Impact of the invasive macroalgae Asparagopsis armata - – an ecotoxicological assessment

Marta Sofia da Cruz Jacinto

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Dissertação para obtenção do Grau de Mestre em Biotecnologia dos Recursos Marinhos

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Title: Impact of the Invasive macroalgae *Asparagopsis armata* – an ecotoxicological assessment

Título: Impacto da macroalga invasora *Asparagopsis armata* – uma avaliação ecotoxicológica

À minha mãe e ao meu pai pela confiança que sempre depositaram em mim,

aos meus irmãos por todo o apoio sempre que necessário,

aos meus avós

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Resumo

Os Oceanos representam o maior sistema de suporte de vida sendo a uma grande fonte de riqueza, oportunidade e abundância. No entanto, a humanidade tem levado este ecossistema ao seu limite com crescentes níveis de poluição e outras pressões antropogénicas. A introdução de espécies não-nativas é reconhecida como uma das maiores ameaças à biodiversidade e a segunda maior causa de extinção das espécies. A macroalga vermelha *Asparagopsis armata* é uma espécie invasora originária da Austrália e que atualmente apresenta uma ampla distribuição em todo o globo devido à sua estratégia oportunista, ausência de predadores e altas taxas de crescimento. Uma questão emergente está relacionada com a capacidade destas espécies invasoras produzirem grandes quantidades de metabolitos halogenados potencialmente tóxicos. Esta característica pode representar um perigo adicional para o equilíbrio ecológico da comunidade invadida.

O presente trabalho teve como objetivo avaliar o potencial ecotoxicológico dos exsudatos de *A. armata* usando um gastrópode, *Gibbula umbilicalis*, como organismo modelo. A macroalga recolhida na costa de Peniche (Portugal) foi colocada em tanques no laboratório, durante 12 h, sendo depois o meio recolhido e filtrado para ensaios posteriores com os exsudatos da alga. No ensaio agudo, observou-se a mortalidade de *G. umbilicalis* que foi exposta a crescentes diluições do exsudato durante 96 h. Adicionalmente, os gastrópodes foram expostos a concentrações não letais do exsudato e analisou-se as respostas bioquímicas recorrendo a biomarcadores relacionados com destoxificação, defesas antioxidantes, danos oxidativos, danos neurotóxicos e metabolismo energético.

Os resultados revelaram que os exsudatos de *A. armata* afetaram significativamente a sobrevivência dos organismos expostos com uma CL_{50} 96h de 5.03% de exsudato da alga. A exposição aos exsudatos da alga também resultou em efeitos bioquímicos e metabólicos ao nível subcelular com resultados significativos na inibição da glutationa-S-transferase (GST), perda de integridade do ADN e níveis crescentes de atividade da lactato desidrogenase (LDH), dando uma indicação dos mecanismos de toxicidade desta alga marinha. Os níveis mais elevados de danos no ADN ocorreram quando a GST apresentou os níveis mais baixos de atividade e esta mesma atividade aumentou quando os danos no ADN diminuíram, em simultâneo com o aumento dos níveis de atividade da LDH, indicando que as necessidades energéticas aumentam devido à necessidade de sintetizar mais enzima.

Conclui-se que a *A. armata* tem capacidade de libertar substâncias tóxicas que podem ter potenciais impactos no ambiente envolvente. Adicionalmente, as respostas

bioquímicas estudadas em *G. umbilicalis* têm potencial para serem usadas como sinais de aviso na determinação dos efeitos provocados pelos compostos libertados por esta macroalga vermelha.

Palavras-chave: Ambientes costeiros, Biomarcadores, Ecotoxicologia, Espécies invasoras, *Gibbula umbilicalis*, Sobrevivência, Stress oxidativo

Abstract

The Oceans represent the largest life support system being a major source of wealth, opportunity and abundance. However, mankind has pushed this ecosystem to its limit with increasing pollution levels and other anthropogenic pressures. The introduction of non-native species is recognized as one of the major threats to biodiversity and the second leading cause of species extinction. The red macroalgae *Asparagopsis armata* is an invasive species native from Australia and currently has a wide distribution across the globe due to its opportunistic strategy, absence of predators, and rapid growth rates. One problematic issue emerging from the invasion of this species is related to its capacity to produce large amounts of halogen metabolites potentially toxic. This characteristic may represent an additional danger to the ecological balance of the invaded community.

This study aimed to assess the ecotoxicological potential of exudates of *A. armata* using a gastropod, *Gibbula umbilicalis*, as test organism. The seaweed collected at the coast of Peniche (Portugal) was left in laboratory tanks, for 12 hours, and afterwards the media was collected and filtered for further testing with algae exudates. In the acute test, *G. umbilicalis* mortality was observed with exposure to increasing dilutions of exudate for 96 h. Additionally, gastropods were exposed to non-lethal concentrations of exudate and analyzed using biochemical biomarkers responses associated with detoxification, antioxidant defenses, oxidative damage, neurotoxicity and energy metabolism.

The results showed that *A. armata* exudates significantly affected the survival of exposed organisms and a 96h LC_{50} of 5.03% of algae exudates as found. Exposure to the algae exudate also disturbed the biochemical and metabolic responses at the subcellular level with significant inhibitions of glutathione-S-transferase (GST), DNA integrity loss and increasing levels of lactate dehydrogenase (LDH) activity, giving an indication of the mechanism of toxicity of this seaweed. The highest levels of DNA damage occurred when the GST had the lowest levels of activity and DNA damages decreased with the increase of GST and LDH activities, indicating that the energy requirements increase due to the necessity to synthesize more enzyme.

In conclusion, *A. armata* is capable of releasing toxic substances with potential severe impacts to its surrounding environment. Also, the biochemical responses studied in *G. umbilicalis* have the potential to be used as early-warning signals to assess effects of the compounds released by this red seaweed.

Keywords: Biomarkers, Coastal Environments, Ecotoxicology, *Gibbula umbilicalis*, Invasive Species, Oxidative stress, Survival

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Table of Contents

List of Figures

Chapter I. General Introduction

Chapter III. Survival and biochemical responses in Gibbula umbilicalis exposed to exudates of the red macroalgae Asparagopsis armata

List of Tables

Chapter I. General Introduction

Chapter III. Survival and biochemical responses in Gibbula umbilicalis exposed to exudates of the red macroalgae Asparagopsis armata

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List of Abbreviations

ACh – acetylcholine
AChE – acetylcholinesterase
CAT – catalase
CbE – Carboxylesterase
CDNB – 1-chloro-2,4-dinitrobenzene
DBA – dibromoacetic acid
DNA – deoxyribonucleic acid
DTNB – 5,5-dithiobis-(2-nitrobenzoic acid)
EDTA – ethylenediamine tetracetic acid
GPx – glutathione peroxidase
GR – glutathione reductase
GSH – glutathione
GSSG – glutathione disulfide
GST – glutathione-S-transferase
Gww – grams of tissue wet weight
H_2O_2 – hydrogen peroxide
IAS – invasive alien species
IOC – Intergovernmental Oceanographic Commission
LC_{50} – medial lethal concentration
LDH – lactate dehydrogenase
LPO – lipid peroxidation
MDA – malondialdehyde
NADH – nicotinamide adenine dinucleotic
O_2^- - superoxide radical
OH^- - hydroxyl radical
POPs – persistent organic pollutants
ppm – parts per million
ROS – reactive oxygen species
SOD – superoxide dismutase
TMP – 1,1,3,3-tetramethoxypropan

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Chapter I.

General Introduction

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1. Pollution on marine ecosystems

The oceans cover more than 70% of the Earth's surface and contain around 99% of the living space on earth. Subsequently, oceans are home to a great source of the World's biological biodiversity (Targett et al., 2002; IOC/UNESCO et al., 2011). Moreover, the oceans play an important role in the regulation of the planet's climate and are responsible for over 35% of the primary production. The significance of marine waters is also economical, with 60% of the total economic value of the biosphere being attributed to the oceans (Costanza and Folke, 1997).

However, there is evidence that the oceans have suffered at the hands of mankind for millennia, with the history of marine environmental pollution dating back to the very beginning of the history of human civilization (Islam and Tanaka, 2004). Human impacts have increased with rapid population growth and substantial developments in technology. However, aquatic pollution did not receive much attention until a threshold level was reached, with possible adverse consequences on the organisms and their ecosystems (Jenssen, 2003). At the present, it has become a major global concern and one of the greatest challenges of the century, and identified by the European Union as one of the main threats to the normal function of ecosystems (Rodrigues et al., 2009; Nota et al., 2010). Many organizations have made several efforts to promote awareness and taken actions to stop biodiversity loss (Islam and Tanaka, 2004).

The United Nations Convention on the Law of the Sea defined pollution as "*the introduction by man, directly or indirectly, of substances or energy into the marine environment, including estuaries, which results or is likely to result in such deleterious effects as harm to living resources and marine life, hazards to human health, hindrance to marine activities, including fishing and other legitimate uses of the sea, impairment of quality for use of the sea water*" (Islam and Tanaka, 2004).

Knowledge of the pollution sources and impacts on ecosystems is important not only for a better understanding on the ecosystem responses to stressors but also to formulate prevention measures. Many of these impacts are generally well known and include a range of threats like oil spills, untreated sewage, heavy siltation, eutrophication, persistent organic pollutants (POP's), heavy metals, acidification, radioactive substances, marine litter, overfishing, and destruction of coastal and marine habitats (McCook, 1999, Nyström et al., 2000; Bellwood et al., 2004). Besides this, new concepts on environmental pollution are emerging, for example biological pollution (e.g. invasive species) (Islam and Tanaka, 2004).

The term "biological pollution" has been growing in recent years with identified impacts of introduction and invasion of species throughout the world (Boudouresque and

Verlaque, 2002). An ever-increasing number of articles, journals and books bearing "invasion" in their titles document that it is an extremely active research area, integrating a diversity of fields such as biogeography, ecology, evolutionary biology, biosecurity, conservation practice, and applied management (Heger et al., 2013).

2. Invasive Alien Species

The European Commission (2002) defined Invasive alien species (IAS) as "*a species whose introduction and/or spread outside their natural past or present distribution threatens biological diversity*". As a result, an introduced species is a species that fulfills the four following criteria: 1) it colonizes a new area where it was not previously present; 2) the extension of its range is linked, directly or indirectly, to human activity; 3) there is a geographical discontinuity between its native area and the new area; and 4) new generations of the non-native species are born *in situ* without human assistance, consequently constituting self-sustaining populations (Boudouresque and Verlaque, 2002).

2.1. Introduction and Establishment

Globalization has combined widely dispersed human communities into a worldwide economy. This process provides many benefits through the movement of people and goods, but also commonly leads to the transfer of organisms among ecosystems that were previously separate (Keller et al., 2011). Species have always used the oceans to move around the planet, but until recently, this process has been moderated, limited by the currents and the winds. However, since people began travelling they have inadvertently carried "pests" with them, including unnoticed marine organisms (Bax et al., 2003).

Invasions usually occur in two major phases: Introduction and Establishment. The first step in the process is the transfer of a species from its native range into a new place. Natural transport, or dispersal, is not considered invasion, only Human-mediated transportation is considered true invasion, which can be intentional or accidental. Intentional transport involves introduction of new species for a purpose, for example as pets, for hunting, or as ornamental species. Accidental transport is where organisms are unintentionally moved out of their home range. However, only a few species manage to survive transit (Namboothri et al., 2012).

The major vectors responsible for the global movement of aquatic organisms include ships' ballast, aquarium industry, aquaculture, the bait industry, and fouling of ships' hulls (Bax et al., 2001). In Table I a more complete source of marine species introduction is presented with the main target associated to each vector.

Table I – Anthropogenic vectors for accidental marine introductions (Adapted from Bax et al., 2003).

Source	Vector	Target
Commercial shipping	Ballast water	Plankton, nekton, benthos in sediment
	Hull fouling	Encrusting, nestling and some mobile species
	Solid ballast (rocks, sand, etc.)	Encrusting, benthos, meiofauna and flora
Aquaculture and fisheries	Intentional release for stock enhancement	Single species
	Gear, stock or food movement	Various
	Discarded nets, floats, traps, trawls, etc.	Various
	Discarded live packing material	Various
	Release of transgenic species	Single species
Drilling platforms	Ballast water	Plankton, nekton, benthos in sediment
	Hull fouling	Encrusting, nestling and some mobile species
Canals	Movement of species through locks due to water motion or active swimming	Various
Aquarium Industry	Accidental or intentional release	Aquarium fauna and flora
Recreational boating	Hull fouling	Encrusting, nestling and some mobile species
Dive practices	Snorkeling and scuba gear	Algal spores, bacteria, some small mobile species
Floating debris	Discarded plastic debris	Encrusting and some mobile species

Once introduced into a different system, the establishment of species in its new habitat depends on several factors, including the richness of the habitat and it ecological quality. Consequently, when an alien species arrives at a new location, several things can happen: 1) it can find its new habitat unwelcoming and die; 2) it can survive with little environmental impact; or 3) it can take over, harming the naturally existing wildlife in a variety of ways. Factors intrinsic to the species that aid in their establishment and spread comprise: high environmental tolerance, short generation times, rapid growth, a broad diet, early sexual maturity, high reproductive output, and rapid dispersal (Namboothri et al., 2012).

However, the establishment of a naturalized population of a non-native species does not imply that the species has become invasive. To be considered invasive, a species has to establish large populations and spread in its new system, causing damages (Namboothri et al., 2012).

2.2. Impacts and Management

Invasive species are causing increasing concern, since they are almost impossible to eradicate, once becoming established (Wallentinus and Nyberg, 2007).

Their impact can be divided into three major areas of influence: environmental, economic, and social (Bax et al., 2003; Zenetos et al., 2005).

These organisms can act as ecosystem engineers, influencing the habitat itself, positively or negatively, directly or indirectly, and physically or chemically. At a first glance they may have a positive impact, by providing places for shelter in previously barren areas or increasing habitat diversity and special heterogeneity, which would increase species richness. However, the opposite effect is most often observed (Wallentinus and Nyberg, 2007).

On a global level, invasive alien species are considered as one of the major threats to biodiversity, both in terrestrial and marine environments (Zenetos et al., 2005; Altamirano et al., 2008; Regulation (EU) No 1143/2014), and the second leading cause of species extinction, along with habitat destruction (Zenetos et al., 2005). According to the National Wildlife Federation approximately 42% of endangered species are at risk owing to invasive species (Pimentel et al., 2005).

The consequences are due to ecological interactions with biota in a variety of ways. For example by competition for resources (including place to settle and spawning grounds, grazing or predation on native organisms, trophic cascading effects, or filling up empty niches), being a reservoir for parasites or a vector for pathogens, by hybridizing with a related species or varieties, by altering the local food web, disrupting pollination services, changing habitat structure, or even being toxic (Zenetos et al., 2005; Wallentinus and Nyberg, 2007). Thereby, the threat extends also to ecosystem functions and services.

Biotic communities throughout the world are being homogenized and restructured through biological invasions. This has the potential to cause large economic losses, particularly in countries that rely on natural and primarily resources of production like agriculture, forestry and fisheries for their development (Namboothri et al., 2012). Human health and economies are also at risk. The impacts on our natural ecosystems and economy cost billions each year.

Approximately 12 000 species in the environment of the European Union (EU) and in other European countries are alien, of which roughly 10 to 15% are estimated to be invasive (Regulation (EU) No 1143/2014). Invasive alien species are estimated to have cost at least €12 billion/year over the past 20 years, and the figure is growing. The risk such

invasive species pose may intensify due to increased global trade, transport, tourism, and climate change (Regulation (EU) No 1143/2014).

In Portugal, over the last two centuries, and especially in more recent decades, the number of exotic species has increased significantly, currently amounting to 670 species, which corresponds to approximately 18% of the total native. Several of the exotic species listed in Portugal are considered invasive and about 8% have invasive behavior (Almeida and Freitas, 2012).

Many organizations around the world are creating laws, legislations and regulations to control and eventually eradicate the invasive species. The most recent is the Regulation (EU) No 1143/2014 on the prevention and management of the introduction and spread of invasive alien species of the European Parliament, which began to be applied in the present year of 2015.

2.3. Examples of successful invasions

The studies about aquatic invasive organisms are numerous, some examples are: the green algae *Caulerpa taxifolia* in the Mediterranean (Meinesz and Hess, 1991) and the zebra mussel (*Dreissena polymorpha*) in the North American Great Lakes and Europe (Carlton, 1996).

The green alga *Caulerpa taxifolia* (Vahl) C. Agardh is one of the most publicized introduced marine species (Thibaut et al., 2004; Theil et al., 2007; Burfeind and Udy, 2009). This seaweed is successful at colonizing low light and nutrient enriched areas, so it can live in lower water quality conditions and assimilate nutrients from the water column and sediment (Burfeind and Udy, 2009). It forms dense meadows from few meters under the surface to over 40 m (Thibaut et al., 2004). It is native to tropical and subtropical areas of Australia, with Moreton Bay being the southernmost extent of its native range (Burfeind and Udy, 2009). *Caulerpa taxifolia* has been recorded from Mediterranean Sea (Croatia, France, Italy, Monaco, Spain and Tunisia), along the coast of California, as well as Japan and eastern Australia (Thibaut et al., 2004; Theil et al., 2007; Burfeind and Udy, 2009). Indeed the invasion in the Mediterranean Sea is a long and well-documented history where its rapid expansion has been observed competing with native sea grass (Burfeind and Udy, 2009).

Zebra mussel is a good example of a successful invasive species. Its rapid expansion and its important ecological and socio-economic effects, have led to numerous studies with this species (Wolfram, 1994; Miller and Watzin, 2007; Costa et al., 2008; Evans et al., 2011; Strayer, 2012; Colomer et al., 2014; Faria et al., 2014; Pain-Devin et al., 2014; Lindim, 2015)

Zebra mussel are freshwater bivalves native from the Caspian and Black Sea basins but expanded along European waters courses in the 19th and 20th centuries and reached the Great Lakes and other water bodies in North America during the last decades of the 20th century. In Europe, zebra mussels are reported to exist in Germany, Great Britain, The Netherlands, Czech Republic, Sweden, France, Italy, and Spain, being the last European country to report sightings of this invasive bivalve (Faria et al., 2014; Colomer et al., 2014; Lindim, 2015). It is considered an opportunistic species with the ability to settle in a wide variety of aquatic habitats, essentially because it has a rapid life cycle featuring a massive reproductive potential, along with a high larval mobility, thus implying great dispersal capability. (Colomer et al., 2014).

Dreissena polymorpha is thus a highly successful colonizer able to influence the new aquatic ecosystem causing several ecological changes (Colomer et al., 2014). Because of their fast growth and high filtration rates, they are able to remove a significant portion of primary production, induce trophic shift, starving out many of the Great Lakes' native mussel populations, and reduce water turbidity (Lindim, 2015). It also impacts water use significantly increasing operating costs and maintenance of hydraulic works, such as in the case of collapsed drains and water pipes, loss of attraction and rejection by tourists of recreation zones associated with fishing, sailing or swimming (Colomer et al., 2014). Hundreds of millions of dollars are spent annually to control their densities in America and just in Ebro River basin in Spain it causes damage amounting to €2 million per year.

2.4. Macroalgae as invasive

Marine macroalgae are considered autogenic ecosystem engineers because they control resource availability to other species through their physical structure (Crooks, 2002). Seaweeds are organisms that largely influence the architecture on both rocky and sediment bottoms (Wallentinus and Nyberg, 2007).

In coastal habitats, macroalgae constitute an important component of introduced biota, ranging from 8 to 38% of the total number of the recorded non-indigenous species (Altamirano et. al., 2008). The Mediterranean and the NE Atlantic support the highest number of seaweed introductions (Pacios et. al., 2011).

Williams and Smith (2007) reviewed the impacts of introduced seaweeds and pointed out that in the majority of studies (55%) there was a negative effect on native species, although in some cases the effect was not detectable (30%) or even positive (enhancement) (15%) (Pacios et. al., 2011; Guerra-García et. al., 2012).

2.5. *Asparagopsis armata*

Asparagopsis armata is a red algae (Rodophyta), belonging to the Order Bonnemaisoniales and Family Bonnemaisoniaceae (Table II). Two species of this Genus are currently known: *Asparagopsis armata* Harvey (1855) and *Asparagopsis taxiformis* (Delile) Trevisan (1845) (Andreakis et. al., 2004; Guerra-García et. al., 2012). Their characteristic aspect of "asparagus" (Asparagus in Latin) gives them the name, but they differ in the presence of conspicuous harpoon-like barbed spines of *A. armata* that are very distinctive and give it the common name of harpoon weed. The word “armata” means “armed” (Chualáin et al., 2004).

Table II – Taxonomic classification of *Asparagopsis armata*.

Kingdom: Plantae
Phylum: Rhodophyta
Class: Florideophyceae
Subclasse: Rhodymeniophycidae
Order: Bonnemaisoniales
Family: Bonnemaisoniaceae
Genus: *Asparagopsis*
Species: *Asparagopsis armata*

This red seaweed has the most elaborate life-cycle of all marine algae. It has a triphasic life cycle, indicating three distinct phases: the gametophyte, the carposporophyte, and the tetrasporophyte (Figure 1). Besides, the life-cycle is diplohaplontic with a haploid gametophyte and a diploid tetrasporophyte (Chualáin et al., 2004; Pacios et. al., 2011).

There are separate male and female gamethophytes that produce the corresponding gametes, which have half the genetic code necessary for the species (n), in specific reproductive organs, spermatangium and carpogonium, respectively. There are no flagellated cells, thus when the spermatia are released they are carried passively by water currents to the trichogyne located on the carpogonium. Once the gametes are fertilized, they growth into a carposporophyte (2n), a separate generation. The carpospores are eventually released into the water column, where they can settle, germinate, and grow into another generation known as the tetrasporophyte (2n). These tetrasporophyte produce four

tetraspores (n), by meiosis that are then released into the water and germinate and grow into more male and female gametophytes. In fact this is a heteromorphic life cycle, that means it has two distinct forms and they are so different that at one time it was considered to be two different species - the gametophyte generation *A. armata* and the tetrasporophyte generation *Falkenbergia rufolanosa* (Figure 2) (Chualáin et al., 2004; Garon-Lardiere, 2004; Kraan and Barrington, 2005). Both phases are capable of vegetative reproduction and fragmentation too.

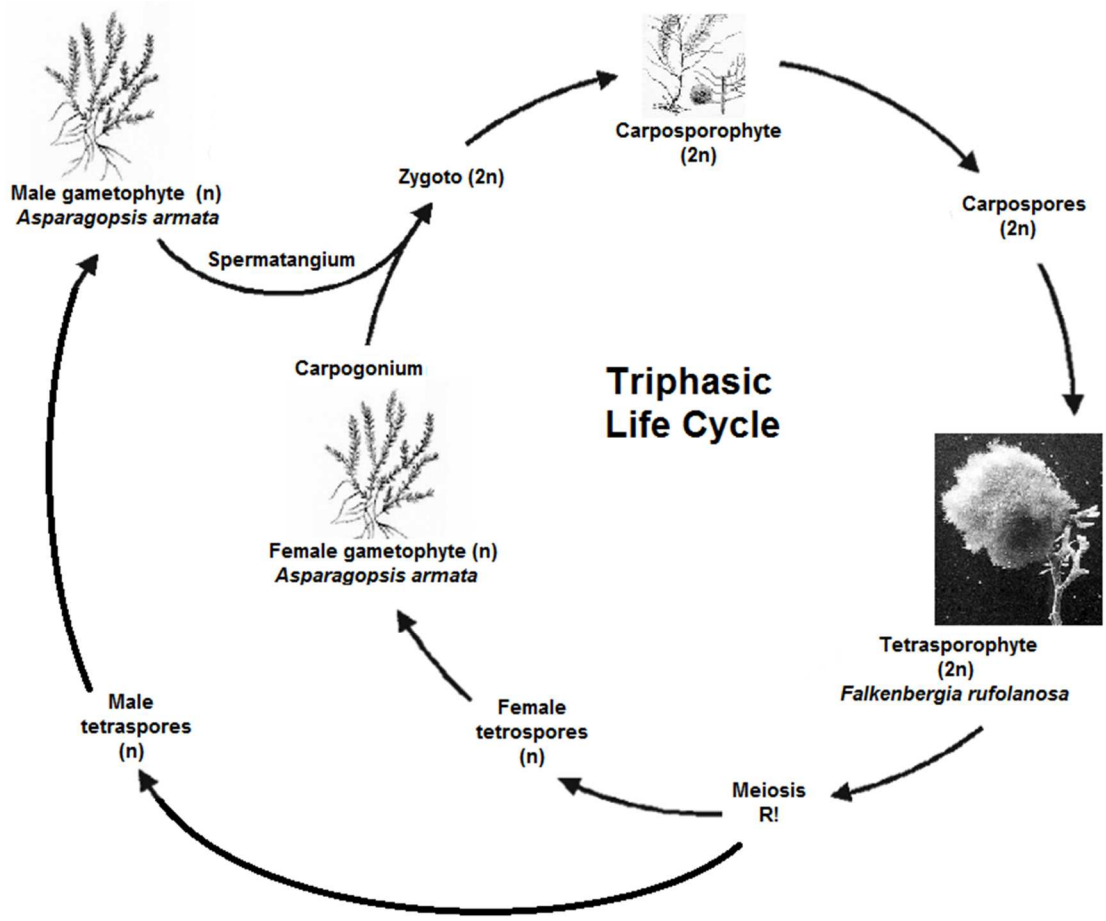

Figure 1 – Schematic representation of the life cycle of *Asparagopsis armata*.

Asparagopsis armata is found in the lower intertidal to shallow subtidal and occasionally in deeper pools, up to 15 meters deep, forming natural vegetation belts on exposed coasts (Altamirano et. al., 2008; Soler-Hurtado and Guerra-García, 2011; Guerra-García et. al., 2012). This species are often found growing on other algae (epiphytically) or, otherwise, can attach to rocky substrates or float freely (most often seen in tetrasporophyte

phase). The gametophyte phase is found mainly from March until August in the Atlantic coast of Iberian Peninsula.

Falkenbergia rufolanosa, tetrasporophyte, is a rosy-pink collection of fine, irregularly branched filaments which appear as small (1 to 3 centimeters in diameter) fluffy pom-pom balls underwater. The sexual gametophyte generation, however, is much different. *Asparagopsis armata* are composed of bushy, paired, spirally arranged branchlets and erect shoots from which numerous side branches develop in all directions. The latter ramify over and over again giving a plumose appearance and branch tips taper into harpoon-like barbs, the characteristic feature of the specie. Its color can range from pale pink through to red and bright purple, but quickly degenerating when removed from the water and becoming distinctly orange (Figure 2) (Andreakis et. al., 2004; Pacios et. al., 2011).

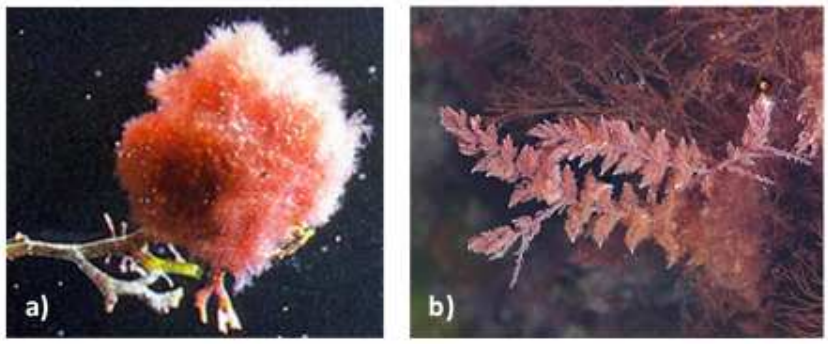

Figure 2 – Photograph of a) tertrasporophyte generation, *Falkenbergia rufolanosa* (Núcleo de Ambiente da Universidade do Algarve, NAMB) and b) gametophyte generation, *Asparagopsis armata* (Fiona Crouch).

Species of the family Bonnemaisoniaceae are well known as sources of halogenated compounds with strong antibacterial, antifungal and antibiotic activity that can be used for cosmetic and pharmaceutical purposes (Genovese et. al., 2009). The halogenated compounds from *Asparagopsis* have a wide range of volatility and solubility. This species also produces sulphated galactans with promising therapeutic applications. *Asparagopsis armata* is also harvested or grown for the production of agar, a firm gel-like substance, that is used as a thickener and stabilizer in many products, for example in food industry, as well as in laboratory work as growth medium for bacterial cultures. In general, the production of biologically-active metabolites is inherently linked to an ability to storage compounds into specialized structures (gland cells) in order to avoid autotoxicity (Mata et al., 2006; Genovese et. al., 2009). The gland cell is internal to the parent cell, but maintains a physical connection with the outer cell wall. This connection appears to be a means for the release of metabolites to the algal surface (Paul, 2006). The pungent aroma of these algae is due

to an essential oil that is composed mainly of bromoform with smaller amounts of other bromine, chlorine, and iodine-containing methane, ethane, ethanol, acetaldehydes, acetones, 2-acetoxypropanes, propenes, epoxypropanes, acroleins and butenones (Genovese et. al., 2009).

Asparagopsis armata has a strong invasive behavior, and is included in the list of the "Worst invasive alien species threatening biodiversity in Europe" and also in the list of the "100 Worst Invasives in the Mediterranean Sea" (Altamirano et. al., 2008). It is native of the Southern Hemisphere, from Australia and New Zealand, where it was discovered by Harvey in 1885 (Chualáin et al., 2004; Soler-Hurtado & Guerra-García, 2011; Pacios et. al., 2011; Guerra-García et. al., 2012). This species was introduced to the Northern Hemisphere and, the first appearances in the Atlantic Oceans and Mediterranean Sea date from 1920's. The first record of its appearance in Europe dates from 1923 (Garon-Lardiere, 2004). Since the spreading, *A. armata* is now found globally, from Western shores of England and Ireland, the Atlantic coast of France, Spain and Portugal down to the Canary Islands and throughout the Mediterranean Sea, Pacific and Indian Oceans. (Garon-Lardiere, 2004; Kraan and Barrington, 2005).

This seaweed is regarded as invasive because it spreads naturally in natural habitats in short time, colonizing a wide area, displacing native species and produces a significant change in terms of community composition (Chualáin et al., 2004; Soler-Hurtado & Guerra-García, 2011). Prolific vegetative reproduction may explain the fast dispersal rates of the species, which also exhibits an attaching system consisting in basal stolons and rhizoids able to facilitate the establishment of the propagules (Altamirano et. al., 2008).

Additionally, *A. armata* can be distributed on tide pools during the low tide and these pools may be roughly seen as a microenvironment because they have approximately six hours between low tide and high tide, they constitute an isolated system without water input or output and are exposed to extreme biotic and abiotic factors. These conditions can get adverse for this seaweed and consequently lead to stress disorders and severe consequences for littoral animals, such as invertebrates and fish larvae (Engström-Öst and Isaksson, 2006). Furthermore, *A. armata* exudates toxicity has been reported in the amphipod *Hyale nigra* (Paul et. al., 2006).

3. Sentinel species

The selection of sentinel species susceptible to study is an equally important part of any environmental assessment study and responds to five recommendations:

- Distribution and nature of the contaminant;

- Ecological and economical importance;
- Abundance and susceptibility to study;
- The existent of previous works;
- Sensibility;

Sentinel species are used to assess the pollution in a habitat through space and time. Thus, research conducted in aquatic ecotoxicology is mainly based on the use of sentinel species (Beeby and Richmond, 2011).

In field studies, these species can be used for rapid risk assessments to provide information on the environmental conditions of an area. So, they are selected for their capability to reveal ecological perturbations and provide insight about environmental changes, centered on their life history and physiological characteristics (Zettler et al., 2013).

A sentinel organism can indicate the presence of toxic substances by the manifestation of unusual symptoms or measureable responses that are not associated to their basal activities. The information about the presence of environmental stressors can be assessed through the study of: the content of certain elements or compounds; the changes on morphological or cellular structure; metabolic/biochemical responses; and behavior or population structure (Beeby and Richmond, 2011). In this line, animal sentinels must have measurable responses to the toxic substance in study, whether that is due to the animal's death, disappearance, behavioral changes, alterations on biochemical processes, among others determinable aspects.

These organisms are ideally un-endangered species and are usually easy to find and handle. Further, the aptitude of a species to function as an indicator might depend on the position and role inside the ecosystem. (Zettler et al., 2013)

3.1. *Gibbula umbilicalis* as a test organism

Over the years, benthic organisms have been especially useful in applied research and in ecotoxicology in particular given that they tend to be good indicators of environmental stress.

Gibbula umbilicalis (Costa, 1778) is a sea snail, marine gastropods of the family Trochidae. It is an eastern Atlantic species with a wide geographical distribution inhabiting temperate waters in the upper intertidal zone on rocky shores where wave energy is low. The preferential habitat is rocky platforms with a dense algal cover but also can be found in pools, under stones, and on upper surfaces of rocks. Their morphologic characteristics are a small top shell and a large round umbilicus, a deep hole on the underside of the shell.

The shell color is cream or greenish in a background with broad stripes of red or reddish-purple. This organism becomes sexually mature at about 18 months with a shell size of 8 to 9 mm. (Williams, 1964; Gaudèncio and Guerra, 1986; Underwood, 1972).

In terms of research, they provide excellent opportunities because they are easily found, collected and maintained. Besides, the facility to identify and count along the relative size and low mobility make this organism an appropriate species for ecotoxicological experiments (Cabecinhas et al., 2015). Previous studies revealed the high tolerance of this organism to sewage discharges (Ali and Bream, 2010), and heavy metals (Cabecinhas et al., 2015). Moreover, Cabecinhas and colleagues (2015) confirmed the potential of *G. umbilicalis* as a marine model to ecotoxicological tests, along with the use of various endpoints such survival, behaviour and enzymatic biomarkers.

Regarding the present study, the fact that the *G. umbilicalis* live around high tide mark and can be distributed by tide pools along the presence of *A.armata* provides an opportunity to test the exposure of this sea snail to the algal exudates and observe its effects.

4. Ecotoxicological bioassays

Ecotoxicological laboratory tests are important bioassays for a first toxicity assessment and as a first tier for Environmental Risk Assessment (ERA) purposes. ERA is a process of predicting whether there may be a risk of adverse effects of chemical substances in the environment, which can be - either synthetic or natural (e.g. human hormones, toxins produced by algae) - or also the effects of other abiotic stressors such as temperature, U.V. light, predation, etc. (Lemos et al., 2010). It is generally based in information derived from research on physical-chemical characteristics of xenobiotic and from laboratory toxicity tests (Moore et al., 2004) focusing on the relationship between toxic substances in the environment and the potential hazards of these if they exceed certain threshold levels (Binelli et al. 2005). One of the core missions of ecotoxicology is to find concentrations of environmental contaminants that exert an adverse effect on the organisms and to understand the mechanisms by which these contaminants perturb normal biological performance in order to develop appropriate measures to prevent adverse outcomes (Connon et al., 2012).

The biological assessments can be divided into three categories: 1) Acute/lethal tests; 2) Chronic/sublethal responses; 3) Biomarkers of biochemical/cellular/molecular responses (Widdows, 1998).

4.1. Classical endpoints

Traditionally, toxic effects are measured using standardized methods, based mainly on acute (e.g. mortality) and chronic responses (e.g. reproduction) of a sensitive biological indicator (Amorim et al. 2005).

Acute tests are short-term exposure experiments (hours or few days) and generally use mortality as endpoint. In acute exposures, organisms come into contact with higher doses of the toxicant in a single event or in multiple events over a short period of time and usually produce immediate effects, depending on absorption time of the toxicant (Widdows, 1998; Connon et al., 2012). The United Nations (2006) define acute tests as those which determine an LC_{50}, Median Lethal Concentration, i.e. concentration causing mortality in fifty percent of exposed organisms. Acute tests are robust and very frequently used given their short time duration, simplicity and unambiguity of the endpoint measured. The results can provide meaningful comparisons of lethality between organisms, toxicants or test conditions (Orchard, 2000).

Avoidance behavior, energy budget, reproduction, feeding and growth rates are also among the most common endpoints used to assess toxicity at an individual level, concerning sublethal effects (Moreira et al., 2006), since reductions in such parameters have been correlated with the presence of toxicants (McLoughlin et al., 2000). However, acute tests are usually employed as a "screening tool" with a broad range of toxic concentrations.

4.2. Biochemical Biomarkers

The biomarkers approach has been incorporated in several pollution monitoring programs in Europe, such as the Water Framework Directive (2000/06/EC). Biomarkers were originally defined, by Depledge in 1994, as any biochemical, histological or physiological alterations or manifestations of environmental stress. Later in 1996, Gestel and Brummelen defined biomarkers in more detail as "*any biological response to an environmental chemical below-individual level, measured inside an organism or in its products (urine, faeces, hairs, feathers, etc.), indicating a departure from the normal status, that cannot be detected from the intact organism*". So, the term is now used in a more restrictive sense, as biological responses at the sub-individual level resulting from exposure to xenobiotics (foreign toxic compound), and many authors follow this approach.

Given that biomarkers are measured at the molecular or cellular level, they may act as "early warning" signals of biological stress and as a result may anticipate changes at higher levels of biological organization (Figure 5, Lemos et al., 2010). In an environmental

context, ideally these early-warning signals could help scientists to better understand to which class of xenobiotics the organisms have been exposed to and if they are causing a toxic effect at critical targets (McCarthy and Shugart, 1990).

Generally, xenobiotic compounds existing in the bodies are lipophilic in nature and, to be efficiently eliminated and excreted, they need to be converted into more water-soluble compounds. There is an extraordinary range of enzymes and biotransformation pathways involved in their detoxification and removal (Chen, 2012). Two main phases in the detoxification process are considered: Phase I and Phase II.

Phase I is usually the first enzymatic defense against foreign compounds, where a non-synthetic alteration occurs, with a functional group being introduced to the chemical structure of the lipophilic compound. It includes reactions such as oxidation, hydrolysis or reduction and produces more water-soluble metabolites by increasing the polarity, making it ready for the next phase. Consequently, this step is defined as “functionalization” (Chen, 2012). For example, the cytochrome P450 enzyme uses oxygen, and as a cofactor NADH, to add a reactive group, being a typical Phase I reaction (Liska, 1998).

Phase II corresponds to a conjugation reaction, where the functional group of the xenobiotic is combined with a chemical group of a small molecule, normally the cofactor of an enzyme (Liska, 1998). Conjugation reactions can be glucuronidation, sulfation, glutathione or aminoacid conjugation and this step increases the solubility of the foreign compound and the excretory potential simplifying its removal from the body (Liska, 1998). Glutathione-S-transferase (GST, EC 2.5.1.18.) is a multigene superfamily of dimeric, belonging to Phase II detoxification enzymes. GST catalyzes the conjugation reaction between xenobiotic substrates (Phase I metabolic products) and sulphydryl group of reduced glutathione (GSH) (Figure 3) (Hayes and Pulford, 1995; Vidal-Liñán et al., 2015). Several studies have been developed on GST activity to assess the toxicity of pollutants (Geracitano et al., 2004; Woo et al., 2009; Faria et al., 2010; Hernández et al., 2013; Cabecinhas et al., 2015; Vidal-Liñán et al., 2015).

In the normal and healthy cell, reactive molecules such as reactive oxygen species (ROS) are produced, including hydrogen peroxide (H_2O_2), the free superoxide anion (O_2^-) and hydroxyl ($OH\cdot$) radicals (Wright and Welbourn, 2002), although usually there is a balance between the generation of ROS and antioxidant defense mechanisms (Moreira et al., 2006). However, as a consequence of stress conditions, an excess production of ROS can occur and overcome antioxidant defenses. These ROS are extremely potent oxidants capable of reacting with critical cellular macromolecules, causing oxidative stress which eventually leads to oxidative damage such as enzyme inactivation, lipid peroxidation (LPO),

DNA damage, protein damage, and ultimately may lead to cell death (Winston and Di Giulio, 1991; Martín-Diaz et al., 2004).

The excess production of superoxide radicals like O_2^- can reduce the cellular antioxidant capacity (Freitas et al., 2012), or cause peroxidation of membrane lipids, resulting in a deterioration of antioxidant enzyme activities by loss of membrane integrity and the inactivation of membrane-bound enzymes (Wright and Welbourn, 2002). The hydrogen peroxide H_2O_2 even though not highly reactive can also inhibit some antioxidant enzymes, mainly creating toxic effects at several different subcellular locations due to its fast capacity to penetrate cell membranes. Additionally, H_2O_2 can be the responsible to produce OH· radicals that can extensively attack every type of macromolecule in living cells comprising lipids, proteins and DNA (Wright and Welbourn, 2002), and can lead to several disease conditions such as cancer, cardiovascular disease and neurological disorders (Chen, 2012).

Antioxidant defense mechanisms against xenobiotics are greater in tissues with functions related to food processing like liver or digestive glands (Livingstone, 2001). The main antioxidant enzymes include: the superoxide dismutase (SOD, EC 1.15.1.1.), catalase (CAT, EC 1.11.1.6.), glutathione peroxidase (GPx, EC 1.11.1.9.), among others (Vicente et al., 2012). These defenses are among the most frequently used subcellular biomarkers.

Superoxide dismutase (SOD) is the primary defense against oxygen toxicity (Ameur et al., 2012). SOD is a group of metalloenzymes responsible to catalyze the conversion of superoxide free radical (O_2-) into H_2O_2, which is formed by the transfer of a single electron to oxygen. This H_2O_2 consequently needs to be detoxified by CAT or GPx (Figure 3) (Stegman et al., 1992; Wright and Welbourn, 2002; Leslie et al., 2013). Catalase (CAT) is a hematin-containing enzyme capable of metabolizing H_2O_2 into oxygen (O_2) and water (H_2O). Glutathione Peroxidase (GPx) catalyzes the metabolism of H_2O_2 into water, involving the oxidation of a cofactor, reduced glutathione (GSH) into its oxidized form (GSSG) (Stegman et al., 1992; Apel and Hirt, 2004). This system acts as the key part to fight against oxygen damage and excess of free radicals produced during Phase I of xenobiotic detoxification (Faria et al., 2009; Yang et al., 2012). There are many studies that establish the toxicity of pesticides and metals based on antioxidant defenses (Geracitano et al., 2004; Richardson et al., 2008; Woo et al., 2009; Douhri and Sayah, 2009; Faria et al., 2010; Comoglio et al., 2011; Costa et al., 2012).

In theory, it is expected, that under oxidative stress the antioxidant enzymes increase their activities. But in practice and due to these enzyme's transient nature, the results can vary between increase (Moreira et al., 2006; Bouraoui et al., 2010; Faria et al., 2010; Benedetti et al., 2012), decrease (Gravato et al., 2010; Ameur et al., 2012, Oliva et

al., 2012) and biphasic responses (Sun and Zhou, 2008; Won et al., 2012). The responses can be influenced by the vulnerability of the organisms exposed, and by the type, number and concentration of chemicals of which the organism was exposed to (Faria et al., 2009).

As mentioned above, a failure of the antioxidant defenses to remove excess of ROS leads to oxidative stress, which may cause significant damage to important macromolecules. Consequences of oxidative stress have been measured biochemically as perturbed redox status, LPO, DNA damage and protein damage.

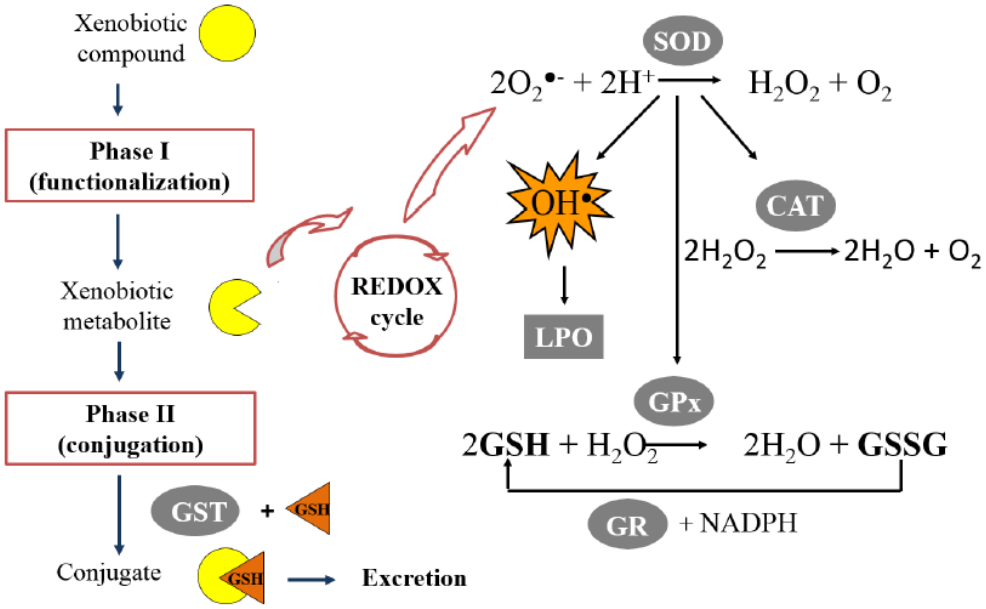

Figure 3 – Enzymatic mechanism involved in xenobiotic biotransformation and antioxidant responses (adapted from Howcroft et al., 2009). GST – Glutathione-S-transferase; SOD – Superoxide Dismutase; CAT – Catalase; LPO – Lipid Peroxidation; GPx – Glutathione Peroxidase; GR – Glutathione Reductase; GSH – Reduced Glutathione; GSSG – Oxidized Glutathione.

Lipid Peroxidation occurs with a chain reaction caused by the presence of a single radical that captures membrane lipids electrons forming an instable fatty acid peroxyl radical, reacting with itself or other fatty acids. LPO breakdown products such as epoxides, ketones, aldehydes, and more importantly, malondialdehyde (MDA) may also produce DNA adducts (Lieibovitz and Siegel, 1980). DNA damage can also be a consequence of the ROS accumulation. Structural damage of DNA, if not repaired, could impair the capability of cells to transcribe their own genes, consequently improving gene mutations, cancer and other diseases (Acharya, 1971). Experiments centered on damage to cells have been widely developed (Livingstone, 2001; Jebali et al., 2007; Richardson et al., 2008; Faria et al., 2009; Faria et al., 2010; Comoglio et al., 2011; Velma and Tchounwou, 2013).

Besides the above mentioned enzymes, there are others not directly related to the detoxification systems but can also play very important roles in the organisms' physiology and be used as biomarkers, to indicate other forms of effects.

Cholinesterases represent a well-known group of serine hydrolases. They are considered ubiquitous enzymes which physiological function is to remove acetylcholine (ACh) from synaptic clefts (Strum et al., 1999). Among them, acetylcholinesterase (AChE, EC 3.1.1.7.) function involved in the regulation of the transmission of nerve impulses becoming one of the most central enzymes for nerve response (Bouraoui et al., 2009). It hydrolyzes ACh, in the post-synaptic membrane of cholinergic synapses where the nerve impulse is conducted to the next axon, producing choline and acetate and interrupting thus the nerve impulse (Figure 4) (Payne et al., 1996). When AChE is inhibited the nerve impulse is not interrupted. ACh will accumulate in the synaptic cleft, because it is not hydrolyzed, leading to an overstimulation of cholinergic receptors, neuromuscular paralysis and uncoordinated movements which could end in the organism's death (Chen, 2012; Pereira et al., 2013). This enzyme is one of the most used biomarker in environmental studies, due to the ability of certain environmental stressors have to inhibit its activity. For example, some pesticides are well-known to inhibit AChE (Nachmansohn and Wilson, 1951). The inhibition of this enzyme by pesticides and metals have been extensively studied (Day and Scott, 1990; Boone et al., 1997; Amitai et al., 1998; Barata et al., 2004; Brown et al., 2004; Frasco et al., 2005; Arufe et al., 2007; Botté et al., 2012; Leomanni et al., 2015).

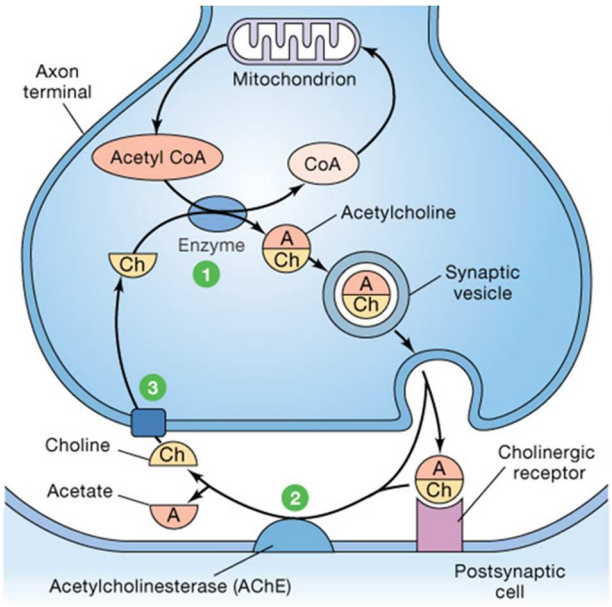

Figure 4 – Mechanism of action of Actetylcholinesterase (adapted from Soreq and Seidman, 2001)

Carboxylesterases (CbE, EC 3.1.1.1.) are another class of serine dependent esterases which hydrolyze a wide range of endogenous and exogenous esters xenobiotics such as organophosphorus and carbamate insecticides, transforming carboxylic acid esters into their corresponding acid and alcohol (Maxwell, 1992; Parkinson, 1996). The mechanisms of action of this enzyme can result in the reduction of the amount of pesticides available for AChE inhibition (Ochoa et al., 2013). It is then possible by combining both carboxylesterase and acetylcholinesterase activities, one may have a more valuable suggestion of exposure.

Other important group of biomarkers is of those related to the energetic budget of an organism. Under normal conditions aerobic organisms use oxygen to generate energy for growth, reproduction and basal metabolism, but under situations of stress a large amount of the energy is used to detoxify and to maintain or compensate basal metabolism activity, leaving less energy available. It is known that xenobiotics are regarded as having effect on the energy consumption of organisms (Calow, 1991; de Coon and Janssen, 2003). Lactate dehydrogenase (LDH, EC 1.1.1.27.) is a cytoplasmic enzyme present in almost all of the body tissues. When oxygen is absent or in short supply, this enzyme catalyzes the reduction of pyruvate to lactate, with the concomitant oxidation of NADH to NAD^+. Alterations in the activity of LDH have been used as indicative of changes in the processes of energy production under stressful conditions (Koenig and Solé, 2013). Moreover, LDH is released from the cytoplasm in response to cell damage. Given its stability in the extracellular environment, LDH quantification has been widely used to evaluate the presence of damage and toxicity in tissues and cells (Cook, et al., 2015; Kaja et al., 2015).

A number of studies have been developed (Koening and Solé, 2013; Cabecinhas et al., 2015; Kaja et al., 2015), regarding the application of this biomarker to assess the impact of xenobiotics in the environment. Principally using LDH as a variable evaluation in toxicity tests (Diamantino et al., 2001) and correlating with oxygen levels (Wu and Lam, 1997).

4.3. Connecting different levels of biological organization – ecological relevance

The great challenge of ecotoxicology is to link the responses at low levels of biological organization (molecular, cellular or individual) to effects at higher levels (population, community or ecosystem), to be able to make predictions on effects before irreversible damages occur (Kammenga et al., 2000; Scott-Fordmand and Weeks, 2000; Vasseur and Cossu-Leguille, 2003; Gravato and Guilhermino, 2009).

The effects comprise all the abiotic and biotic changes which exceed the natural level or frequency, and in extreme circumstances can affect the functioning of entire

ecosystem. In the perspective of ecosystem functioning the most important feature is the interaction between stressors and biodiversity (Connon et al., 2012). To protect the environment and prevent the effects of xenobiotics in it is necessary to understand how different foreign compounds can disturb the ecosystems. However, there are a large number of xenobiotics that coexist in the environment and can have combined and complex effects, along with biotic and abiotic factors (Lemos et al., 2010)

Chemical characterization alone does not provide detailed biological information about possible effects on organisms. In an ideal methodology, cellular level responses linked with responses at whole organism, population, community and ecosystem would be required for a comprehensive assessment of ecotoxicological risk, but such methodologies are difficult and costly. Therefore, the identification of important links between responses at different levels of biological organization with ecosystem functioning can be a suitable step to building more fundamental and effective approaches on ecotoxicology (Connon et al., 2012). In this line, a selection of biological indicators, bioassays of standard whole organisms, biomarkers and *in vitro* tests is needed to assess the toxic effects of pollution to ecosystems (Valavanidis and Vlachogianni, 2014), because a pollutant stress situation usually provokes a cascade of biological responses.

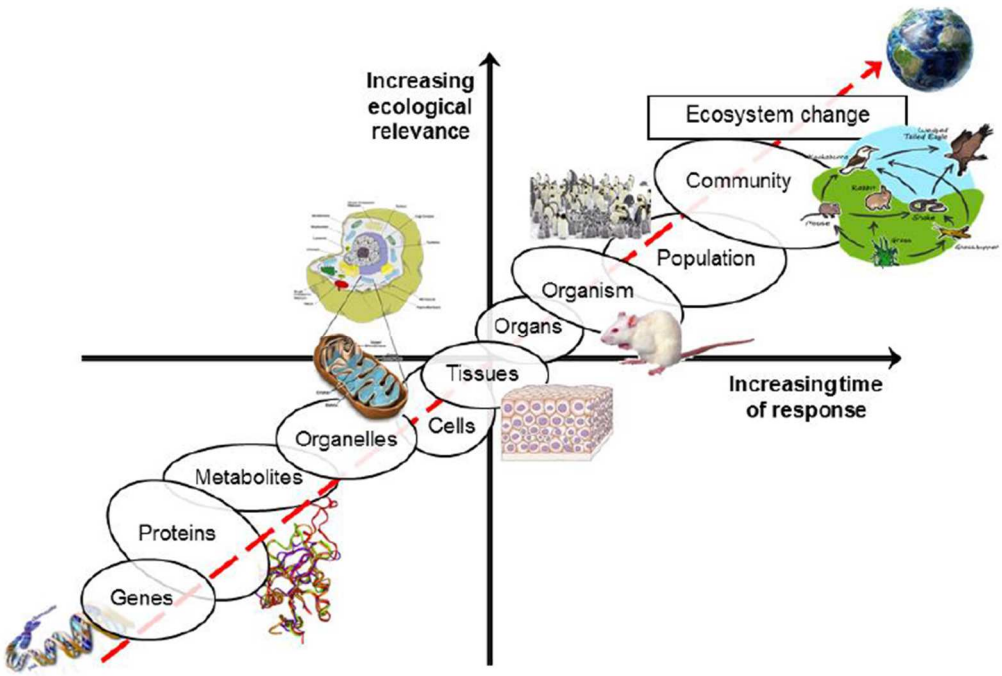

Figure 5 – Relationship between ecological relevance, time of response and the different levels of biological organization after stress exposure (adapted from Lemos et al., 2010).

Most of the studies integrate at least one endpoint at higher levels of biological organization with lower levels, for example associating mechanistic responses with survival. Since effects at higher hierarchical levels are preceded by variations in biological processes at lower levels of organization, the “early warning signals” biomarkers or the individual approaches can be used in a predictive way in order to detect contamination before it hinders a community or even an entire ecosystem, allowing thus the initiation of management strategies of toxicants (Figure 5) (Lemos et al., 2010).

These early responding endpoints at lower biological levels have the potential to become powerful tools for rapid diagnoses of toxicity, and greater the integration of the above endpoints is, the greater will be our understanding of the adverse effects caused by foreign compounds.

5. Aims of the study

The main purpose of this work was to assess the potential ecotoxicological impact of exudates from red macroalgae *Asparagopsis armata* in the marine gastropod *Gibbula umbilicalis*, under laboratory conditions, to evaluate the hazard of these invasive species for the ecosystem they are invading.

To fulfill this objective, the following steps were developed:

(a) Development of a protocol for obtaining exudates from *A. armata*.

(b) Assessment of survival effects of the algae exudates on *Gibbula umbilicalis* performing acute ecotoxicological tests: lethal concentrations (LC_X) were calculated to evaluate the effects of the individual biological level.

(c) Assessment of the effects of non-lethal concentrations of *A. armata* exudates over biochemical responses of *G. umbilicalis*. For this, optimization and evaluation of the potential of biomarkers involved in detoxification, oxidative stress responses, neurotoxicity and energetic functions were performed, since these biomarkers have already been considered in other studies as suitable tools for ecological effects assessment.

In sum, the outcome of this study was expected to become a first step tool to understand the toxicological capacity of *A. armata*, mainly for intertidal organisms, to unravel its mechanisms of toxic action, identify potential impacts and recognize the general potential toxicity of this macroalgae exudates.

Chapter II.

Survival and biochemical responses in *Gibbula umbilicalis* exposed to exudates of the red macroalgae *Asparagopsis armata*

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Survival and biochemical responses in *Gibbula umbilicalis* exposed to exudates of the red macroalgae *Asparagopsis armata*

1. Introduction

The oceans are the largest life support systems responsible for sources of wealth, opportunity, and abundance. However, the interaction between mankind and this ecosystem has pushed oceans to their limit, for example with overfishing and pollution (Islam and Tanaka, 2004). Biological invasions are a form of pollution that involves two main issues: the human-mediated transport of a species to an area where it does not naturally occur, and its influence on the economy and the environment (Namboothri et al., 2012). These species are designated invasive alien species (IAS) and are considered as one of the major threats to biodiversity (Altamirano et al., 2008; Regulation No 1143/2014), and the second leading cause of species extinction (Zenetos et al., 2005). This biodiversity loss is caused mainly by the fact that these IAS can compete with native species for available resources. In some cases, such as the case of algal exudates, the competitive behavior is through the release of toxic substances, which are harmful to the surrounding species (Paul, 2006). Until date, little is known about the impact that invasive species have over native ones, and thus deserve further attention.

Asparagopsis armata is a red macroalgae (Rodophyta) globally recognized as an invasive species. It is found in the intertidal to shallow subtidal areas, forming natural vegetation belts on exposed coasts (Altamirano et al., 2008), mainly from March until August in the Atlantic coast of Iberian Peninsula. Intertidal ecosystems represent areas where various parameters are subjected to unexpected changes, in a small spatial and temporal scale. These factors are further modified by regular events, such as tides (Soler-Hurtado and Guerra-García, 2011).

This red macroalgae produces secondary compounds that are stored in and released from specialized gland cells (Paul, 2006). For example, the pungent aroma of these algae is due to an essential oil that is composed mainly of bromoform with smaller amounts of other bromine, chlorine, and iodine-containing methane, ethane, ethanol, acetaldehydes, acetones, 2-acetoxypropanes, propenes, epoxypropanes, acroleins, and butenones (Genovese et. al., 2009). Over 100 halogenated compounds are found in the genus *Asparagopsis* alone (Paul, 2006). Considering the fact that this seaweed lives in the intertidal zone, it is distributed along tidal pools during low-tides. Generally, these pools may be seen as microenvironments, since they may last for proximately six hours, between low

tide and high tide, as an isolated system without water input or output. Additionally these microenvironments are usually subjected to extreme biotic and abiotic factors that may stress *A. armata*, and thus increase its potential to produce toxic substances that may have dramatic consequences to the other species in the microenvironment.

In this sense, there is an urgent need for employing well known biological monitoring tools and ecotoxicological research to improve the understanding of the biological impacts of algal exudates (Lewis and Watson, 2012), such as those of *A. armata*.

Survival has been one of the most common endpoints used for the assessment of toxicity, and it is usually employed as a "screening tool" with a broad range of toxic concentrations (Martín-Díaz et al., 2004). On the other hand there are some tools of fast detection based on early biological responses at lower levels of biological organizations, such as biochemical biomarkers, that can be very useful and help to foresee a possible outcome in the organism (Payne et al., 1996; Binelli et al., 2005). The use of such biomarkers has also the advantage of improving the knowledge on the modes of action of the toxic compounds as well as the mechanisms of the organisms' responses to the stressor.

Antioxidant enzymes and small molecular-weight antioxidants (such as glutathione) are used to neutralize, remove, or scavenge reactive oxygen species (ROS) that are formed as a byproduct of normal oxidative metabolism or during detoxification of xenobiotics (foreign chemical substances) (Ameur et al., 2012). ROS are chemically reactive molecules, such as OH^- or O_2^-. A dramatic increase of its levels causes oxidative stress which may result in significant damage to cell structure.

Groups of antioxidant enzymes that can oppose oxidative stress induced by ROS include superoxide dismutase (SOD) and catalase (CAT) enzymes among others. SOD metabolizes the superoxide anion (O_2^-) into molecular oxygen and hydrogen peroxide (H_2O_2), which is then further broken down into water and oxygen by CAT or GPx (Howcroft et al., 2009) Another well-known antioxidant is glutathione (GSH) that can act in the antioxidant defense through different pathways: 1) being the substrate for GPx which metabolizes hydrogen peroxide (H_2O_2); 2) being a scavenger of pro-oxidants (like transition metals); or 3) being conjugated by glutathione-S-transferase (GST) during the phase II detoxification metabolism to electrophilic xenobiotic metabolites resulting from phase I metabolism (Meister, 1995; Saint-Denis et al., 1999; Novais et al., 2011). Once oxidized, glutathione is recycled back to its reduced form by glutathione reductase (GR). However, under stress conditions, as pollution, ROS may overcome antioxidant defenses leading to oxidative damage in cellular macromolecules such nucleic acids, lipids and proteins (Patetsini et al., 2013).

Another well-known group of biomarkers are the esterase enzymes, such as acetylcholinesterase (AChE) and carboxylesterase (CbE). Acetylcholinesterase is involved in the regulation of the transmission of nerve impulses through the hydrolysis of neurotransmitter acetylcholine (ACh) into choline and acetate and is known to be inhibited by environmental stressors like pesticides (Day and Scott, 1990; Barata et al., 2004; Arufe et al., 2007; Botté et al., 2012). Carboxylesterase is a serine dependent esterase that hydrolyzes carboxylic acid esters into their corresponding acid and alcohol (Bouraoui et al., 2009). Since the mechanisms of actions of CbE can result in the reduction of the amount of pesticides available for AChE inhibition (Faria et al., 2010), a combined monitoring of carboxylesterase and acetylcholinesterase activities can be useful to evaluate toxicity of chemicals.

Enzymes linked to energy metabolism can also suffer changes in their activities under stress environments. Under normal conditions, aerobic organisms use oxygen to generate energy for their growth, reproduction and basal metabolism but in case of stress, extra and rapid energy is needed since a large amount is used to deal with chemical detoxification and to compensate basal metabolism. Lactate dehydrogenase (LDH) catalyzes the reduction of pyruvate, final product of glycolysis, to lactate with the concomitant oxidation of NADH to NAD^+, in the absence or when low concentrations of oxygen are available (De Coen and Janssen, 2003). So, changes in the processes of energy production under stressful conditions have been accessed by alterations in the activity of LDH (Faria et al., 2014). The knowledge of its mode of action has been widely used to detect and measure biological effects of toxics in the marine environment (Botté et al., 2012).

Benthic organisms have been very often used in ecotoxicology given that they tend to be good indicators of environmental stress. The marine gastropod, *Gibbula umbilicalis* is a good candidate for studying the effects of *A. armata* exudates given that it has a wide geographical distribution inhabiting the upper intertidal on rocky shores where is coexists with *A. armata* and therefore is often exposed to the algal exudates. Moreover, previous studies have shown the sensibility of this species to environmental stressors and promising biochemical responses to these stressors were also found, which makes this gastropod a suitable test species to assess effects of contaminants in intertidal marine ecosystems (Silva et al., 2014; Cabecinhas et al., 2015).

Thus, the main goals of this study were: 1) to address the effects of *A. armata* exudates on the survival of *G. umbilicalis*; 2) to investigate the effects of non-lethal concentrations of *A. armata* exudates in *G. unbilicalis* by using a battery of biochemical responses related with the detoxification metabolism, oxidative stress, neurotoxicity and

energetic metabolism. In sum, the main objective was to address the toxic capacity and effects of this macroalagae, to unravel its mechanisms of toxic action and to identify its potential impacts.

2. Material and Methods

2.1. Test Organisms

2.1.1. *Asparagopsis armata* – Collection and preparation of exudates

Asparagopsis armata were collected manually during low tide at Portinho da Areia Norte of Peniche (39°22'08.2"N, 9°22'41.4"W), a rocky beach in the Portuguese coast. Samples were immediately transported to the laboratory, sorted, and cleaned of other species and sediment particles. After this process, which took approximately one hour, the algae were immediately used to obtain the exudates. One Kg of *A. armata* was placed in each tank containing 10 L of filtered seawater (35 ppm of salinity), for 12 h in the dark, at 20°C±1 with no aeration. Four replicates of these tanks were performed. Seawater was previously filtered through 0.45 µm cellulose acetate membrane filters. After incubation, the algae were removed and the solution from the different replicas was pooled. The algal exudate was obtained by filtering the resulting solution through a 0.45 µm cellulose acetate membrane filter. This exudate constituted the stock for the entire experiments and was stored at -20°C until further use.

2.1.2. *Gibbula umbilicalis* – Collection and culture conditions

Adult sea snails (*G. umbilicalis*) of similar size (10 ± 1 mm of shell diameter) were captured during low tide at Carreiro de Joannes in Peniche (39°21'17.9"N, 9°23'40.4"W). Selected specimens were immediately transported to the laboratory and allowed to recover during 15 days in 100L tank system filled with physical and biological filters and aerated seawater. During acclimation, organisms were kept at 20°C±1, with a photoperiod of 16h:8h (light: dark) and fed, *ad libitum*, with the green macroalgae *Ulva sp.* Salinity, pH, ammonia, nitrates, and nitrites were monitored regularly. Prior to testing, organisms were kept for 24h fasting.

2.2. Exposures design

All experiments were conducted at 20°C±1, 16h:8h (light-dark) photoperiod, with saturated air humidity to reduce medium evaporation. Replicates consisted of 60 mL glass flasks, with one organism each, enclosed with mesh tissue to prevent organism escape and

assure constant submersion. For each endpoint, a control was made with filtered sea water only. During exposures, no food was added and exudate solutions were renewed every 24h to avoid excreta saturation and possible loss of volatile compounds.

2.3. Acute assay - Lethality

Two survival tests were performed: 1) first screening of the effects of the exudates with a wide range of exudate percentages, with one individuals of *G. umbilicalis* per condition, using nine different percentages (0.1%; 0.2%; 0.4%; 1%; 2%; 5%; 10%; 25%; 50%) of *A. armata* exudate and exposing the organisms for 96 h; and 2) final acute assay where the sea snails were exposed to increasing percentages of *A. armata* exudates ranging from 0.5% to 10% of exudate (0.5%; 0.8%; 1.5%; 2.5%; 4%; 6%; 10%) for 96 h, with 8 replicates per treatment.

2.4. Subcellular level responses – biochemical biomarkers

Based on the acute exposures, the previously assessed LC_{10} was used as the highest concentration/exudate percentage tested for subcellular responses assessment. The dilutions applied were: 0.05%; 0.1%; 0.2%; 0.4%; 0.7%; 1.4%; 2.7% of *A. armata* exudates. Sixteen replicates per condition were used and the organisms were exposed for 96h. After exposure, test organisms were broken with a vise in order to collect the soft tissues and immediately frozen and stored at -80°C until further use.

2.4.1. Tissue preparation

For the biochemical analysis, 2 organisms from each treatment were pooled together, making a total of 8 biological replicates per condition. Each replicate was homogenized in 2 mL of ice-cold homogenization buffer (100 mM phosphate buffer pH 7.4, containing 100 mM KCl and 1mM EDTA) using an electrical homogenizer. An aliquot of tissue homogenate (150 µL) was transferred to a microtube containing BHT (2,6-dieter-butyl-4-metylphenol) 0.01% to avoid tissue oxidation for further determination of lipid peroxidation (LPO). Another aliquot (50 µL) of tissue homogenate was collected for DNA damage assessment. The remaining homogenate was centrifuged for 20 min at 10000 g (4°C). The resulting supernatant (S9 fraction) was aliquoted and stored at -80°C for further measurements of total protein quantification and measurement of SOD, CAT, GST, AChE, CbE and LDH activities. All steps were performed on ice to prevent enzyme degradation.

In all assays, blanks were made using homogenization buffer instead of the sample and all samples were measured in quadruplicates. All of the spectrophotometric

measurements were made in a Synergy H1 Hybrid Multi-Mode microplate reader (BioTek® Instruments, Vermont, USA).

2.4.2. Protein quantification

The protein concentration was determined according to the Bradford method (1976), adapted from BioRad's Bradford microassay set up in a 96 well flat bottom plate, using bovine y-globuline as a standard. In each well of the microplate 10 µL of each sample (diluted 30x) or standard was added along with 290 µL of Bradford reagent (in quadruplicates). After 15 min of agitation at 150 revs/min, absorbance was measured at 600 nm and results were expressed in mg of protein/mL.

2.4.3. Phase II detoxification enzyme activity

Glutathione-S-transferase (GST) activity was determined following an adaptation of the procedure described by Habig et al. (1974). In each well, 100 µL of sample (previous diluted to a protein concentration of 0.8 mg/mL) was added along with 200 µL of K-phosphate (100 mM, pH 7.4) containing GSH (1.5 mM) and CDNB (0.25 mM). The formation of the thioether glutathione dinitrobenzene, a product of the reaction between GSH and CDNB, was followed at 340 nm for 5 min. GST activity was calculated using molar extinction coefficient of $9.6x10^3$ $M^{-1}cm^{-1}$ and expressed in nmol/min/mg of protein.

2.4.4. Oxidative stress parameters

Superoxide dismutase (SOD) activity was measured using an adaptation of the method described by McCord and Fridovic (1969), based on the measurement of the inhibition of cytochrome C reduction which normally occurs through O_2^- released by the xanthine oxidase/xanthine reaction. In each well the following quantities were added: 50 µL of S9 fraction, 100 µL phosphate buffer (50 mM, pH 7.8, 0.1 mM EDTA), 50 µL of cytochrome C (0.06 mM), 50 µl of xanthine (0.14 mM) and 50 µl of xanthine oxidase (0.01 U/ml). Samples were diluted in homogenization buffer to a previously optimized protein concentration. The reaction was measured at 550 nm during 10 min, and after blank discount, the SOD content (U/mL) was determined by quadratic regression of the standard curve (SOD units were determined using a standard curve of 0 to 1.5 SOD units/mL). Final results were normalized by total protein content and expressed as U/mg of total protein.

Calase (CAT) activity was determined following the procedure of Clairbone (1985), analyzing the decay in the H_2O_2 concentration. Samples were diluted to a protein concentration of 0.8 mg/mL and into each microplate well 50 µL of sample was added, along

with 100 µL of K-phosphate (50 mM; pH 7.8) and 150 µL of H_2O_2 (30 mM). Absorbance was read every 10 seconds for 1 minute, at 240 nm. The CAT activity was expressed in µmol/min/mg of protein, using a molecular extinction coefficient of 40 $M^{-1}cm^{-1}$.

Lipid peroxidation (LPO) levels were assessed by measuring the content of malondialdehyde (MDA), using the method described by Esterbauer et al. (1991) based on the reaction of a chromogenic compound, 1-methyl-2-phenylindole, with MDA. In sum, 650 µL of 1-methyl-2-phenylindole (7.72 mM) was added to 200 µL of sample followed by 150 µL of HCl 37%. After incubation for 40 min at 45°C to catalyze the reaction and give raise to a chomophore, the samples were centrifuged for 10 min at 15000 g (4°C). The supernatant was then divided in microplate wells and the absorbance was read at 586 nm. A standard curve of 1,1,3,3-tetramethoxypropan (TMP) ranging between 0 and 2 µM, determined under the same conditions as the samples, was used to extrapolate results that were then normalized by sample weight and expressed as nmol of MDA equivalents/g of wet weight (gww).

The DNA strand breaks were quantified using an alkaline precipitation assay (Olive, 1988), adapted by De Lafontaine et al. (2000). Tissue homogenates (50 µL) were incubated with 500 µL of SDS solution (2%) containing 50 mM NaOH, 10 mM Tris and 10 mM EDTA plus 500 µl of KCl (0.12 M) at 60°C for 10 min. After incubation, samples were cooled on ice for 15 min to induce the precipitation of SDS associated nucleoproteins and genomic DNA and then centrifuged for 4 min at 8000 g (4°C). Finally, 50 µL of supernatant was added to each well of a microplate and mixed with 200 µL of Hoesch dye (1µg/mL, bisBenzimide). Fluorescence was measured using 360:450 nm to excitation:emission wavelength filters. Calf thymus DNA was used as a standard to extrapolate DNA concentration that remained in the supernatant and constitutes the damage DNA. Results were expressed as µg of DNA/g of tissue wet weight (ww).

2.4.5. Esterases determination

Acetylcholinesterase (AChE) activity was measured following Ellman's method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996). Activity was measured with 50 µL of sample (adjusted to 0.8 mg/mL of total protein) in the presence of acetylthiocoline iodide (2mM) and DTNB [5,5-dithiobis-(2-nitrobenzoic acid)] (0.33 mM), to 300 µL of final volume. The increase in absorbance was measured for 5 minutes at 414 nm and the AChE activity was calculated using the molar extinction coefficient of $13.6x10^3$ $M^{-1}cm^{-1}$. Results were expressed as nmol/min/mg of protein. Acetylthiocoline was used as substrate based on prior substrate preference experiments (results not shown).

The Carboxylesterase (CbE) activity was measured based on the method of Ellman and colleagues (1961) adapted to microplate. CbE activity was determined by incubation for 5 minutes, 50 µL of sample with DTNB (0.33 mM) in phosphate buffer (100 mM, pH 7.4). The substrate, S-Phenyl Thioacetate (3 mM) was then added (300 µL final volume) to start the enzymatic reaction and the increase in the absorbance was recorded at 414 nm for 5 min. Results were calculated using the molar extinction coefficient of $13.6x10^3$ $M^{-1}cm^{-1}$ and expressed as nmol/min/mg of protein.

2.4.6. Lactate dehydrogenase activity

LDH activity was measured according to the method described by Vassault (1983) with adaptations of Diamantino et al. (2001), which is based on the measurement of the oxidation of NADH when pyruvate is converted to lactate by LDH. 50 µL of sample was incubated for 5 minutes in phosphate buffer (100mM, pH 7.4) containing NADH (18 mM) and pyruvate (18 mM), to a final volume of 300 µL. The decrease in absorbance was followed at 340 nm. LDH activity was calculated using the molar extinction of $6.3x10^3M^{-1}cm^{-1}$ and was expressed as nmol/min/mg protein.

2.5. Data analysis

Lethal concentration (LCx's) and corresponding 95% confidence limits were determined by the probit regression model using Minitab statistical package (Minitab Inc., 2005).

Regarding the biochemical parameters, one-way analysis of variance (ANOVA) was applied to determine significance differences between treatments. When differences were found, Dunnett's post hoc test was employed to determine significant differences between percentages of algal exudates and the control. Data was log_{10} transformed to improve normal distribution and homogeneity of variance. The correlation between biochemical responses was achieved by Pearson correlation. For all statistical tests, the significance level was set at $p<0.05$ and performed with SigmaPlot software for Windows, version 11.0 (Systat Software Inc. 2008).

3. Results

3.1. Acute assay - Lethality

Lethal concentrations/percentages were calculated for the acute exposures of *G. umbilicalis* to *A. armata* exudates. After the 96 h of exposure, the obtained LC_{50}, with 95%

confidence interval, was 5.03% (4.09 - 6.62) of exudate content and LC_{10} was 2.70% (0.86 - 3.66) of exudate content. No mortality was found in the control (Figure 6).

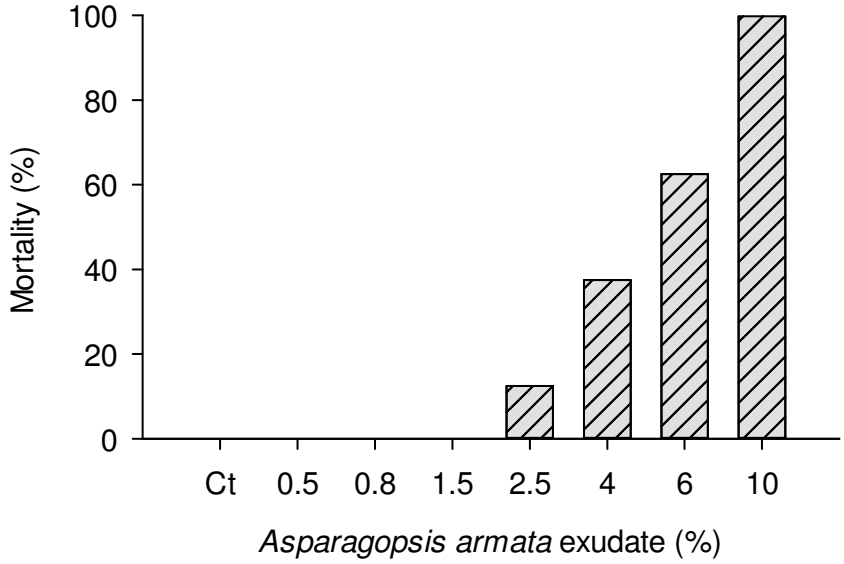

Figure 6 – Mortality of *Gibbula umbilicalis* caused by exposure to *Asparagopsis armata* exudates for 96 h, n=8.

3.2. Subcellular level responses – biochemical biomarkers

3.2.1. Antioxidant and detoxification enzymes

Considering the enzymatic activities related with oxidative stress, CAT and SOD showed no statistically significant differences between treatments (CAT: $F_{7,53} = 2.068$, $p = 0.205$; SOD: $F_{7,32} = 1.494$, $p = 0.063$). However, while the algae exudates did not influenced CAT activity (Figure 7A), there was a trend for lower SOD activities with increasing exudate percentages (Figure 7B).

On the other hand, the activity of the phase II detoxification enzyme (GST) was strongly affected by the increase of exudate concentration, i.e. with the increment of exudate percentage the GST activity values decreased. The inhibition of this enzyme was statistically significant ($F_{7,55} = 24.002$, $p < 0.001$) for percentages higher than 0.1% (Figure 7C).

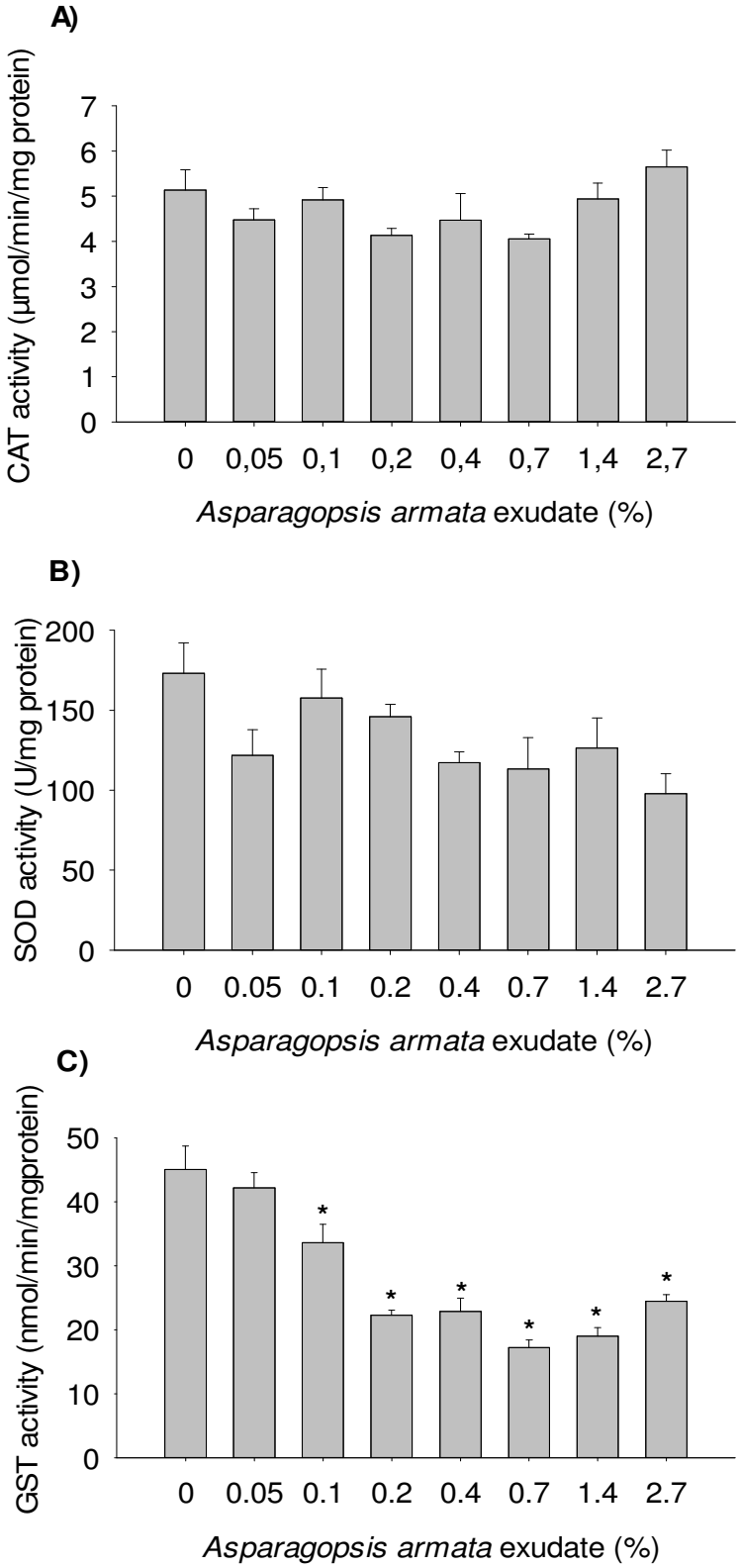

Figure 7 – Enzymatic activities of antioxidants (A - CAT and B – SOD) and phase II detoxification (C – GST) defenses of *Gibbula umbilicalis* when exposed to different percentages of *Asparagopsis armata* exudate for 96 h. Bars represent Mean + standard error (SEM). n=8, each replicate consisting of a pool of 2 organisms. (*) indicates statistically significant differences between exposed groups and control (ANOVA, Dunnett's post hoc multiple comparison test, $p<0.05$).

3.2.2. Oxidative damage

No oxidative damage on lipids was observed after the 96 h of exposure. Actually, the results showed high inconsistency across exudate concentrations due to unknown factors (Figure 8A).

In general, DNA damage levels increased in parallel with algal exudate doses (Figure 8B). The observed increase was statistically significant from 0.1% of *A. armata* exudate concentration ($F_{7,54}$ = 2.927, p = 0.011). For percentages higher than 0.7% the levels of DNA damage start to show a new decrease.

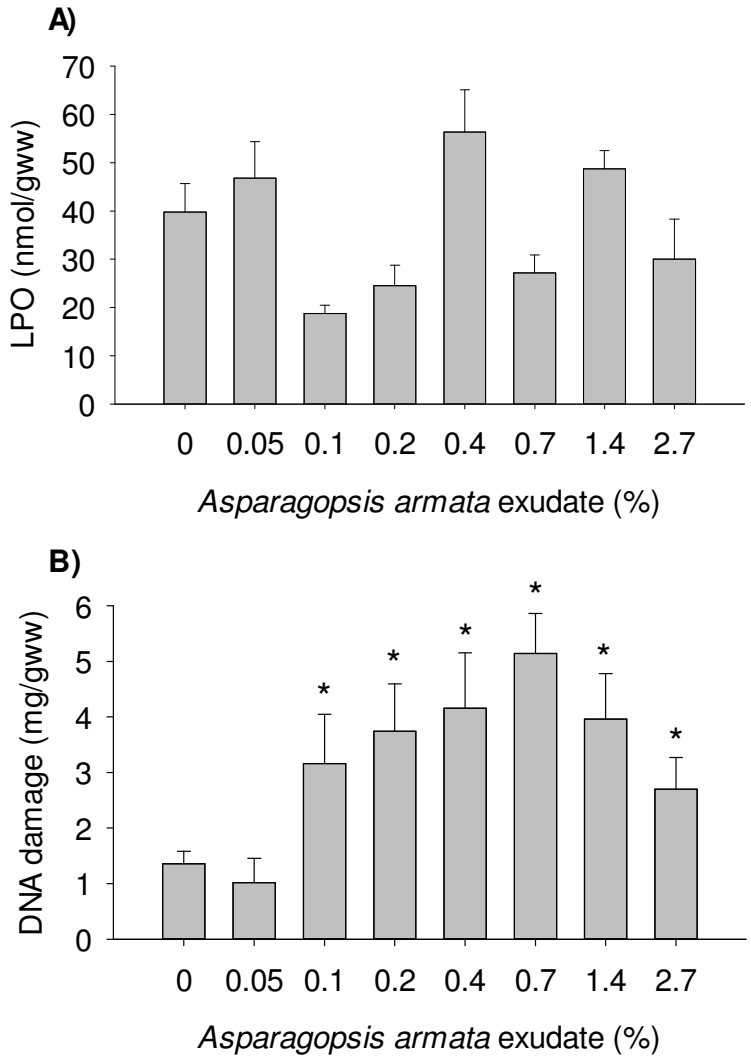

Figure 8 – Oxidative damage measured in *Gibbula umbilicalis* exposed for 96h to different percentages of *Asparagopsis armata* exudates by means of A) Lipid peroxidation (LPO) levels and B) DNA damage levels. Results are presented as Mean + standard error (SEM). n=8, each replicate consisting of a pool of 2 organisms. (*) indicates statistically significant differences between exposed groups and control (ANOVA, Dunnett's post hoc multiple comparison test, p<0.05).

3.2.3. Esterases and lactate dehydrogenase activities

No statistically significant effects were observed in both studied esterases activities, AChE and CbE, of *G. umbilicalis* in the presence of *A. armata* exudates (AChE: $F_{7,54}$ = 1.464, p = 0.200; CbE: $F_{7,54}$ = 1.900, p = 0.088) (Figure 9).

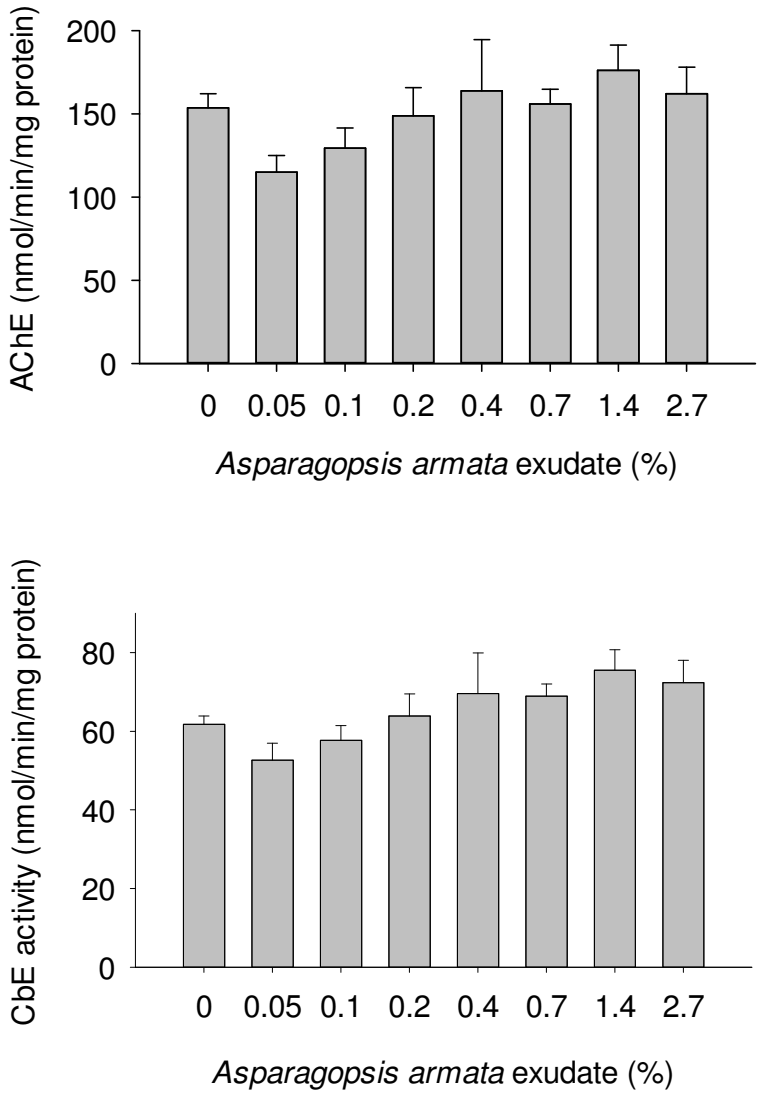

Figure 9 – Esterases activities of *Gibbula umbilicalis* exposed for 96h to different percentages of *Asparagopsis armata* exudates. A) Acetylcholinesterase (AChE) and B) Carboxylesterase (CbE). Bars are Mean + standard error (SEM). n=8, each replicate consisting of a pool of 2 organisms.

Regarding LDH activity, this enzyme was in general unaffected by the presence of algae exudate with the exception of 1.4% concentration, where statistically significant

differences were observed ($F_{7,56}$ = 2.628, p = 0.02) (Figure 10). A second highest peak, though not found statistically different was at 0.4% of exudate. High variability observed could have probably prevented to see a clearer discrimination across treatments.

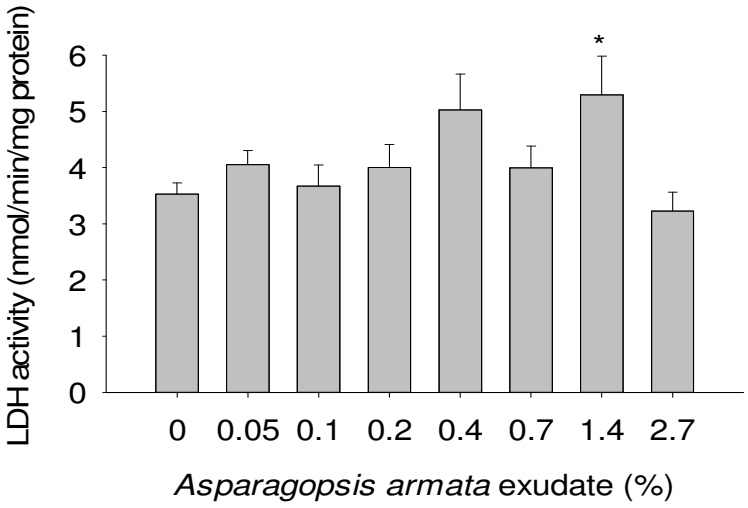

Figure 10 – Lactate dehydrogenase (LDH) activity of *Gibbula umbilicalis* exposed to *Asparagopsis armata* exudate for 96 h. Results are presented as Mean + standard error (SEM). n=8, each replicate consisting of a pool of 2 organisms. (*) indicates statistically significant differences between exposed groups and control (ANOVA, Dunnett's post hoc multiple comparison test, p<0.05).

3.2.4. Correlation between biochemical biomarkers

Correlations between biomarkers that presented statistically significant differences were assessed and the results are described in Table III.

Table III – Pearson correlation between biochemical responses that presented statistically significant differences in this study. The correlation coefficient, positive or negative, of the significant correlations (p<0.05) is underlined and emphasized in bold.

	GST	***DNA damage***	***LDH***
GST			
DNA damage	**-0.41**		
LDH	**-0.26**	0.14	

GST = Glutathione-S-transferase; LDH = Lactate dehydrogenase.

4. Discussion and conclusions

Acute ecotoxicological testing revealed that exposures to exudates of *A. armata* affected the survival of *G. umbilicalis* where, after the 96 h of exposure, a medium with only 5% of this algae exudates was enough to reduce the survival of the snails in 50%. Previous experiments have demonstrated that this seaweed also affects marine crustaceans, such *Echinogammarus sp.*, and primary producers, such green algae *Ulva sp.*, where the crustacean model showed effects in survival when exposed to *A. armata* exudates and the green algae revealed the influence of this exudates in growth and color variation in a 3-demensional scale (Jacinto et al., 2013). Given these results and the fact that *A. armata* can reach high densities in sheltered areas, increasing the likelihood that exudates could become concentrated enough to be lethal, such as in pool tides, it can be concluded that this invasive species is likely to represent a real threat on biodiversity of coastal environments.

The lethal effects observed in this study can be a result from natural organic toxins exuded into the water by *A. armata*. Toxic properties are more often associated with microalgal blooms than with macroalgal blooms, although herbivory inhibitions are well documented with macroalgae, and inhibition of invertebrate development by extracts from a macroalgae has also been suggested (Nelson et al., 2003). The fact that inhibitory chemical compounds are produced by macroalgae has been known for some time, particularly with regard to substances which inhibit bacteria, fungi and other algae (Paul et al., 2006; Genovese et al., 2009; Oumaskour et al., 2013; Jacinto et al., 2013; Pinteus et al., 2015). There is also evidence that macroalgae produce compounds affecting animals, but most of these studies are confined to their potential to modify the habitat characteristics (Wallentinus and Nyberg, 2007) due to antifouling capacity and modifications on animal assemblages. Examples of works that have been done on assemblages with macroalgae are related to their influence on growth and survival of corals (Box and Mumby, 2007), anti-herbivory investigations (Vergés et al., 2008), the impact on crustaceans communities (Pacios et al., 2011; Soler-Hurtado and Guerra-García, 2011; Guerra-García et al., 2012), macroalgal assemblages (Vaz-Pinto et al., 2013), and experiments about antifouling defenses (Gama et al., 2014). Others studies have confirmed the production of toxic exudates by seaweeds and its detrimental effects to invertebrates, as the case of *Ulva lactuca* (Johnson and Welsh, 1985).

Seaweeds contain a large variety of biologically active compounds (Nelson et al., 2003), and red algae in particular, have been recognized since the late 1800's to be a rich source of halogens, predominantly bromine and iodine (Paul, 2006). Traditionally, marine secondary metabolites have been seen as defensive compounds against herbivore grazing

(Murphy, 2003) but also as inhibitors of surface colonization by microorganisms (McConnell and Fenica, 1977). In the case of *A. armata*, the major natural products known are numerous halogenated metabolites, including halomethanes, halocarbons, haloacids and haloacetones, which possess a wide range of volatility and solubility (McConnell and Fenica, 1977). The overall toxicity of halogen-containing compounds seems to be derived from their abilities as alkylating agents (McConnell and Fenica, 1977). Haloacetones, for example, are well-known enzyme inhibitors which are capable of cross- linking serine residues in various proteins and some halomethanes are strong biocides (McConnell and Fenica, 1977). Halocarbons such as bromoform and short chained haloacids such as dibromoacetic acid (DBA) have relatively high levels of production and release and have been suggested as the main antibacterial agents of *A. armata* (Paul, 2006). Since bromoform is characterized as "likely to be carcinogenic to humans" following the EPA Guidelines for Carcinogen Risk Assessment (USEPA, 1999) and DBA is known to produce defects in spermatogenesis and fertility (Kaydos et.al, 2004), these major halogenated metabolites are likely to be some of the responsible compounds for the toxicity observed in other organisms.

Early warning effects of stressors might be more easily detected in the organisms at lower levels of biological organization such as the cellular level, given that effects at these levels are expected to occur earlier. A useful approach based on early biological responses produced by an organism is the use of biochemical biomarkers. The use of a wide battery of these biomarkers in the present study allowed detecting physiological effects of *A. armata* exudates in the gastropod species at sublethal conditions. Previous laboratory studies conducted with *G. umbilicalis* exposed to mercury showed similar basal levels of enzymatic enzymes compared with the present study (SOD - 100U/mg protein; CAT - 6 µmol/min/mg protein; GST – 50 nmol/min/mg protein; and LDH – 3 nmol/min/mg protein) (Cabecinhas et al., 2015).

It is well known that under stress conditions there is a consequent cellular production of pro-oxidant free radicals like ROS and this was expected after the exposure to the exudates since this ROS production can be enhanced by exposure to halogenated compounds (Murphy, 2003). The first line of antioxidant enzymatic defense against ROS involves the enzymes SOD and CAT (Faria et al., 2009), which are expected to increase their activities under oxidative stress conditions. However, no effects were seen in the CAT activity after exposure to the algae exudates and SOD had instead an apparent trend for decreasing activities with increasing percentages of exudate. These results are in line with a study with fluoride, a halogenated compound, where inhibitions of SOD have been

reported in different exposed organisms (Lawson and Yu, 2003). Inhibition of this enzyme may be due to a competitive binding in the active site.

Like mentioned above, it is expected that antioxidant enzymes increase their activities if they are under oxidative stress conditions, but in practice the responses can be varied. Previous studies that include antioxidant enzymes demonstrated different results caused by the presence of foreign compounds with increases (Lima et al., 2006; Vlahogianni et al., 2007; Yi et al., 2007; Sampaio et al., 2008; Verlecar et al., 2008; Lou and Liu, 2011; Danion et al., 2014; Trevisan et al., 2014; Martins et al, 2015), decreases (Lawson and Yu, 2003; Chandran et al., 2005; Ameur et al., 2012; Jiang et al., 2015), and also with biphasic responses (Sun and Zhou, 2008; Wang et al., 2009; Won et al., 2012). The type of response can be influenced by numerous factors according to the susceptibility of the exposed species, the intensity of exposure and the characteristics of foreign substances, if it is a single or a mixture and its bioavailability (Gomes et al., 2014).

Glutathione-S-transferase is a family of detoxifying enzymes that catalyze the conjugation of reduced glutathione (GSH) with a group of compounds having electrophilic centers (Clark et al., 1986), being essential for detoxification of many toxic xenobiotics. GST activity has been widely used as a biomarker of exposure to PAHs, PCBs and trace metals both in fish and invertebrates (e.g. Fitzpatrick et al., 1997; Funes et al., 2006), and has been recently identified as a suitable biomarker for monitoring chemical pollution in highly productive marine coastal ecosystems (Vidal-Liñán et al., 2015). In the present study, the activity of this enzyme was significantly decreased in exudate percentages higher than 0.1%, after 96 h of exposure. It has been suggested that GST inhibition can happen directly through the action of the compounds on the enzyme or indirectly through different ways: 1) ROS produced during phase I detoxification can interact with the enzyme; 2) the reduction of the levels of its substrate (GSH); or 3) due to a down regulation of GST coding genes (Cunha et al., 2007; Novais et al., 2011). If the phase II of detoxification is not efficient this can lead to the production and accumulation of more ROS and form electrophilic intermediates, which can in its turn be responsible to inactivate other enzymes or, under more dramatic situations, lead to damages on lipids or DNA (Novais et al., 2011).

The oxidative damage is then a consequence of the failure of antioxidant and detoxification defenses to remove ROS leading a disruption in the balance of the antioxidant/prooxidant system. This oxidative damage is a toxicity phenomenon widely demonstrated in field conditions, which involve the increase levels of LPO or/and the loss of DNA integrity (Faria et al., 2010). In the present research study, no effects on LPO were observed but the amount of DNA strand breaks in the sea snail increased significantly with increasing exudate percentages until reaching a highest at 0.7%. However, with the two

higher percentages of exudate there was a decrease in DNA damage, although the levels were still significantly higher than in the control. One possible explanation is that the defense mechanisms were increased at this point, to further control the pro-oxidant levels and avoid very high levels of the DNA damage. This is in line with the observation of GST where a decrease in its activity was seen until 0.7% of exudates with a subsequent increase in the two higher concentrations. In fact, there is a significantly negative correlation between DNA damage and GST activity (Pearson correlation, r^2= - 0.411, p = 0.001). This pattern of DNA damage recovery has also been reported in a recent study of exposure to chromium in goldfish, *Carassius auratus* (Velma and Tchounwou, 2013).

Regarding both esterases, it can be concluded that *A. armata* exudates do not affect AChE and CbE enzymes in *G. umbilicalis* since no significant differences were observed in their activities across exudate doses.

Energy metabolism related enzymes, such as lactate dehydrogenase (LDH), are involved in cellular respiration and the production of energy, being particularly important when a considerable amount of additional energy is rapidly required. The determination of LDH activity is also a measure of the anaerobic capacity and status of the cell (Faria et al, 2014). In this study, LDH activity in *G. umbilicalis* showed an increase when exposed to *A. armata* exudates, with statistically significant differences at 1.4% of exudate content. This result suggests the organisms' need of a rapid and supplemental energy from anaerobic metabolism to cope with the stress condition. Curiously, this is the condition at which the organisms seem to be able to increase their GST activity in comparison with the previous and lower exudate percentages, which can explain the demand for extra energy. Indeed, there is significant negative correlation between LDH activity and GST activity (Pearson correlation, r^2= - 0.261, p = 0.039). Changes in this enzyme activity have been related with the process of energy production under stressful conditions by several authors (e.g. Diamantino et al., 2001; Gravato et al., 2010; Cabecinhas et al., 2015).

Additionaly, it is also important to mention that some points observed during the experiments should be taken into consideration too, such as the water turning reddish within minutes, after immersion of macrolagae, which is in accordance to Nelson and collegues (2003). Another example is a decreased ability of the organisms to adhere to the walls of the bottles in the highest exudate concentrations and the formation of a "gelatin" type barrier on the top of the bottles (interface water-air), at higher concentrations. The formation of a "gelatin" type barrier in interface water-air may result in an oxygen tension. Engström-Öst and Isaksson, (2006) mentioned that algal exudates and hypoxia have severe consequences for littoral animals, such as various invertebrates and fish larvae. These

potentially synergistic effects could be critical, for example Johnson and Welsh (1985) demonstrated that under high levels of oxygen the toxins exuded by algae required a time-scale of days to affect the exposed organisms, but at low oxygen levels the toxins required only a few minutes. In this way, a negative correlation between LDH and oxygen levels for some marine organisms suggests a possible biochemical adjustment in response to the lower oxygen levels (Diamantino et al., 2001). This probably occurs also in situations of chemical stress and, consequently, this enzyme may be a sensitive criterion both in laboratory and biomonitoring studies with invertebrates.

The alterations on GST and LDH activities, along with the observed damages provoked in DNA, are effects likely to be projected towards alterations at higher levels of biological organization to the individual level such as behavior or reproduction, among others.

In summary, the present results represent a first insight into the effects caused by exudates from *A. armata* on *G. umbilicalis*. Furthermore, the impairment in endpoints performed in *G. umbilicalis* in this study show the high suitability of this organism as ecotoxicological biomonitor, as well as the evaluated biological responses demonstrated to be potential tools for environmental risk assessment. Some suitable biomarker candidates, such as GST, DNA damage and LDH, were identified with this work for future research studies with these macroalgae exudates.

Chapter III.

General Discussion and Concluding remarks

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Marine ecosystems are continuously exposed to diverse forms of pollution predominantly with anthropogenic origin that can cause the decline of our oceanic resources (Matthiessen and Law, 2002). Biological invasions can be considered as a form of anthropogenic pollution because they are mediated by human transport where a species is introduced to an environment where it does not naturally occur and where it becomes an invasive species (Namboothri et al., 2012), which is already recognized as one of the major threats to biodiversity (Altamirano et al., 2008; Regulation (EU) No 1143/2014).

Asparagopsis armata is one of such invasive species with the aggravating circumstance of being spread worldwide. Dealing with other non-native species, competition with natural communities is normally observed leading to local population losses and niche contraction of native species. In fact, with respect to the epifauna, various studies have demonstrated modification of habitat complexity due to proliferation of invasive species (Pacios et. al., 2011). However, little is known about the effects of this macroalgae on the environment regarding its exuded products and this is highly relevant since algae products can be natural contaminants.

In an ideal approach for a better comprehensive assessment of ecotoxicological risk, systematic responses on the cellular level should be linked to a whole organism, population, community or ecosystem effects and services. However, with the difficulty and expensiveness of such methodology, it is crucial to identify key links between responses at different levels of biological organization (Connon et al., 2012). To evaluate the effects of pollutants on organisms, various methods have been developed extending from the molecular to the ecosystem level of biological response. However, when effects are detected at higher levels of biological organization it usually means that ecologically important alterations have already occurred (e.g. death or impaired organismal function). To determine early effects, individual and subcellular level responses, such as biochemical biomarkers, can be more adequate since they detect more quickly and eventually more specifically the presence of several toxic compounds, allowing earlier identification of changes before deleterious effects reach higher levels of biological organization (Cajaraville et al., 2000; Martín-Díaz et al., 2004; Monserrat et al., 2007). Thereby, some efforts have been made by several authors to relate some biochemical biomarkers that act at the subcellular level and parameters in individual level (Novais et al., 2011).

The objective of this study was to understand the overall effects and gain insight on the mechanisms behind the responses in *G. umbilicalis* exposed to exudates from *A. armata*. To fulfill this goal, experiments were conducted which aimed at determining effects on various endpoints under similar conditions: survival, antioxidant defenses, oxidative

damage, neurotoxic status and energetic alterations. In the outcome of this study the first results of *G. umbilicalis* biochemical responses when exposed to exudates of *A. armata* were obtained.

Traditional ecotoxicological bioassays applied in the present work allowed the determination of effect concentrations of the exudates on the *G. umbilicalis* survival and consequently to conclude that the invasive algae truly exudates toxic compounds for the sea snail. These particular results further indicate that under real field scenarios, and because the organisms are sessile or have low mobility, they are not able to easily avoid this compound that affects their survival and disturbing effects are expected to occur at the population level. Thus, the potentially harmful effect that this macroalgae has to the surrounding marine life was confirmed since even a low amount of the exudate obtained under laboratory conditions was enough to affect the survival of the exposed organisms.

On the other hand, with the biochemical biomarkers studied in the gastropods in the presence of sublethal concentrations of *A. armata* exudate, it was possible to identify alterations in the detoxification mechanisms and energetic metabolism as well as genotoxicity. Impacts like these can also imply harmful effects to the surrounding species, impairing the biodiversity of the invaded area. Moreover, the invasive macroalgae *A. armata* can be easily found confined in tide pools and these puddles can reveal “micro-environments" with high biodiversity where the presence of this macroalgae becomes critical, especially with the constant increase of populations of this invasive algae.

Additionally, the present results demonstrate the importance of testing a range of concentration to better understand the extent of the biochemical and metabolic effects taking the maximum mechanistic information which is essential to develop such tools for quick diagnosis of exudates toxicity. The proposed test species, *G. umbilicalis*, showed to be sensitive to *A. armata* exudates which make it a suitable species to be used in ecotoxicological testing of exudates in marine ecosystems.

Although chemical characterization of the exudates itself would not provide specific biological information about potential hazards to organisms, for future research it would be interesting and important to do such characterization, as a complement to the toxicity assays, to better understand the impacts and the mechanistic action of the *A. armata* exudate compounds. The chemicals identified could be used in future experiments using single and combined exposures assays. Also, the application of other individual bioassays that could give indication of behavior changes, such as avoidance, feeding or mobility tests (Cabecinhas et al.,2015) would be important as they could reveal possible alteration in terms of food seeking, predator avoidance, migration or even possible reproduction impairment, thus being endpoints of great ecological relevance. Regarding molecular

endpoints the application of “omics” methodologies, such as transcriptomics, genomics, proteomics, or lipidomics would also be interesting to consider and of utmost value to amplifying the knowledge of the mechanisms of toxic action of these algae exudates.

The present study is an important step in the research of toxic exudates released to the environment essentially due to the premise that they can affect the surround organisms and most certainty influence their invaded ecosystems. Besides, the results here obtained represent important data of toxicity of *A. armata* exudates in marine invertebrates and, although much more work can and need to be done to deepen the knowledge of its effects, this study represents an important starting point for future research.

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Chapter IV.

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