made to evaluate the chronic toxicity effects of pharmaceutical drugs, the vast majority of the studies are relative to the short-term exposures where the concentrations tested and the EC_{50} determined are in the mg/L range, not being representative of the concentrations that occur in the environment.

In this way, in the present work it was studied the acute and chronic toxic effects of environmental concentrations of CBZ (0.03-9.00 μg/L) in bivalves, namely *V. decussata* and *V. philippinarum*, being a contribution to improve the knowledge on the environmental implications of the presence of pharmaceutical drugs on the aquatic ecosystem, specifically in the Ria de Aveiro. Moreover, it was applied the directive competitive immunoassay ELISA to quantify, directly, the CBZ present in the clams' tissues.

The application of ELISA demonstrated to be an efficient tool to analyze the occurrence of pharmaceutical residues in environmental matrices not only in water samples but also in complex matrices as clams' tissues. In this case, allowed to observe the uptake of CBZ by clams and to quantify the CBZ accumulated in their tissues with good recoveries percentages (between 91 and 118%), not requiring sample pre-treatment such as a pre-concentration, neither being affected by matrix interferences that could easily react with the antibody. In opposition to the reference techniques used to quantify pharmaceutical drugs in environmental samples, this assay also permitted to save time, to reduce the amount of materials (reagents and equipment) used and to reduce losses of the compound which could occur during the sample pre-treatment or the sample degradation.

Overall, the biomarkers applied indicated that CBZ impaired the health status and performance of the species studied here through an oxidative stress mediated process. Although the two species accumulated low levels of CBZ, presenting a low bioconcentration factor (approximately 1), these was enough to trigger variations in biochemical and physiological parameters indicative of a stress response. Moreover, it is known that the pharmaceutical drugs can exert its pharmacological action even at low concentrations.

The two species had a different response to the presence of CBZ in the aquatic medium. Overall, *V. philippinarum* presented a diminished activity of the enzymes involved in the oxidative stress combat and thus a higher oxidation state in their cells, promoting the oxidative damage of

the unsaturated lipids belonging to the cellular membranes or proteins. In the other hand, *V. decussata* seemed to be more capable to cope with CBZ due to the better antioxidant defense mechanisms and, in consequence, due to a better efficiency to neutralize the reactive oxygen species triggered by CBZ accumulation.

The chronic toxicity test didn't run as expected, with a higher percentage of death on the control condition, reason why the results obtained must be interpreted carefully and represent merely a preliminary study. Nevertheless, it was possible to observe a higher toxicity exerted by CBZ in the clams after a long-term exposure (28 d). This was mainly reflected as a lower activity/content in the mechanisms involved in the antioxidant defense system and thus, a lower capability to lead with oxidative stress induced by CBZ. The similar accumulation of CBZ comparing with the acute test at the highest concentration suggested that some type of carrier is involved in the uptake of CBZ at higher concentrations and that for lower concentrations no regulation in the uptake of this drug seemed to occur.

These data obtained in the present work is of relevant importance for the Ria de Aveiro since *V. decussata* and *V. philippinarum* are competing for the same habitat. As was reported before, *V. decussata* is a native species in the Ria de Aveiro whereas *V. philippinarum* was introduced later being an invasive species. Although previous studies have been reporting that *V. philippinarum* is more tolerant to environmental stressors and thus have a better capacity to occupy new habitats, in this study we found the opposite, where *V. decussata* seemed to be more tolerant to the presence of CBZ than *V. philippinarum.* This study also demonstrated that the species *V. philippinarum* and *V. decussata* are good sentinel species to evaluate contamination by CBZ.

The risk quotient determined in this work, for the presence of CBZ in the Ria de Aveiro was above 1, meaning that an ecotoxicological risk is suspected, being necessary to perform a better ecotoxicological assessment of the presence of CBZ in this lagoon. These studies should include more chronic tests, with more specific endpoints, taking into account the target organ of CBZ and that it can act on evolutionarily conserved targets. The CBZ risk assessment should also be performed using aquatic organisms, especially the ones that are harvested for human consumption, being of concern the potential for bioaccumulation and transference of CBZ along food chain, ultimately impacting humans.

5.2. Future works

In future works I hope to repeat the chronic toxicity test, and thus obtain more reliable results for the toxic effects after a prolonged exposure to CBZ. As a hypothesis about the possible inadequate depuration period was raised, the optimal conditions need to perform an acute and chronic toxicity tests must be optimized first. It should also be assessed the effect of tissues on the biomarkers analyzed, since some tissues could be more adequate than others to study the CBZ effects. The study of specific targets of CBZ must also be performed. CBZ exerts its action through voltage-dependent inhibition of Na^+ and Ca^{2+} channel currents (Ambrósio et al., 2002; Martin-Diaz et al., 2009b), reason why the study of these channels must be included in the next works. Moreover, since sediments can act as a reservoir for contaminants, the interference of sediments in the accumulation of CBZ by clams should also be addressed. Given the current widespread contamination of aquatic systems by numerous pollutants, not only pharmaceutical drugs, organisms will most likely be exposed to mixtures of compounds and thus, the effect of these mixtures should be evaluated when assessing the effects of CBZ.

Chapter 6

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Chapter 7

Annexes

7. Scientific divulgation

7.1. Papers on peer reviewed journals

Almeida A, Calisto V, Esteves VI, Schneider RJ, Soares AMVM, Figueira E, Freitas R. Presence of the pharmaceutical drug carbamazepine in coastal systems: effects on bivalves. Accepted in Aquatic Toxicology 2014. IF 2012: 3.730, Percentile 97%

7.2. Participation in scientific events

7.2.1. Oral communication

Almeida A, Freitas R, Calisto V, Esteves VI, Soares AMVM, Figueira E. Presence of the pharmaceutical carbamazepine in the clam *V. decussata*: comparison of acute and chronic toxicity tests. Accepted for ICEH-CISA 2014, International congress on environmental health. 24-26 September 2014, Porto, Portugal

7.2.2. Poster

Freitas R, **Almeida A**, Calisto V, Esteves VI, Soares AMVM, Figueira E. Presence of carbamazepine in coastal systems: effects on bivalves. SETAC Europe 24th Annual Meeting. 11-15 May 2014, Basel, Switzerland