Universidade de Aveiro
Ano 2021

Carla Ofélia Ferreira da Silva

Impacto da alga invasora *Asparagopsis armata* em ambientes costeiros

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Ciência, Tecnologia e Gestão do Mar, realizada sob a orientação científica do Doutor Marco Filipe Loureiro Lemos, Professor Adjunto do Politécnico de Leiria e co-orientação do Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro, e do Doutor Carlos Barata Martí, Investigador do Departamento de Química Ambiental (IDAEA-CSIC), Barcelona

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio através da atribuição de uma bolsa de doutoramento atribuída a Carla Ofélia Ferreira da Silva (SFRH/BD/87030/2012)

“Caminante, son tus huellas
el camino y nada más;
Caminante, no hay camino,
se hace camino al andar.
Al andar se hace el camino,
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.
Caminante no hay camino
sino estelas en la mar”

António Machado

o júri

presidente

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agradecimentos

Ao Doutor Marco Lemos, Doutor Amadeu Soares e Doutor Carlos Barata por terem tornado possível a concretização deste doutoramento.

Um especial agradecimento ao meu orientador Marco Lemos por todo o apoio durante o meu doutoramento. À Sara Novais, por toda a ajuda prestada. Sinto que tive um grande crescimento científico ao vosso lado.

Assim como alguns investigadores que contribuíram para a realização desta tese, como João Neto, Rui Gaspar e Tiago Simões.

Aos colegas que me ajudaram na grande apanha de alga, e restantes saídas de campo, às poças, mostrando sempre um grande espírito de camaradagem.

Um obrigado às amizades de Peniche, e no quão bem me receberam. Desde o pequeno GIRM, crescemos tanto! Um orgulho fazer parte deste grupo que juntos vimos crescer o MARE-IPLeiria! Os meus colegas do Campus do Mar, Celso e Susete que juntos íamos tirando dúvidas do programa doutoral mesmo com a distância Aveiro – Peniche, mas tudo superamos.

Aos restantes colegas e amigos que fui conhecendo no Cetemares, com boa disposição, companheirismo e sorrisos, tudo se torna mais fácil!

Às amizades do Norte e de sempre, que sempre provaram que nada mudou.

Amizades importantes especialmente numa etapa final e de confinamento: vocês sabem quem são!

À minha família, pais, irmãos e avô. Todos os seus esforços feitos para que eu pudesse prosseguir a minha formação. Difícil expressar o quão grata estou por todo o vosso apoio. São a minha bússola e o meu aconchego.

À minha avó Margarida, a quem dedico, com muita saudade.

À Fundação para a Ciência e Tecnologia pelo financiamento através da minha Bolsa de Doutoramento (SFRH/BD/87030/2012), e dos projetos de investigação MARINE INVADERS - Impact of the invasive macroalga *Asparagopsis armata* on coastal environments (PTDC/MAR-EST/6226/2014) e SmartBioR- Smart Valorization of Endogenous Marine Biological Resources Under a Changing Climate (Centro-01-0145-FEDER-000018). De igual forma, à Comissão Europeia pelo financiamento concedido através do projeto de investigação europeu AMALIA (EASME/EMFF/2016/1.2.1.4/03/SI2.750419).

palavras-chave

Biomarcadores bioquímicos, perfil de ácidos gordos, efeitos subletais, espécies invasoras, comunidades intertidais, invertebrados marinhos, diferentes níveis de organização biológica

resumo

A introdução de espécies invasoras tem aumentado com a globalização e é reconhecida como uma das principais ameaças aos oceanos e a segunda causa da perda de biodiversidade. A alga vermelha *Asparagopsis armata* exibe um forte comportamento invasor induzindo mudanças significativas na comunidade invadida. O estudo do impacto desta invasora em espécies ecologicamente relevantes é crucial para a avaliação de risco. A avaliação de parâmetros ao nível suborganismal pode, portanto, fornecer indicadores precoces da exposição de *A. armata* e seus possíveis impactos nas populações naturais. Os resultados enfatizam a importância de se considerar respostas específicas *in situ* (biomarcadores), bem como respostas mais generalizadas e ecologicamente relacionadas, para identificar e avaliar os efeitos biológicos de *A. armata* no campo. Para validar a ferramenta de perfil de ácidos gordos (PAG), o caracol marinho *G. umbilicalis* foi exposto a três metais: cádmio, níquel e mercúrio, e mediu-se o teor de lipídios totais, peroxidação lipídica e PAG. A análise PAG sugeriu uma mudança no metabolismo dos ácidos gordos e indicou uma ligação entre a exposição a metais e adaptação homeoviscosa e resposta imune. Em particular, cinco ácidos gordos (ácidos palmítico, eicosatrienóico, araquidónico, eicosapentaenóico e docosahexaenóico) mostraram-se bons indicadores das respostas de *G. umbilicalis* aos metais utilizados, tendo, portanto, potencial para serem utilizados como biomarcadores de contaminação por metais em esta espécie. No uso continuado desta espécie, houve também a necessidade de a caracterizar bioquimicamente. Para avaliar as diferentes formas enzimáticas presentes no caracol marinho, foram utilizados diferentes substratos e inibidores seletivos. Além disso, os efeitos *in vitro* e *in vivo* do pesticida clorpirifos (CPF) sobre a atividade da AChE foram investigados, juntamente com os efeitos sobre o comportamento de caracóis. Os resultados obtidos mostraram que *G. umbilicalis* possui colinesterases com características de AChE. Além disso, o CPF inibiu a atividade da AChE tanto *in vitro* quanto *in vivo*, e a inibição da AChE foi positivamente correlacionada com o teste de viragem.

Para compreender os mecanismos de toxicidade da invasora *A. armata*, os efeitos letais e subletais de *A. armata* foram investigados e as respostas de biomarcadores bioquímicos associadas ao metabolismo energético foram analisadas.

resumo
(Cont.)

Os resultados mostraram comprometimento do estado fisiológico dos invertebrados após a exposição a este exsudado de algas. As concentrações mais altas de exsudado aumentaram significativamente o conteúdo de lípidos em ambos os organismos. No camarão, o teor de proteína, ETS e LDH também aumentaram significativamente. Ao contrário, esses parâmetros diminuíram significativamente em *G. umbilicalis*. Efeitos comportamentais foram observados em *G. umbilicalis* exposto ao exsudado de *A. armata*, com redução no consumo de alimento e aumento do tempo de viragem. As defesas antioxidantes, dano oxidativo e parâmetro neuronal, bem como o perfil de ácidos gordos foram avaliados após exposição ao exsudado de *A. armata*. Os resultados revelaram diferentes respostas metabólicas entre as espécies, indicando que o exsudado de *A. armata* afetou os organismos por diferentes vias. Apesar de estudos anteriores indicarem que o exsudado afetou a sobrevivência e o comportamento de *G. umbilicalis*, isso não parece resultar de stress oxidativo ou neurotoxicidade direcionada. Para *P. elegans*, a inibição da AChE e a diminuição da capacidade antioxidante com o aumento da LPO, sugere neurotoxicidade e stress oxidativo como mecanismos de toxicidade do exsudado para esta espécie. Para os ácidos gordos, houve diferenças mais pronunciadas para *P. elegans* com um aumento geral de PUFA, o que comumente significa um mecanismo de defesa que protege da ruptura da membrana. PUFAs ómega-3 ARA e DPA foram aumentados em ambos os invertebrados. Para avaliar os efeitos desse invasor em um cenário mais realista, foram avaliadas as variações nas comunidades nativas de algas marinhas intertidais e macroinvertebrados habitando poças rochosas com e sem a presença da macroalga invasora *A. armata*. Os resultados mostraram diferentes padrões na composição de macroalgas das comunidades, mas não para as comunidades macrobentónicas. *Ellisolandia elongata* foi a principal espécie de algas afetada pela invasão de *A. armata*. As poças invadidas tenderam a apresentar menor riqueza de espécies, apresentando uma estrutura mais constante e conservadora, com menor variação de sua composição taxonómica do que as poças sem *A. armata*. Este trabalho baseou-se na informação ecotoxicológica e ecológica para fornecer uma visão de como *A. armata* afeta invertebrados específicos e em comunidades costeiras no geral, abrangendo métodos em diferentes níveis de organização biológica que preparam o caminho para o teste de várias hipóteses na avaliação da toxicidade do exsudado de *Asparagopsis armata*.
As análises de biomarcadores bioquímicos contribuem para o conhecimento dos efeitos subletais e impactos ecológicos nas comunidades. Auxiliando assim na compreensão abrangente dos mecanismos que levam a respostas de nível superior.

keywords

Biochemical biomarkers, fatty acid profile, sub-lethal effects, Invasive species, Intertidal assemblages, marine invertebrates, different levels of biological organization

abstract

The introduction of invasive species has been increasing with globalization and is recognized as one of the main threats to the oceans and the second cause of biodiversity loss. The red alga *Asparagopsis armata* exhibits a strong invasive behavior inducing significant changes in the invaded community. The study of the impact of this invasive on ecologically relevant species is crucial for risk assessment. Assessing sub-organismal endpoints may therefore provide early indicators of *A. armata* exposure and their possible impacts on natural populations. The results emphasize the importance of considering specific (biomarkers) as well as more generalized and ecologically related *in situ* responses to identify and evaluate biological effects of *A. armata* in the field. To validate the fatty acid profile (FAP) tool, the marine snail *G. umbilicalis* was exposed to three metals: cadmium, nickel and mercury, and the total lipid content, lipid peroxidation and FAP were measured. The FAP analysis suggested a change in fatty acid metabolism and indicated a link between exposure to metals and homeoviscous adaptation and immune response. In particular, five fatty acids (palmitic, eicosatrienoic, arachidonic, eicosapentaenoic, and docosahexaenoic acids) proved to be good indicators of the responses of *G. umbilicalis* to the metals used, thus having the potential to be used as biomarkers for contamination by metals in this species. In the continued use of this species, there was also a need to characterize it biochemically. To assess the different enzymatic forms present in the sea snail, different substrates and selective inhibitors were used. Additionally, *in vitro* and *in vivo* effects of the pesticide chlorpyrifos (CPF) on AChE activity were investigated, along with effects on snails' behaviour. The results obtained showed that *G. umbilicalis* has cholinesterases with characteristics of AChE. In addition, CPF inhibited AChE activity both *in vitro* and *in vivo* conditions, and AChE inhibition was positively correlated with flipping test.
To understand the mechanisms of toxicity of the invasive *A. armata*, lethal and sublethal effects of *A. armata* were investigated and biochemical biomarkers responses associated with energy metabolism were analyzed.

abstract (cont.)

Results showed invertebrates' physiological status impairment after exposure to this algae exudate. Highest concentrations of exudate significantly increased lipid content in both organisms. In the shrimp, protein content, ETS, and LDH were also significantly increased. On the contrary, these parameters were significantly decreased in *G. umbilicalis.* Behavioural impairments were observed in *G. umbilicalis* exposed to *A. armata* exudate, with reduction in feeding consumption and increased flipping time. Antioxidant defences, oxidative damage and neuronal parameter as well as the fatty acid profile were evaluated after exposure to *A. armata* exudate. Results revealed different metabolic responses between species, indicating that *A. armata* exudate affected the organisms through different pathways. Despite previous studies indicating that the exudate effected *G. umbilicalis'* survival and behaviour, this does not seem to result from oxidative stress or addressed neurotoxicity. For *P. elegans*, an inhibition of AChE and the decrease of antioxidant capacity with the increase of LPO, suggests neurotoxicity and oxidative stress as mechanisms of exudate toxicity for this species. For fatty acids, there were differences more pronounced for *P. elegans* with a general increase in PUFA, which commonly means a defence mechanism protecting from membrane disruption. Omega-3 PUFAs ARA and DPA were increased in both invertebrates. To evaluate the effects of this invasive in a more realistic scenario, Variations on native intertidal seaweed and macroinvertebrate assemblages inhabiting rock pools with and without the presence of the invasive macroalgae *A. armata* were assess. Results showed different patterns in the macroalgae composition of assemblages but not for the macrobenthic communities. *Ellisolandia elongata* was the main algal species affected by the invasion of *A. armata.* Invaded pools tended to show less species richness, showing a more constant and conservative structure, with lower variation of its taxonomic composition than the pools not containing *A. armata*. This work relied on the ecotoxicological and ecological information to provide insight in how *A. armata* affects specific invertebrates and in general coastal communities, embracing methods at different biological organization levels which prepare the way for various hypotheses testing in assessing *Asparagopsis armata* exudates toxicity. The analyses of biochemical biomarkers contribute to the knowledge of the sub-lethal effects and ecological impacts on communities. Thus, aiding the comprehensive understanding of the mechanisms that lead to higher level responses.

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Chapter I

General Introduction

Chapter I – General introduction and thesis outline

1. Invasion ecology

The scientific interest about invasion biology has grown enormously in recent decades and is now a subdiscipline of ecology. In the 19th century, several pioneering naturalists: Charles Darwin, Alphonse de Candolle, Joseph Hooker and Charles Lyell mentioned invasive species in their writings. Since Charles Elton's book in 1958 – "The ecology of invasions by animals and plants"-, many debates and discussions have recently taken place about biological invasions. Undoubtedly, his book stimulated an enormous research in the area of invasion ecology (Richardson, 2010). Elton wrote: "[there are] two rather different kinds of outbreaks in populations: those that occur because a foreign species successfully invades another country, and those that happen in native or long-established populations. This book is chiefly about the first kind, the invaders" (Elton, 1958).
The topic of biological invasions has received much interest due to the rapidly accelerating number of introduced species. Nowadays, thousands of articles are published in indexed journals about this subject. At the present moment, in 2020, around three thousand including the word "invasive" or "invasion" in their titles can be found on Web of Science database. Non-indigenous species (NIS), alien, exotic, non-native, are often used to signify that a species is not native to a particular location. Those are species introduced outside their natural range (past or present) distribution, intentional or unintentional, due to human activities (Richardson *et al*., 2000).
Some NIS have neutral or even beneficial impacts on native species and ecosystems, while others become invasive (Olenin *et al.*, 2010). So, invasive alien species (IAS) are established NIS that have spread and have negative consequences on biological diversity, ecosystem functioning, socio-economic values, and/or human health in their new environment (Unep, 2002). Natural invasions, extinctions and speciation, have shaped the diversity patterns of ecosystems. Invasive species act as environmental engineers, as they often cause impact on their receiving environment. At the same time, invaders can work synergistically as the

environmental modifications caused by one species provide increased opportunities for more alien species to invade. This is called “invasional meltdown hypothesis” (IMH) (Braga *et al.*, 2018). This theory describes the process whereby the establishment of one type of invasive species in a new environment can facilitate the invasion of other.

Prompted by increasing levels of trade and travel, biological invasions now pose a major threat to biodiversity of ecosystems and lead to vast economic losses worldwide (Mooney *et al*., 2005). Due to widespread and profound changes in ecosystems, biological invasions have also been recognized as a significant part of global environmental change (Vitousek *et al.*, 1997).

1.1 The invasion process

The invasion process can be divided into a series of stages. For each stage, introduced species must overcome certain barriers that will define the success of the invasion and if the introduced species will become an invasive species.

These stages include the transportation, introduction, establishment, and the spread/invasion of the successful invader, before they are able to inflict ecological or economic harm (Theoharides & Dukes, 2007). Some factors may influence this success: propagule pressure, abiotic conditions and community interactions. For example, if the introduced species invade new areas, it means that this species creates itself a new niche or it is a superior competitor, using resources better than native species.

During the process of invasion, NIS pass through various ecological barriers at different stages of invasion before impact economically and ecologically.

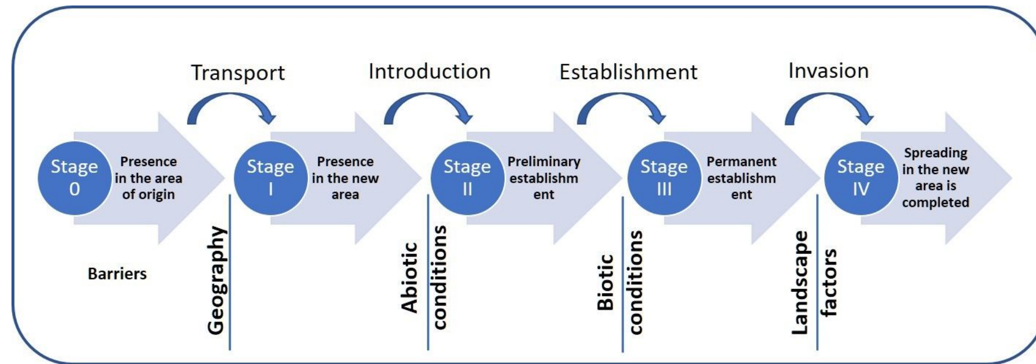

Figure 1 - Stages of biological invasions. Potential invaders have to pass through barriers that may stop the passage to subsequent stages (Adapted from Colautti & MacIsaac, 2004)

In the stages 0 and I, in general, biological invasions begin with the uptake of a resident potential invaders propagules from a donor region by a transport vector, e.g., ship ballast water, aquaculture, aquarium trade, scientific research, and moved over a distance further than their natural biogeographical barriers. The overall success of the transportation depends of the NIS traits. In stage II, propagules that survive the transport are released and thus introduced into a new region. Here, the introduced species is considered non-indigenous species (NIS). In stage III, during establishment, interactions between the introduced and resident species in the community should become increasingly important and determine if the NIS reaches the next stages to become widespread. This capacity to establishment success in the new host habitat is often referred to as species invasiveness (e.g., better competitive performance, greater enemy resistance than native species) (Lloret *et al*., 2005). The stage of integration and spread (stage IV) represents the stage where NIS have a regular reproduction and develop sustainable widespread and dominant populations (Colautti *et al*., 2006). NIS reaching this stage is commonly referred as 'invasive', a definition also used in this thesis.

These invasion mechanisms that allow some introduced species to establish and become abundant in their new environments are, however, still poorly understood. A good explanation of mechanisms that determine the capacity of a native community to resist an invader have led ecologists to propose ecological hypotheses to explain the invasion success. The Biotic Resistance Hypothesis proposed by Elton (1958) predicts that species-richer communities are more resistant to invasions than species-poorer communities.

Through his studies of native and exotic species on oceanic islands, Elton (1958) showed evidence that richer communities were more stable, less vulnerable to disturbances and thus less likely to be invaded.

The Enemy Release Hypothesis (ERH), states that the introduced specie will have a competitive advantage over native due to reduced control by natural enemies, by being unrecognized or unpalatable to native enemies (Keane & Crawley, 2002). On the other hand, the Evolution of Increased Competitive Ability (EICA) hypothesis states that after the loss of specific competitors, NIS may reallocate the energy originally dedicated to defense, to the growth, and reproduction (Blossey & Notzold, 1995). Interactions between NIS and native species may also modify chemical traits as it is argued by the Novel Weapon Hypothesis (NWH). Callaway & Ridenour (2004) suggest that some NIS may have an advantage over native competitors since they may contain potent defense mechanisms. Novel weapon is limited to biochemical released from NIS that affect native species. Although this concept arises from terrestrial plant ecology, competitive suppression of natives via allelochemicals has been documented in other organisms, e.g., toxic substances produced by the gastropod *Nucella* and phenolic compounds from seaweeds (Svensson *et al*., 2013).

2. Marine invasions

The arrival of NIMS (non-indigenous marine species) from other seas and oceans is a global phenomenon. The natural biogeographic oceans barriers contributed to the organisms and ecosystems speciation. With the globalization of economy and the increase of maritime traffic, these barriers are losing their effectiveness and many organisms have been transferred due to human activities from one part of the world to another through trade, transport, travel and tourism, either deliberately or accidentally. Among marine ecosystems, coastal areas and estuaries are particularly vulnerable to biological invasions due to the impact of numerous introduction vectors and human activities (nutrient enrichment, maritime trade, and others). Interactions between NIS and other anthropogenic stressors may greatly influence colonization and distribution patterns as well as the effects of marine invaders. Among the human–mediated transfers, shipping and

aquaculture are the most frequently vector systems involved in marine introductions (Williams *et al.*, 2013). Shipping, as an essential part of world trade, facilitates the movement of sessile organisms as seaweeds, sponges, mussels, barnacles and other "fouling" species which can attach to hulls and other gear in the first stage of invasion process. Also, free living organisms and planktonic propagules may be carried over large distances in ballast water tanks. The loading and discharge of ballast water by large vessels is an important primary introduction vector of marine NIS (Gollasch, 2002), however, fouling of ship hulls has been recognized as a more important vector for seaweeds than ballast water (Johnson & Chapman, 2008).

Nowadays, NIS species of almost all phyla are known. Galil *et al.* (2014) point out that the phyla that most contribute to the list of NIS species on the Atlantic coast are mollusks, followed by crustaceans, fish, algae, and annelids. The success of NIS introduction depends of the introduction vector, the ability of the specie to adapt to habitat receptor conditions, and the susceptibility of the recipient habitat to new introductions. Marine NIS are a component of global change and determining interactive effects of anthropogenic agents of disturbance with natural processes should be the focus of ecological research. Moreover, global climate change is increasing water temperatures in northern-latitude and this may cause seasonally stressful conditions for cold water-adapted species but may implement suitable thermal conditions to allow non-native warm-water species to succeed in these habitats (Sharma *et al*., 2007).

Nevertheless, the impacts of marine introductions remain poorly understood. Impacts are documented in fewer than 30% of globally recognized NIMS, with even fewer impacts quantitatively assessed (Davidson *et al.*, 2015).

2.1 Macroalgae as marine invaders

Marine macroalgae are a significant component of NIMS, with current global estimates of introduced macroalgae ranging from 163 to >300 species, and its introduction and spreading increased over the last 20 years (Davidson *et al*., 2015). In the Mediterranean sea, several species have caused significant ecological and economic impacts [e.g.,

Caulerpa taxifolia, *Codium fragile*, *Sargassum muticum* and *Undaria pinnatifida* (Boudouresque & Verlaque, 2002)].

The increase in biomass of invasive macroalgal blooms often result in the decline or even complete disappearance of perennial species, while opportunists (indigenous species) take advantage mainly of changes in environmental conditions (e.g., organic enrichment), which give them competitive supremacy over other indigenous peoples. When the invaders demonstrate opportunistic behavior, the damage may be greater. However, invasive macroalgae can cause serious impacts: they may change ecosystem structure and function by monopolizing space and acting as ecosystem engineers and can altering competitive interactions and trophic networks (Thresher, 2000). Macroalgae can alter light availability to other species, change nutrient cycling, affect herbivory intensity (Britton-Simmons, 2004; Sánchez *et al*., 2005; Yun & Molis, 2012), modify ecosystem properties, and ultimately, may decrease native diversity (Didham *et al*., 2005).

In the northwest coast of the Iberian Peninsula (northern Portugal and Galician waters), the number and distribution range of non-native macroalgal species have increased over the last years. In Portugal, 86 NIS have been registered in mainland Atlantic waters, 39 in Madeira and 64 in Azores (Chainho *et al*., 2015)

3. Chemical contamination in marine ecosystems

Coastal ecosystems are among the most relevant and dynamic systems in terms of productivity and biodiversity (Beaumont *et al.*, 2007). Further to their ecological importance, these systems also provide important economic resources, serving as food provision for economically relevant species and humans (Beaumont *et al*., 2007).

However, coastal systems are subjected to a large amount of contaminants from natural and anthropogenic sources, contributing to their deterioration (Tornero & Hanke, 2016). Chemical contamination of the marine environment (both estuarine and coastal areas) is a highly complex problem. Up to 15 different groups of pollutants including metals and pesticides and emerging organic contaminants can be simultaneously present at different

levels in marine ecosystems. All of them make a "cocktail" of hazardous substances that may pose negative consequences for the aquatic environment, human health (through the possible ingestion of contaminated seafood), and related coastal activities such as fishing, aquaculture, or recreational activities (Álvarez-Muñoz *et al*., 2016). Human activities are responsible for the great majority of the contamination of marine environments, and consequently, for the decline of their resources (Derraik, 2002). Metals are one of the most common environmental pollutants and their occurrence indicates the presence of natural or anthropogenic sources associated with industrial or domestic effluents (Masindi & Muedi, 2018). Heavy metals like cadmium (Cd), mercury (Hg) and nickel (Ni) can all be found in aquatic environments and once accumulated by marine organisms, can induce the formation of reactive oxygen species, inducing excessive oxidative stress which can lead to damage on important macromolecules like proteins, lipids or DNA (Canli & Atli, 2003; Tchounwou *et al*., 2012; Jaishankar *et al*., 2014). Also, Silva *et al*. (2017), demonstrates that metal exposure influences the FA profile, with mechanisms that may act as potent inducers of fatty acid modulation in the marine snail *G. umbilicalis*. Pollutants like organophosphorus (OPs) compounds are an important group of pesticides in current use in developing countries, being generally accepted as the most effective means for protecting crops against insects (Costa, 2018). OPs are extremely toxic, being able to easily permeate cells and severely modify the neurological responses of organisms (Cao *et al*., 1999). The toxicity of OPs is based on inhibition of the enzyme Acetylcholinesterase which cleaves the transmitter acetylcholine, thereby interfering with proper neurotransmission in cholinergic synapses and neuromuscular junctions. Chlorpyrifos [CPF; O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate], the active ingredient in the commercial formulation Dursban®, is known to be a potent inhibitor of acetylcholinesterase (AChE) in the marine snail *G. umbilicalis,* which can result in neurotoxicity impacts mediated by cholinergic synapses (Silva *et al*., 2019)

4. Ecotoxicology, risk assessment and environmental biomarkers

Truhaut, 1977 defined ecotoxicology as “a branch of toxicology concerned with the study of toxic effects, caused by natural and synthetic pollutants, to the constituents of ecosystems (animals including human, vegetable and microbial), in an integrated context”. Paracelsus proclaimed that the dose makes the poison: "All things are poison and nothing is without poison: the dose alone makes a thing not poison."

Besides the findings of toxicology and ecology, ecotoxicology integrates and utilizes the results of physiology, chemistry, mathematics, geology, genetics, microbiology, among many other disciplines, providing a new approach.

To understand how contaminants affect organisms, ecotoxicologists have the necessity to develop specific tools for pollution biomonitoring studies. Biomarkers are a proactive tool that can help scientists to predict which contaminants the organism has been exposed to, in which tissues they have been accumulated, and if they are causing a toxic effect at critical target (McCarthy & Shugart, 1990). Risk assessment cannot be exclusively based on chemical analysis of environmental samples because this approach does not indicate any deleterious effects of contaminants on the biota. Comparison of the chemical levels determined with reference values is often limited because for many contaminants reference values is still not available (especially ECs) and different species and populations may exhibit differential sensitivity towards environmental contamination (Cajaraville *et al.*, 2000).

Biomarkers can provide an early warning for relevant effects occurring at higher levels of biological organization. This is an advantage because effects at higher levels are usually only measurable after a significant or permanent damage has occurred (Forbes *et al*., 2006).

The general route of contaminants in an organism starts with their uptake from the environment, passes through detoxification mechanisms where the xenobiotics are converted to more soluble-water compounds, and finally undergoes excretion and/or bioaccumulation. Through circulation, contaminants enter the cells, where they reach target site(s), by crossing membranes (lipophilic contaminants), diffusion (polar molecules)

and endocytosis (hydrophilic contaminants), where the chemical is engulfed by the membrane and form a vesicle. These processes are dependent on the contaminant (e.g., physico–chemical characteristics, concentration reaching the target sites/receptors, duration of the exposure) and the organism (e.g., affected species, genetic background, life cycle stage, nutritional and reproductive status) (Newman, 2009). Reactive oxygen species (ROS) are generated during regulated physiological processes, and these are cytotoxic through oxidative damage to DNA, proteins, and lipids. Preventing these damages, major enzymes act as ROS scavengers, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx, selenium dependent), and glutathione-S-transferase (GST, selenium independent). Superoxide dismutase integrates the first line of defense by catalyzing the dismutation ($O_2^{-}\bullet$) of the superoxide radical into oxygen and hydrogen peroxide. Following, CAT detoxify hydrogen peroxide into water and oxygen while GPx catalyses the metabolism of H_2O_2 to water, involving a concomitant oxidation of reduced GSH to its oxidized form – GSSG (Ameur *et al.*, 2012). A failure of the antioxidant defenses to remove excess of ROS leads to oxidative stress, and damage to important macromolecules related to enzymatic activities, DNA damage, and lipid peroxidation (LPO), which levels have the potential to be used as biomarkers (Winston & Di Giulio, 1991). Also, other important group of biomarkers is of those related to the energetic budget of an organism: Calorimetric measurements of available energy (protein, lipid, and carbohydrate content) and energy consumed (electron transport system activity; ETS). When organisms live in suboptimal environments, there will be some difficulty in dealing with stress in terms of metabolic resources (Smolders *et al*., 2004). Under situations of stress a large amount of the energy is used to detoxify and to maintain or compensate basal metabolism activity, increasing energy consumed and leaving less energy available. On the other hand, Lactate dehydrogenase (LDH) is a key enzyme in the anaerobic pathway of energy production, being particularly important for muscular physiology in conditions of chemical stress when high levels of energy may be required in a short period of time). 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. Iso-citrate dehydrogenase (IDH) on the other

hand, catalyzes the reduction of iso-citrate, which is a crucial step in the aerobic citrate cycle (De Coen *et al*., 2001)

Acetilcholinesterase (AChE) is an important nervous system enzyme responsible for hydrolyzing the neurotransmitter acetylcholine into choline and acetic acid, an essential process for both neuronal and motor capabilities (Huggett, 2018). AChE activity can be compromised by several compounds commonly found in the environment, such as organophosphates (OPs) and carbamates (CBs) pesticides that act as neurotoxic molecules, being thus designated as anticholinesterasic compounds. Different types of cholinesterases (ChEs) have been already described and are differentiated by their substrate preference and by their specific inhibition profiles in the presence of particular inhibitors. Since the properties of ChE may differ between species, it is important to characterize the type of enzyme present in the species studied before its use as a biomarker (Kristoff *et al*., 2006).

4.1 Fatty acid profile

The use of lipid biomarkers is adequate for understanding the changes that the accumulation of xenobiotics can cause in lipid profiles of contaminated organisms (Testai, 2002). FAs in particular, and lipids in general, are sensitive to environmental stress (Gonçalves *et al*., 2017). Membrane lipids, especially fatty acids (FAs) have great structural diversity and high biological specificity. FAs have been used as quantitative markers for nutrition or health biomarker and as food-web tracers (Rude *et al*., 2016). Moreover, changes in lipid metabolism and FA profiles have been used to better understand how pollution affects organisms in aquatic food-webs and as a biochemical response to pollutant exposure. Since many of these xenobiotics appear to be fat soluble, the use of lipid molecular biomarkers is often adequate to understand the changes that their accumulation may cause in the lipid profiles of contaminated organisms (Chyczewski, 2001). Moreover, the importance of lipids in cellular metabolism, given their structural and energy storage function (among others), justifies looking at the potential effects of contaminants, particularly xenobiotics, on lipid profiles.

Different studies for the application of biomarkers to assess the impact of xenobiotics in the environment have been developed, while novel biomarkers need to be assess based on their ability to respond to toxic exposure, but also to to indicate physiological impairments.

4.2 Behavioral ecotoxicology

Behavior is an organism-level effect characterize as the reaction or functioning of a system under a set of specific circumstances (Hellou, 2011). It results from the combination of conditions to which the organisms are exposed and represents an acute cumulative effect (Hellou, 2011). A change in behavior can be a direct result of pollutants, but also a method used by the organism to protect itself against the pollutant. Thus, behavior is the final integrated result of a diversity of physiological processes interacting with the surrounding abiotic and biotic components (Amiard-Triquet *et al.*, 2012). According to the concept of a hierarchical cascade of biological responses to pollutants, behavior could be the key marker, providing predictive assessment of pollution at the population level (Amiard-Triquet *et al.*, 2012). Studying behaviour alterations allows the integration of individual physiological processes and mechanisms with the environmental stimuli that are causing them (Dell'Omo, 2002). Behavioural tests are, in addition to being sensitive, relatively fast, simple to perform, noninvasive, cheap and, as described in many studies, with a high ecological relevance (Hellou, 2011).

Flipping test is a behavior endpoint that consists in recording the time that a snail (e.g.,) takes to flip back to an upright position (Cabecinhas *et al.*, 2015). Some studies have already shown that the organisms under normal/control situations take only a few seconds to turn, so flipping time is a simple yet valuable and ecologically relevant novel tool to assess contaminant effects on snails, permitting a quantitative measurement of an ecologically relevant behavioral endpoint with potential implications for population dynamics despite the need for further validation under different exposure scenarios. A slowed righting ability has been already previously demonstrated in gastropods under stressful circumstances (Cabecinhas *et al.*, 2015; Silva *et al.*, 2019). Hellou *et al.*, 2009 stated that snails display a

higher susceptibility to physical stress, with an increased number unable to twist from being on their shell to their foot, and with longer righting time.

Also, foot detachment is a component of escape behaviour in marine snails that isn't a turnover test like flipping but has already test also in snails where it´s measure the observed foot loosening and subsequent detachment. They describe the lateral edges of the snail's foot lifting off the substrate, then portions of the "sole" detaching progressively until locomotion stopped. Pedal waves and foot movements occurred, but the snails could not reattach. They called this response "sole detachment" (Fong & Hoy, 2012; Fong & Molnar, 2013). Other behavioural parameters are used in aquatic organisms exposed to contaminants like swimming velocity in fishes (Kennedy & Farrel, 2006), amphipods (Xuereb *et al*., 2009) and also in shrimps (Oliveira *et al*., 2012). Avoidance behaviour are based on the ability of an organism to move away from contaminated sites and is also used in many ecotoxicological studies (Oliveira *et al*., 2013; Da Luz *et al*., 2004; Loureiro *et al*., 2005)

Feeding is also is a useful and sensitive endpoint to detect sub-lethal impacts on individual organisms with relevance to higher levels of organisation. Feeding determines the health of a population because altered growth and reproduction can be investigated by an effect on feeding (Agatz *et al.*, 2013). Feeding activity is used as a short-term sublethal endpoint due to its rapid and easy measurement and its sensitivity, but mainly because of its biological significance because the energy intake determines the energy available to important life functions.

5 Background on the study species

5.1 *Asparagopsis armata*: distribution and its biology

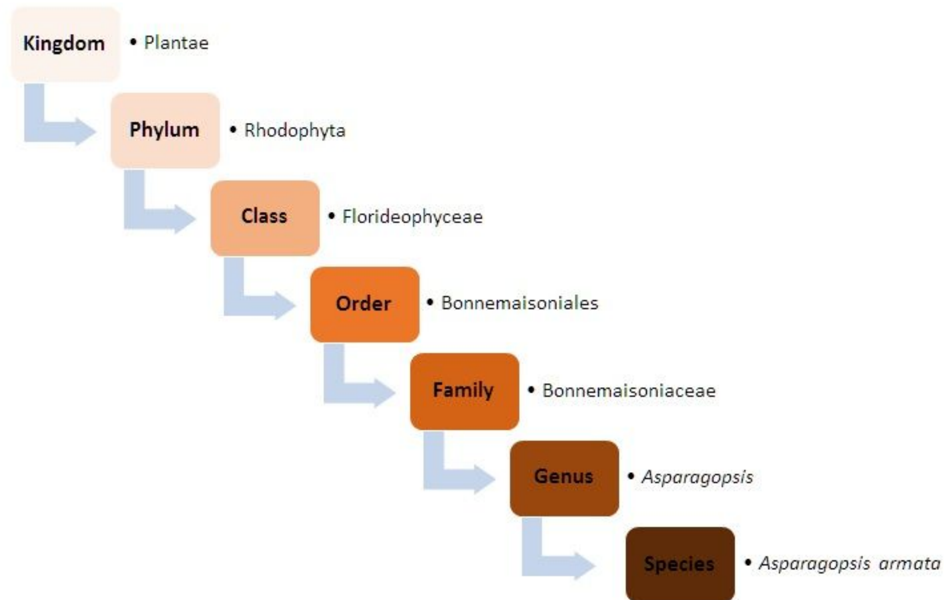

Figure 2 - *Asparagopsis armata* taxonomy

The genus *Asparagopsis* (Montagne, 1840) contains two invasive species: *Asparagopsis armata*, Harvey (1855) and *Asparagopsis taxiformis* (Delile) Trevisan 1845, both characterised by heteromorphic, diplo-haplontic life histories (Figure 3) (Feldmann & Feldmann, 1939; Feldmann, 1942), whose respective diploid phases (sporophytes) were first described as two separate species: *Falkenbergia rufolanosa* (Harvey) F.Schmitz 1897 (*A. armata*) and *Falkenbergia hillebrandii* (Bornet) Falkenberg, 1901 (*A. taxiformis*).

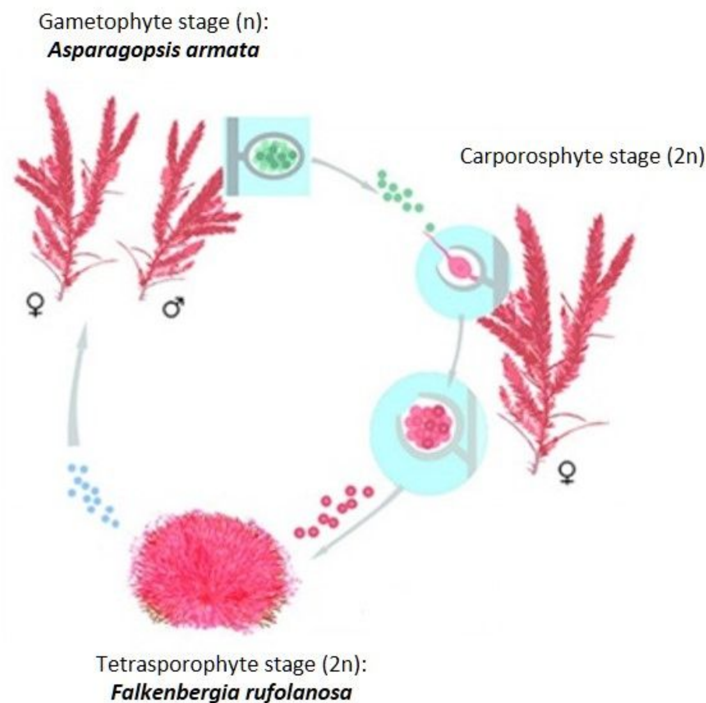

Figure 3 - *Asparagopsis armata* life-cycle (adapted from IUCN – Species report *Asparagopsis armata*)

The diploid tetrasporophyte is known as 'Falkenbergia', that is included in both species' life history, is composed by branched filaments with a narrow axial cell and three periaxial cells, forming pinkish-reds tufts, sometimes described as pink "pompoms" (Bolton *et al*., 2011). *Asparagopsis armata* is morphologically distinct from *A. taxiformis* in that it possesses long stolons bearing harpoon-like hooks. *Asparagopsis taxiformis* has a more compact rhizoida system, lacks spiny branches, and forms more patchy tufts.

Figure 4 - *Asparagopsis armata* gametophytic thalli. Branches provided with spine-like branclets are visible. (Photo: Carla Silva)

Asparagopsis armata is common in temperate seas and is native to southern Australia and New Zealand (Horridge, 1951) and is now found from the British Isles, passing through Portugal, the Canary and Salvage Islands to Senegal. It was introduced in Mediterranean Sea and is considered a Lessepsian immigrant, first reported from the Algerian coasts in 1923 (Feldmann, 1942). Along the Iberian Peninsula, the species is distributed throughout the Atlantic and Mediterranean coasts. This species was detected in the Iberian Peninsula on the first half of the last century (Miranda, 1934). Presently, this seaweed has managed to settle largely on both Atlantic and Mediterranean coasts and seems to have a continuous expansion. In Portugal, it shows a discontinuous distribution along the north and north-west Atlantic coast of the Iberian Peninsula. In the north of Portugal, it is present close to Minho river (Rubal *et al*., 2018)), then, using modelling tools (Blanco *et al*., 2020), *A. armata* has low probability of presence till Peniche, where it is very abundant and frequent, forming blooms in summer season (Figure 5). On the southwest coast, there are records of this species from Sines to Odeceixe. Then in the south, in Albufeira (MACOI 2008)

Both *Asparagopsis* species are considered among the "worst invasive alien species threatening biodiversity in Europe" (EEA 2007) and among the "100 worst invasive seaweeds in the Mediterranean Sea" (Streftaris & Zenetos, 2006).

Intrinsic traits such great dispersal potential due to a free-floating tetrasporophyte in its life cycle, its defense system in the release of halogenated compounds that increase resistance to herbivory (Paul *et al*., 2006), have been cited as responsible for the success of *A. armata* as invader. Also, *A. armata* possesses long hooked stolons that gets entangled with others macroalgae or attachment to other floating structures, allowing the development of stalks through large areas.

Figure 5 - *Asparagopsis armata* blooms in Porto da Areia Norte, Peniche. (Photo: Carla Silva)

Several hypotheses have been formulated to understand the ability of *Asparagopsis armata* to expand and adapt to new environmental conditions (Monro & Poore, 2009; Zanolla *et al.*, 2015). Up to now, morphological and physiological traits have been studied mainly in order to assess the potential of invasion (Zanolla *et al.*, 2015; Greff *et al.*, 2017).

5.2 Production of secondary metabolites

Many of the ecological interactions involving macroalgae are mediated by compounds that are not modeled by primary metabolism. Such compounds are known as secondary metabolites and have a range of structures, including terpenes, alkaloids and phenolics (Fenical 1982). These compounds have significant biological activities, and they are maintained into specialized storage structures in order to avoid autoxicity (Fenical 1982). Members of Bonnemaisoniaceae form specialized structures that include gland cells and cells with refractile inclusions, such as *corp en cerise* located in surface cell layer (Young *et al*., 1980).

The chemistry of *Asparagopsis armata* is well characterized, with over 100 halogenated compounds found, including haloforms, haloacids, and haloketones (McConnell & Fenical, 1977). These halogenated volatile hydrocarbons containing one to four carbons are known to include compounds having antimicrobial, antifeedant and cytotoxic properties (Pinteus *et al.*, 2015; Paul *et al.*, 2006; Zubia *et al.*, 2009). Chemical analysis of the major halogenated metabolites of in gametophyte of *Asparagopsis armata* are listed in Table I (Paul *et al*., 2006a).

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 (Burreson *et al*., 1976). This alga is unpalatable to herbivores but the sea hare *Aplysia parvula* is an opisthobranch mollusk capable to eat this chemically defended red seaweed (Pereira *et al.*, 2013).

Table I - Quantitative analysis of the major halogenated metabolites of *Asparagopsis armata* gametophyte was performed by gas chromatography-mass spectrometry (GC-MS). Units expressed as mass compound per unit algal dry weight. (DW). Paul *et al*., 2006a.

Metabolite	**Mean internal level (% DW)**
Bromoform ($CHBr_3$)	1.67% DW (± 0.16 SE)
Dibromoacetic acid (DBA)	0.25% DW [±0.06 SE)
Bromochloroacetic acid (BCA)	0.08% DW [± 0.01 SE)

Dibromochloromethane ($CHBr_2Cl$)	0.03% DW [± 0.003 SE)

6 Life in intertidal pools

Asparagopsis armata is mainly found in the low intertidal zone (Fa *et al.*, 1997), extending to the first meters of the subtidal. It commonly grows at depths of 0-10 m but has occasionally been found as deep as 30 m, but mainly well established in open sandy pools, on rock or epiphytic (mainly on *Ulva spp*.) (Womersley 1996).

The space that is continuously covered and uncovered by the tide is known as the intertidal zone and is home to a rich ecosystem that helps sustain life in the water and on land. In tide pools, algae grow in the abundant sunlight and support an entire food chain of animals. There´s also some challenges that species faced in periodic exposures to water level changes between high and low tide, due to its environment is always fluctuating, the intertidal zone can be a difficult place to live (Adey & Loveland, 2007). Since the strength of tides vary day to day, three subzones have been created within the intertidal zone: the high, mid, and low intertidal zone. The high intertidal zone, where the species are relatively dry, is located directly under the splash zone and is only covered with water during the highest of high tide. The middle tide zone is covered by all high tides and is exposed by all low tides, and the low intertidal tide is usually wet. These exposed areas, the tide pools, may be roughly seen as microenvironment because they have approximately six hours between low and high tide, and they constitute an isolate ecosystem without water input or output and are exposed to extreme biotic and abiotic factors (Miller, 2008). Intertidal habitats, in general, are suitable for experimental studies due to their accessibility and critical position at the interface between the terrestrial and the marine field, thus being exposed to a range of anthropogenic disturbances from both environments. Shallow coastal waters are among the habitats where the impacts of climate warming will be apparent faster, which makes these areas useful natural laboratories (Vinagre *et al*., 2018). Among shallow-water habitats, tide pools are probably the best sentinels to assess the impacts of anthropogenic and biological pollution on marine assemblages. In fact, many

rocky shores are subjected to an array of stresses caused by human activities, and these anthropogenic stresses are superimposed on the stress caused by natural environmental factors such as emersion and dissecation due to the tides and wave action (Raffaelli & Hawkins, 1996).

6.1 Invertebrates as sentinel species

Invertebrate communities of rocky shores function as integrators of ecological processes over a time scale (Kroncke, 1997). Invertebrates constitute >90% of the living species and play a major role in ecosystem functioning. Most of the marine fauna are sessile or have little motility as adults compared to plankton and many taxa with long life spans (Beuchel, *et al*., 2006). These characteristics makes them suitable bioindicators to assess ecological change due to anthropogenic actions.

Intertidal invertebrates and macroalgae occupy low trophic levels and respond quicker to alterations in local conditions than species at higher trophic levels (Jenouvrier *et al*., 2003). They are the first warning in a cascade of effects up the food chain and are therefore important sentinels of climate change impacts (Johnson *et al.*, 2011).

The littoral shrimp *Palaemon elegans* (Rathke, 1837) is found in the intertidal zone, in sea grass beds or in tidal pools along rocky shores, present in medium-litoral or even supra-litoral zone (Morais et al., 2002).

It has a large distribution, ocorring along the Atlantic coasts of Europe and Africa, and also in the Mediterranean and Black Seas (Janas et al., 2010)

Among aquatic organisms, crustaceans have a key-role in the environment for their central position in the food web and also for their wide distribution and high density. For this reason in ecotoxicological testing, several crustacean species have been proposed (Luigi *et al*., 2015) and are having a wide employment in marine ecotoxicology.

Invertebrates that feed on the shore, either feed on plankton by sieving water passing through or near them (barnacles and bivalve molluscs), rasp young seaweeds and other plant material from the rock surface, or nibble the fronds of older seaweeds (limpets, top shells *Gibbula* and *Littorina* species, e.g.).

The marine gastropod *Gibbula umbilicalis* (Da Costa 1778) 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. It is easy to identify, count and measure, has low mobility, and has been used for the detection and monitoring of contaminants (Cabecinhas *et al.*, 2015; Silva *et al.*, 2017).

6.2 Laboratory and Field experiments

Coastal marine environments are affected by a wide range of pollutants. And assessing these types of environments can be challenging due to the high numerous variables encountered and the chemical mixtures levels found in the environment. The majority of studies assessing the effects of contaminants on organisms are done under laboratory settings where such variables as concentration, exposure time, and environmental factors can be controlled.

However, studying organisms in the field is not as simple or as organized. With field organisms being exposed to a variable environmental conditions and input of compounds, determining the exact effect of a chemical compound in nature is often difficult, and can lead to natural variations in response. These variations include altered physiological, metabolic, and detoxification pathways (Barata *et al.*, 2002).

Aims and outline of the thesis

The main aim of this thesis was to evaluate the toxicity of the invasive species *Asparagopsis armata* exudates and its impact in coastal key species and its potential influence on the ecosystem structure and diversity.

For that, this work was done considering an integrative approach. Effects seen at higher levels of biological organization (populations and communities) are the consequence of the sum of effects on individuals, which result from impacts at the cellular and molecular levels. The goal was to explore the possibility of gaining more and ecologically relevant information on the toxic responses of this biological pollution type, using effects on various endpoints.

This thesis is divided in seven chapters, including the current general introduction (Chapter I), five chapters describing the experimental studies to achieve the main goal (Chapters II to VI), and a final chapter that summarizes the general conclusions of this study (Chapter VII). In detail:

In Chapter II: "Fatty acid profile of the sea snail *Gibbula umbilicalis* as a biomarker for coastal metal pollution", published in Science of The Total Environment, the non-lethal effects of a range of metals were assessed on *Gibbula umbilicalis* and lipid related endpoints were measured: total lipid content; lipid peroxidation; and fatty acid profile (FAP). The various endpoints were compared with the goal of assessing whether FAP could be used as biomarker tool in coastal environments to understand changes in the fatty acids profiles to stress.

In Chapter III: "Linking cholinesterase inhibition with behavioural alterations in the sea snail *Gibbula umbilicalis*: effects of the organophosphate pesticide chlorpyrifos", published in Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, effects of the sublethal concentrations of the pesticide chlorpyrifos (CPF) were assessed. Biochemical properties of ChEs were first unraveled in *Gibbula umbilicalis* and effects of CPF on AChE activity were investigated, along with effects on snails' behavior. Both characterization of

ChEs and behaviors were fundamental to address potential neurotoxicity of *A. armata* exudate.

In Chapter IV: "Impacts of The Invasive Seaweed *Asparagopsis armata* Exudate on Rockpool Invertebrates", effects on biomarkers responses associated with energy metabolism (lactate dehydrogenase, LDH; electron transport system activity, ETS; content in lipids, proteins and carbohydrates) and behavioral responses (feeding and flipping) were assessed after exposure to non-lethal concentrations of *Asparagopsis armata* exudate in the shrimp *Palaemon elegans* and the marine snail *Gibbula umbilicalis*, to understand the mechanisms of toxicity of the invasive *A. armata.*

In Chapter V: "*Asparagopsis armata* Exudate Cocktail: The Quest for the Mechanisms of Toxic Action of an Invasive Seaweed on Marine Invertebrates", effects of *A. armata* exudate on marine snail, *Gibbulla umbilicalis* and the shrimp *Palaemon elegans* with respect to antioxidant defences superoxide dismutase (SOD) and glutathione-S-transferase (GST); oxidative stress parameters lipid peroxidation (LPO) and DNA damage; neuronal parameters as acetylcholinesterase (AChE); as well as the fatty acid profile (FAP) were evaluated. In this chapter we further address potential mechanisms of action involved in the previously observed effects (Chapter IV).

In Chapter VI: "The effects of the invasive seaweed *Asparagopsis armata* on native rock pool communities: evidences from experimental exclusion", variations on intertidal seaweed and macroinvertebrate assemblages inhabiting rock pools with and without the presence of the invasive macroalgae were evaluated, in order to understand the effects of the invader *A. armata* on native assemblages.

In Chapter VII: "General Discussion", the major findings from the previous chapters are highlighted and discussed, and future research perspectives formulated.

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Chapter II

Fatty acid profile of the sea snail *Gibbula umbilicalis* as a biomarker for coastal metal pollution

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Chapter II – Fatty acid profile of the sea snail *Gibbula umbilicalis* as a biomarker for coastal metal pollution [1]

Abstract

Metals are among the most common environmental pollutants with natural or anthropogenic origin that can be easily transferred through the food chain. Marine gastropods are known to accumulate high concentrations of these metals in their tissues. *Gibbula umbilicalis* ecological importance and abundant soft tissues, which enables extent biochemical assessments, makes this particular organism a potentially suitable species for marine ecotoxicological studies. Fatty acids are carbon-rich compounds that are ubiquitous in all organisms and easy to metabolize. Their biological specificity, relatively well-studied functions and importance, and the fact that they may alter when stress is induced, make fatty acids prospect biomarkers. This work aimed to assess fatty acid profile changes in the gastropod *G. umbilicalis* exposed to three metal contaminants. After a 168h exposure to cadmium, mercury, and nickel, lipid related endpoints were measured: 1) total lipid content; 2) lipid peroxidation; 3) fatty acid profile (FAP). FAP suggested an alteration in the fatty acid metabolism and indicated a link between metals exposure and homeoviscous adaptation and immune response. In particular, five fatty acids (palmitic, eicosatrienoic, arachidonic, eicosapentaenoic, and docosahexaenoic acids), demonstrated to be especially good indicators of *G. umbilicalis* responses to the array of metals used, having thus the potential to be used as biomarkers for metal contamination in this species. This work represents a first approach for the use of FAP signature as a sensitive and informative parameter and novel tool in environmental risk assessment (ERA) of coastal environments, using *G. umbilicalis* as model species.

Keywords: Biomarkers, Cadmium, Lipid peroxidation, Marine environmental risk assessment, Mercury, Nickel

[1] Silva, C. O., Simões, T., Novais, S. C., Pimparel, I., Granada, L., Soares, A. M., ... & Lemos, M. F. Published in Chemosphere, Science of the Total Environment, doi.org/10.1016/j.scitotenv.2017.02.01

1. Introduction

Metals are one of the most common environmental pollutants and their occurrence indicates the presence of natural or anthropogenic sources associated with industrial or domestic effluents (Ramadan *et al*., 2010). In the natural environment, organisms living in chronically polluted sites are exposed to low concentrations of metals for long periods, but in some cases, organisms may be suddenly exposed to high levels of metals upon the outfall of a pollutant in coastal waters, primarily due to weathering or anthropogenic input (Pinto *et al*., 2003). Metals, such as cadmium, nickel, and mercury, due to their oxidative potential, may induce the formation of reactive oxygen species (ROS) that may result in damage such as lipid peroxidation (LPO) (Valavanidis *et al*., 2006). Lipid peroxidation can induce structural and chemical alterations on cellular membranes, which involve a chain of reactions triggered by the ROS accumulation on the membrane bilayer that further contribute to the change in its structure, damaging biological membranes (degeneration) and impairing cellular processes (loss of activity) (MacFarlane *et al*., 2006). This ROS accumulation leads mainly to the breakdown of polyunsaturated fatty acids (PUFA) that are relatively sensitive to oxidative reactions (Livingstone *et al*., 1990; Geret *et al*., 2002).

Sea snails of the genus *Gibbul*a are amply distributed in the marine environment, having a wide geographical distribution throughout rocky shores (Williams, 1964). *Gibbula umbilicalis* is a species easy to find, collect and maintain under laboratory conditions. Additionally, because they are easy to identify, measure, and have a low mobility and relative large size (abundant soft tissues to be used in biochemical assessments), they can be considered as potential test species to be used in marine effect assessment studies (Cabecinhas *et al*., 2015).

Fatty acids (FA) are major cell constituents, playing several essential roles in biological systems, as sources of energy, as membrane constituents, or as metabolic and signalling mediators (Pereira *et al*., 2013), being determinant for ecosystem stability. They are present in a great structural variety throughout marine ecosystems associated with the vast biological diversity of marine life, and are susceptible to oxidative damage leading to

cytotoxicity and a decrease in membrane fluidity (Parrish, 2013). Studies have suggested that changes in fatty acid constituents or profiles in marine organisms are mostly associated with diet, stage of larval development, growth, environmental change, and exposure to contaminants (Napolitano *et al*., 1997; Turner and Rooker, 2005). As lipid components are very sensitive to stressors and environmental changes, many organisms have developed mechanisms to maintain the appropriate fluidity of membrane lipids. These mechanisms include changes in the proportions and types of lipids and alterations in the lipid/protein ratio (Rodríguez-Vargas *et al*., 2007).

To our knowledge, most of the information available regarding FA determination in molluscs refers to bivalve species, such as oysters (Abad *et al*., 1995) and mussels (Freites *et al*., 2002; Signa *et al*., 2015), and there is little information available on the biochemical components of other mollusc species. Particularly, there are only few reports on both lipids and fatty acid composition of gastropods, and scarce information about how contaminants may affect them (Go *et al*., 2002; Saito *et al*., 2014).

Fatty acids have been used as quantitative markers for nutrition or health biomarkers for several years and have long been used as food-web tracers (Kris-Etherton *et al*., 2000; Rude *et al*., 2016). Furthermore, changes in lipid metabolism and FA profiles have been used to better understand how pollution affects keystone organisms in aquatic food-webs (Colin *et al*., 2016), and as an integrative biochemical response to pollutant exposure and accumulation in marine organisms (for review see Filimonova *et al*., 2016), where several studies with metals are included (Fokina *et al*., 2013; Thyrring, *et al*., 2015), presenting them as promising biomarkers to assess stress exposure.

This study aimed to address general FA composition in the gastropod *G. umbilicallis* and assess the potential of FA profile to be used as a biomarker of metal contamination while also evaluating other commonly used lipid related endpoints such as LPO and the total lipid content. The final goal is to propose this FA profile as a novel tool to be used in ecological risk assessment of coastal environments using a sea snail as model species.

2. Material and methods

2.1. Test organisms

Gibbula umbilicalis (Costa, 1778), of similar size (10 ± 1mm) were collected by hand from Carreiro de Joannes, a rocky beach in Peniche, central Portugal (39.354887º, -9.394574º); location with no historical chemical contamination. The organisms were acclimated during 7 days in the laboratory prior to each experiment in natural seawater (3,60% salinity and pH of 8,15) filled aquaria at 20 ±1 °C, with a 16 h:8 h (light – dark) photoperiod. During this period they were fed *ad libitum* with the green macroalgae *Ulva lactuca* (Linnaeus, 1753). Prior to testing, organisms were kept fasting for 24 hours.

2.2. Media contamination and chemical analysis

All test solutions and experiments were prepared in plastic material in order to avoid metal adsorption. Natural filtered seawater (0.45 µm pore) was used for the experiments.
Stock solutions were prepared by dissolving the pure compound in ultra-pure water to a concentration of 10 g L^{-1} in the case of $CdCl_2$ (purity ≥99%; Sigma-Aldrich, USA), 1 g L^{-1} for $NiCl_2.6H_2O$ (purity ≥98%; Merck, Germany), and 0.1 g L⁻1 for $HgCl_2$ (≥99.5%, Sigma-Aldrich, USA).
Working solutions were prepared in seawater, kept cold in the dark, and were used to prepare the final nominal concentrations to start the experiments and renew the media daily. Cadmium, mercury, and nickel concentrations were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS), using a Thermo X-Series ICP-MS spectrometer (Thermo Fisher Scientific Inc., MA, USA).

2.3. Exposure conditions

Experimental conditions were as previously described for *G. umbilicalis* maintenance (see 2.1). Tests were conducted in a climatic room, and experimental replicates consisted of 150 mL plastic flasks, with one organism each, and covered with a plastic mesh to prevent

organism to escape and to assure constant submersion. No food was added during exposure, and media was renewed every 24 hours to prevent from excreta saturation (and parameters kept at ranges: pH 8.15-8.21; 3.56-3.63% salinity; DO 77.8-87.8%; and ammonia < 0,25 mg L^{-1}) and any potential volatilization (O'Driscoll *et al*., 2007).

2.4. Endpoints measured

2.4.1. Acute assay - Lethality

Sea snails were exposed to the following analytical concentrations of the different metals: 1) cadmium – 1.50, 1.73, 1.99, 2.28, 2.63, 3.02, 3.48, and 4.00 mg of Cd L^{-1}; 2) mercury – 0.1, 0.13, 0.16, 0.20, 0.25, 0.32, 0.40, and 0.5 mg L^{-1}; 3) nickel – 1.0, 1.39, 1.93, 2.68, 3.73, 5.18, 7.20, and 10.0 mg L^{-1}. Exposures lasted 7 days and mortality was assessed daily. Eight replicates per treatment were used, including a control treatment with filtered seawater only.

2.4.2. Sublethal Endpoints

Based on the acute exposures, the previously assessed LC_{10}s were used as the highest concentrations tested for the 168 h chronic bioassays together with the concentration ten times lower than the LC_{10}. The analytical concentrations used for Cd were: 0.17 and 1.87 mg of Cd L^{-1}; for Hg: 0.02 and 0.27 mg of Hg L^{-1}; and for Ni: 0.26 and 2.79 mg of Ni L^{-1}.
Seven replicates per treatment were used and, at the end of the exposure period, organisms were sacrificed and kept at -80 ºC until further analysis.
Organisms' shell was broken with a vise and the soft tissues were taken from the test organisms and dissected on ice for operculum removal. Each snail, corresponding to an individual replicate, was homogenized in 750 µL of Buffer K-Phosphate 0.1 M, pH 7.4. The homogenate was then divided into 3 microtubes: 1) 150 µL for total lipid quantification; 2) 150 µL for lipid peroxidation (LPO); and 3) 350 µL for fatty acid profile.

2.4.2.1. Total lipid quantification

Lipid content was extracted and further quantified according to the methodologies described by Bligh and Dyer (1959) and De Coen and Janssen (1997). Briefly, the extraction was performed by adding 500 µL of both chloroform and methanol to each sample followed by the addition of 250 µL of Mili-Q water and a centrifugation at 1000g for 5 min. After centrifugation, the organic phase was used for lipid quantification. After adding 500 µL of H_2SO_4 to 100 µL of this lipid extract, the samples were incubated for 15 min at 200 ºC and further diluted in 1.5 ml of Mili-Q water. Total lipid content was determined by measuring the absorbance at 375 nm using Tripalmitin (Sigma-Aldrich, USA) as standard.

2.4.2.2. Lipid peroxidation

Endogenous LPO levels of samples were determined by measuring the thiobarbituric acid-reactive substances (TBARS) according to Ohkawa (1979) and Bird and Draper (1984), with some adaptations. Briefly, 500 µL of 12% trichloroacetic acid, 400 µL of Tris–HCl (60 mM with 0.1 mM DTPA, pH 7.4) and 500 µL of 0.73% 2-thiobarbituric acid (TBA) were added to the 150 µL of sample. The mixture was heated at 100 ºC for 1h and then cooled to room temperature before centrifuging it at 11500 g for 5 min. Absorbance of the supernatant was measured at 535 nm and LPO expressed as nmol TBARS mg^{-1} of fresh tissue using a molar extinction coefficient of $1.56x\ 10^5\ M^{-1}\ cm^{-1}$.

2.4.2.3. Fatty acid profile

The aliquoted homogenate for fatty acid profile was saponified overnight in 0.6 M KOH (67% (v/v) ethanol) at 90°C before derivatization by acid catalysis. The reaction mixture was cooled to room temperature, diluted 1:1 with water, and acidified with 6 M HCL. Fatty acids were extracted with hexane (Fleschner and Cenedella, 1997). The preparation of the FA for their identification was performed according to Lepage and Roy (1986) and Masood *et al*. (2005). To the FA fractions isolated after saponification, 5 mL of acetyl chloride:methanol (1:19 v/v) solution was added and heated at 80 ºC for 1 h. Afterwards, 1 mL of Mili-Q water and 2 mL of *n*-heptane were added and this solution was vortex-stirred for 1 min prior to a

centrifugation of 1500 g for 5 min. The upper organic phase was recovered and analysed by gas chromatography (GC). A Finnigan Ultra Trace gas chromatograph equipped with a Thermo TR-FAME capillary column (60 m x 0.25 mm ID, 0.25 µm film thickness), an auto sampler AS 3000 from Thermo Electron Corporation and a flame ionization detector (FID) were used to analyse the fatty acids methyl esters.
The injector (operating in splitless mode) and the detector temperatures were set at 250 and 280 °C, respectively. The column temperature was initially set at 100 °C for 1 min, then raised at 10 ºC min^{-1} to 160 ºC and held for 10 min followed by an increase at 4 ºC min^{-1} to 235 ºC and maintained for 10 min. Helium was used as carrier gas at a flow rate of 1.5 mL min^{-1}. Air and hydrogen were supplied to the detector at flow rates of 350 and 35 mL min^{-1}, respectively. Fatty acid methyl ester mixes (PUFA No1 from Marine source and PUFA No 3 from Menhaden oil) were used as GC standards (Supelco, Bellefonte, Pa., U.S.A.).

2.5. Statistical analysis

LC_x values and the correspondent 95% confidence intervals were calculated by fitting the probit regression model (Finney, 1971) using Minitab statistical package (Minitab, 2005). All data was checked for normality using q-q plots and Shapiro-Wilk test and for homoscedasticity using Levene test. When these assumptions were met, a One-way analysis of variance (ANOVA) was used to determine significant differences between treatments of each metal concentration for total lipids, lipid peroxidation and for every fatty acid detected. When differences were found, a Dunnett's post-hoc test was employed to determine significant differences between the different concentration of the metal and the control treatment using SigmaPlot software for Windows, version 11.0 (SigmaPlot, 1997). When assumptions of normality and homoscedasticity were not met, a Kruskal-Wallis was applied followed by Dunn's post-hoc test. A PCA (Principal Component Analysis) was also performed to assess correlations and possible patterns between the variables (fatty acids) and the treatments (metals), and to make the degree of divergence between metal concentrations and FA profiles visible. The principal components (PC1 and PC2) provide information on the most meaningful parameters, which describe a whole data set affording data reduction with minimum loss of original information. Although only the

results concerning the first two components are presented (based on the amount of explained variance; Pearson, 1901), the others were also analysed. PCA was performed with CANOCO software, version 4.5 (Ter Braak & Smilauer, 1998). For all statistical tests, the significance level was set at $p<0.05$.

3. Results and discussion

3.1. Lethality

The tested controls presented no mortality. The calculated 168 h LC_{50} [95% CI] for each metal were as follows: 2.67 [2.33 – 3.08] mg of Cd L^{-1}; 0.41 [0.34 – 0.54] mg of Hg L^{-1}; and 4.27 [3.46 – 5.65] mg of Ni L^{-1}. Given these LC_{50} values, the most toxic metal tested for *G. umbilicalis* was Hg and the less toxic was Ni. Toxicity studies performed with Cd in other marine molluscs reported a 96 h LC_{50} of 0.221 mg L^{-1} in the mussel *Modiolus philippinarum, a* 96-h LC_{50} of 9.193 mg L^{-1} in the girdled horn snail *Cerithidea cingulata* (Ramakritinan, 2012), and a 96 h LC_{50} of 2.44 mg L^{-1} and of 2.64 mg L^{-1}, respectively, for the sea snail *Monodonta lineata* and the dogwhelk *Nucella lapillus* (Cunha, 2007). Using similar exposure periods then in the present study (168 h) Eisler, (1977b) found an LC_{50} of 0.150 mg of Cd L^{-1} for the clam *Mya arenaria*, suggesting that *G. umbilicalis* is less sensitive than other molluscs to cadmium exposure.

Ramakritinan (2012) found a 96 h LC_{50} of 0.053 mg of Hg L^{-1} for the snail *Cerithedia cingulate* and 0.007 mg L^{-1} of Hg L^{-1} for the mussel *Modiolus philippinarum*, and even other study with the same 168h of exposure to Hg in the snail *Nassarius obsoletus* showed an LC_{50} of 0.004 mg of Hg L^{-1}. These LC_{50} results are between 10 and 100 times lower than the one found in the present study (0.41 mg of Hg L^{-1}), suggesting that *G. umbilicalis* is more tolerant to that metal.

Regarding Ni, the 96 h LC_{50} found for freshwater mussel *Lamellidens marginalis* was 2.27 mg of Ni L^{-1} (Andhale *et al*., 2011) which is within the same order of magnitude of the168 h LC_{50} found in this study (4.27 mg of Ni L^{-1}). However, other authors found higher LC_{50} values for other species, e.g., 16 mg L^{-1} for the mud-snail *Nassarius obsoletus* and 112 mg

of Ni L^{-1} for the clam *M. arenaria* (Eisler, 1977a), suggesting that, contrary to the other metals, *G. umbilicalis* is more sensitive to Ni then other molluscs.

In general, the acute results indicate that *G. umbilicalis* as a more tolerant species to these metals' contamination, also because most of the examples found in the literature for other species were related to 96 h of exposure (shorter than in the present study) and it is expected that the toxicity increases with longer exposure periods (lower 168 h LC_{50} values).

3.2. Total Lipid Content

Total lipid content, as other energetic reserves, is a factor normally affected by metal contamination. However, in this study there were no significant differences observed between total lipid content in the control and in the different Hg and Cd treatments, meaning that the 168 h exposure to these contaminants was not enough to significantly affect the total lipid reserves of *G. umbilicalis* (Fig. 1). Exceptionally, for Ni effects, it was observed that in the lower concentration of 0.25 mg L^{-1} lipid amount was significantly higher in the organism's soft tissues (Dunnett's, $p=0.006$) (Fig. 1). These results are somehow unexpected since without a food source supply and under stress conditions, normally lipids are not allocated but rather metabolized as energy source and their levels tend to decrease. In this particular lower Ni treatment, it is possible that the organisms somehow lowered their lipid metabolism in order to sustain their energetic levels, either solely metabolically or indirectly through behaviour alterations.

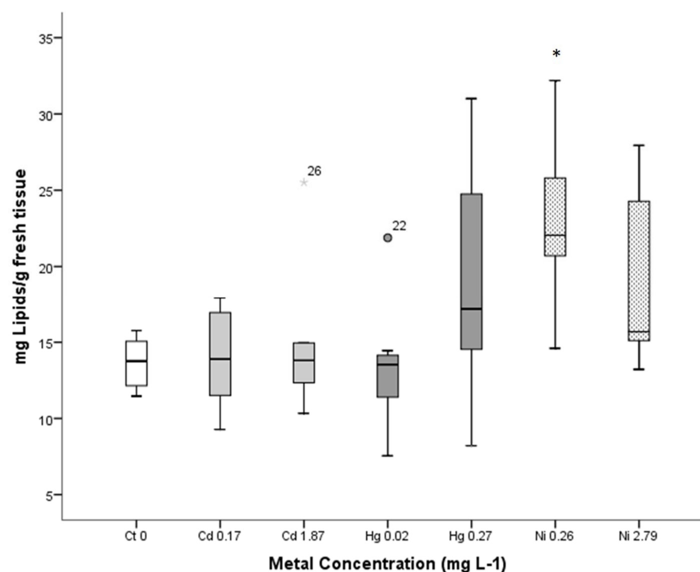

Figure 1 – Total lipid content in *Gibbula umbilicalis*, expressed in terms of mg lipids per g of fresh tissue exposed to cadmium (Cd), mercury (Hg), and nickel (Ni) for 168-h. An asterisk denotes significant differences comparing to control (One-way ANOVA, Dunnett's $p<0.05$).

3.3. Lipid Peroxidation

Transition metals (like Cd, Hg, and Ni) are known to stimulate the peroxidation of membrane lipids through oxidative stress (Knight *et al.*, 1990). This process results in the production of lipid radicals and the formation of lipid degradation products, which are extremely toxic for the cells, due to their high affinity for thiol and amino groups of peptides, enzymes, and nucleic acids (Viarengo, 1989). However, neither of the tested metal concentrations during the 168h of exposure significantly induced LPO in *G. umbilicalis* (Fig. 2).

A number of studies have evidenced that lipid peroxidation increases with increasing metal exposure, including Cd (e.g. MacFarlane *et al.*, 2006; de Almeida *et al.*, 2004). According to a study carried out with clam (*Ruditapes decussatus*), Cd can replace calcium homeostasis and indirectly stimulate the production of ROS as nitric oxide (non-Fenton reaction), a radical known to induce lipid peroxidation of cell membranes (Geret *et al.*, 2002). Some authors have demonstrated that Cd exposure can also induce oxidative stress in molluscs resulting in higher LPO levels (Geret *et al.*, 2002; Goswami *et al.*, 2014; Company *et al.*, 2006). In the present study with *G. umbilicalis*, although no significant, the tendency for an increase in LPO with Cd may reflect an early sign of further lipid peroxidation in the snails continuously exposed to Cd, although at this stage this can only be speculative.

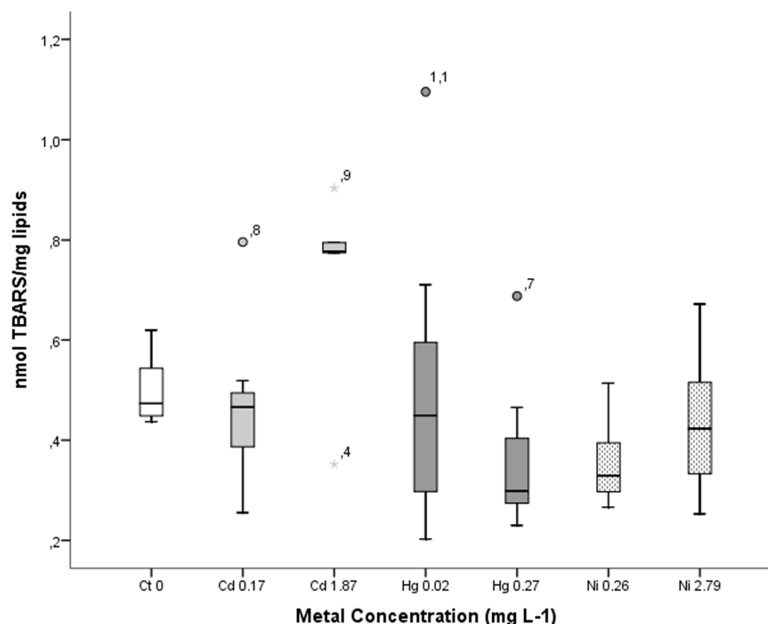

Figure 2 – Lipid peroxidation levels in *Gibbula umbilicalis*, expressed in terms of nmol TBARS/ mg lipids, when exposed to cadmium (Cd), mercury (Hg) and nickel (Ni) for 168h.

3.4. Fatty acid profile

Changes in lipid metabolism and fatty acid profile in animals can be protective strategies under environmental stress. Alterations in saturated and essential fatty acids, along with changes in membrane fluidity have been reported as an effect or an adaptive response of exposure to contaminants (particularly metals) in marine species, also under starvation (e.g. Fokina *et al*., 2013; Rocchetta *et al*., 2006). Fatty acids, as wide units of the membrane phospholipids and lipid storage units, are involved in a variety of functions, particularly the formation and dynamic properties of biological membranes and as fuel for energy production (Vance and Vance, 2008).

In general, the tissues of *G. umbilicalis* presented a FA profile ranging from lauric acid (12:0) to nervonic acid (24:1 n9), in a total of 40 different FAs (Table S1, supplementary material). To have a first and integrative overview on the effects of the tested metals in FA profile of *G. umbilicalis*, a PCA was performed (Figure 3).

Figure 3 – Biplot from principal component analysis integrating all the fatty acids measured in the different metal treatments. Ct = control (0 mg L^{-1}); Hg1 = 0.02 mg of mercury L^{-1}; Hg2 = 0.27 mg of mercury L^{-1}; Ni1 = 0.26 mg L^{-1}; Ni2 = 2.79 mg L^{-1}; Cd1 = 0.17 mg L^{-1}; Cd2 = 1.87 mg L^{-1}. Colour/shape code: Yellow triangles = cadmium treatments; red diamonds = mercury treatments; purple squares = nickel treatments.

The PCA showed that 70.7% of total variance was explained by the first two principal components (PC) (Fig. 3). The first axis, which explained up to 44.6% of data variance separate control from metal treated samples having the highest concentration of Hg the highest scores. Most FA had positive scores for axis PC1. The second PC2 only explained 26.1% of data variance and separated most treatments of Ni, Cd and Hg samples. These results show that metal treatments (and especially Hg and Ni) had a demarked different FA composition in comparison to control. It can also be seen that the mechanisms of toxic action of the higher Hg concentration result in induction of different FA comparing to Cd, Ni and the lowest Hg concentration. Although not fully described, Hg is reported in the literature to induce several metabolic perturbations at the cellular level (Filimonova *et al.*, 2016), some possibly related to lipids and FA. Furthermore, comparatively to control, Cd

was the metal inducing less differences in the FA pattern. This can also be observed by the individual analysis of significant changes in FAs in comparison to control presented in Table 1, where major and essential FA contents are pointed out. In comparison to Cd and Ni, Hg exposed organisms presented the most significant differences, indicating that the mechanisms of action of Hg had a stronger effect in FA profile, already observed from the PCA analysis (Fig. 3).

Table I – Fatty acid profile in *Gibbula umbilicalis* when exposed to cadmium, mercury and nickel for 168h, plus control. Data are expressed as percentage of major fatty acids (mean ± standard deviation); * Significant differences from the control (Dunnett's or Dunn's, p<0.05), highlighted in bold. SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

		Cadmium (mg L^{-1})		Mercury (mg L^{-1})		Nickel (mg L^{-1})	
Fatty Acid	**Control**	**0.17**	**1.87**	**0.02**	**0.27**	**0.26**	**2.79**
14:00	3.33±1.92	2.21±0.77	2.08±1.06	2.71±0.87	2.73±1.08	3.01±1.20	3.16±1.75
15:00	0.23±0.08	0.29±0.07	0.26±0.07	**0.37±0.09***	**0.40±0.10***	**0.38±0.11***	0.32±0.06
16:00	28.07±2.53	**36.52±3.45***	**33.44±3.72***	**35.14±3.43***	29.92±5.53	**33.93±5.58***	**33.77±3.31***
18:00	28.52±5.65	27.40±1.99	27.09±5.32	28.32±4.32	21.91±9.82	28.61±6.06	26.18±4.04
∑SFA	64.40±7.61	60.39±1.18	65.08±7.22	68.68±3.81	56.61±14.61	68.97±5.41	66.63±3.96
16:1*n*-7	0.79±0.67	0.73±0.72	1.20±1.05	1.44±1.25	2.69±1.88	1.54±0.59	1.64±1.37
18:1*n*-7	0.79±0.74	1.33±0.50	1.27±0.28	1.41±1.01	1.67±1.16	1.46±1.31	1.61±1.08
18:1*n*-9	12.92±8.81	7.68±2.37	7.96±2.86	6.99±0.86	13.70±9.67	6.43±1.44	7.65±2.98
∑MUFA	15.50±8.50	10.78±2.80	11.67±3.28	11.28±2.92	19.75±11.39	10.82±3.93	12.33±3.15
16:2*n*-4	0.05±0.05	0.09±0.04	0.10±0.13	0.12±0.14	**0.31±0.24***	0.18±0.18	0.22±0.22
18:2*n*-6	6.05±4,06	4.93±1.79	6.66±5.40	**3.28±0.35***	3.94±0.88	3.42±1.11	3.88±1.69
18:3*n*-6	0.08±0.12	0.12±0.17	0.15±0.14	0.09±0.07	0.37±0.33	0.20±0.23	0.23±0.18
18:3*n*-3	1.02±1.41	0.70±0.47	0.96±0.45	0.64±0.30	0.83±0.50	0.56±0.40	0.76±0.36
18:4*n*-3	0.04±0.05	0.03±0.01	0.08±0.07	0.12±0.09	0.46±0.42	**0.13±0.13***	0.10±0.13
20:3*n*-3	0.45±0.62	**1.65±0.39***	**1.64±0.27***	**1.50±0.61***	1.25±0.95	1.51±1.15	**1.81±0.70***
20:4*n*-6	0.00±0.00	0.02±0.01	0.03±0.03	**0.04±0.04***	**0.10±0.06***	**0.05±0.04***	**0.08±0.05***
20:5*n*-3	1.04±1.17	**2.43±0.89***	1.72±0.53	1.33±0.53	**3.88±2.89***	1.44±0.94	1.66±0.84
22:6*n*-3	0.20±0.15	0.43±0.30	0.86±0.77	0.24±0.09	1.66±2.04	0.25±0.23	**0.58±0.62***
∑PUFA	13.03±4.75	15.33±1.99	15.58±5.53	12.33±2.02	17.30±5.61	13.54±2.39	14.89±1.90
Unsat./Sat	0.46±0.20	0.38±0.07	0.45±0.18	0.35±0,09	0.76±0.52	0.36±0.11	0.41±0.10
n3	3.76±2.65	7.13±1.44	6.58±1.11	5.14±1.96	**9.47±4.88***	5.28±3.22	6.52±2.68
n6	8.24±4.25	7.03±2.10	8.97±5.08	5.74±1.56	6.13±0.56	5.94±2.64	6.88±2.39
n7	2.03±1.55	2.59±1.22	3.03±1.65	3.59±2.23	4.99±2.92	3.77±2.92	1.34±0.58
n9	13.23±8.80	7.95±2.40	8.23±2.86	7.30±0.85	14.27±10.15	6.73±1.48	7.98±2.98
n3/n6	0.57±0.54	1.12±0.46	0.88±0.35	0.97±0.38	**1.52±0.75***	1.57±0.93	1.13±0.67

Regarding the FA composition in the control group, SFA were the major group present, accounting for an average of 64% of total fatty acids. Palmitic (PA; 16:0) and stearic acids

(18:0) were the most abundant (Table 1). Unsaturated fatty acids represented 28.5% of total FA with monounsaturated and polyunsaturated forms contributing almost equally (16 and 13% respectively) to the total unsaturated FA content. The major unsaturated FAs were linoleic (LA; 18:2n-6) and oleic acid (OA; 18:1n-9), with 6% and 13% respectively.

In general and in comparison to control, there was a tendency for a decrease in MUFA and increase in PUFA contents with metal exposure, although the differences between the sums of these groups of FA were not significant. Additionally, there was also a trend for increasing MUFA and PUFA contents with increasing metal concentrations, especially with Hg. The *n3*-PUFA, along with the SFA appeared to be the most affected FA classes by metal contamination in this study. Furthermore, there was also a clear trend for an increasing PUFA-*n3/n6* ratio in the metal contaminated groups. Recent studies have demonstrated that marine invertebrates, including gastropods and bivalves, possess an endogenous ability for the biosynthesis of PUFA and Long-Chain (LC)-PUFA (Surm *et al*., 2015; Monroig *et al*., 2013). This PUFA increasing trend in *G. umbilicalis*, especially related to *n3*, is indicative that the snails might be synthesizing these FAs as a response to the stressors. Supporting this, is also the fact that arachidonic acid (ARA; 20:4n-6) was only detected in the contaminated groups for the three metals in the different concentrations tested. To our knowledge, this is the first report on this possible biosynthetic ability of *G. umbilicalis*. In addition, the increase in PUFA content can probably be due to homeoviscous adaptation and activity of the membrane-bound enzymes and pumps as they act to promote membrane permeability (Filimonova *et al*., 2016; Vance and Vance, 2008). The increase in PUFA content can be considered a defense mechanism, which aims to protect the membranes from oxidation disruption caused by adverse environmental impacts, which was previously reported in different studies (Fokina *et al*., 2013; Maazouzi *et al*., 2008).

Significant differences were observed for some particular FAs (15:0, 16:0, 16:2 *n4*, 18:2 *n6*, 20:3 *n3*, 20:4 *n6*, 20:5 *n3*, and 22:6 *n3*) in punctual remarks. However, as stated before, a common trend was observed for several FAs (mainly for LC-PUFA) with the different metal exposures. Essential and metabolically important FAs (regularly described in literature for membrane organization and immune responses) were selected for a more in depth discussion and their levels in control and contaminated groups are shown in Figure 4. Most

of the selected FAs show a tendency to increase in the presence of metal contamination, with the exception of LA (discussed below; Fig. 4). Additionally, a signature dose-response can be recognized for 5 FAs (POA, ETE, ARA, EPA and DHA), which was evident among the different tested metals and concentrations. The increase in concentration resulted, in most cases, in an increment of these FAs.

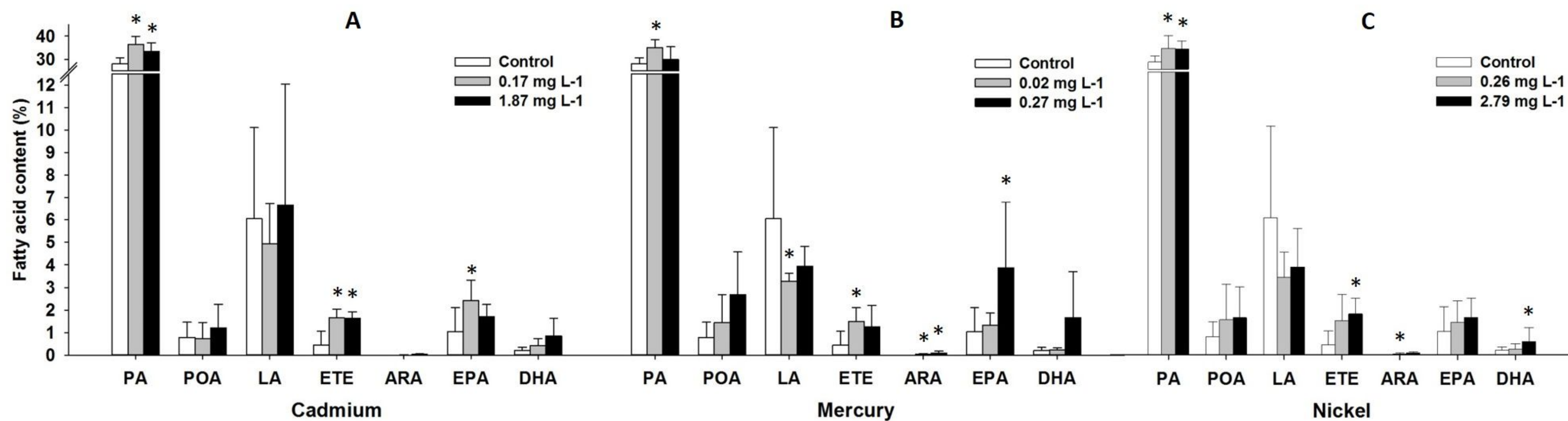

Figure 4 - Selected fatty acid contents (%) of *Gibbula umbilicalis* exposed to different concentrations of cadmium (**A**), mercury (**B**) and nickel (**C**) during 168h. Significant results in comparison to control are indicated with an asterisk (*) (Dunnett's or Dunn´s, $p<0.05$). Results express mean ± standard deviation. PA = Palmitic acid (16:0); POA = Palmitoleic acid (16:1n-7); LA = Linoleic acid (18:2 n6); ETE = Eicosatrienoic acid (20:3 n3); ARA = Arachidonic acid (20:4 n6); EPA = Eicosapentaenoic acid (20:5 n3); DHA = Docosahexaenoic acid (22:6 n3).

Palmitic acid (16:0, PA) is the most common SFA found in marine animals (Martino *et al*., 2004), which is also in accordance with the results obtained for *G. umbilicalis* in this experiment. Levels of this FA were significantly increased in snails exposed to the different metals (Fig. 4). PA is the first fatty acid produced during fatty acid biosynthesis and the precursor of both saturated and unsaturated fatty acids (Mahanty *et al*., 2015). Moreover, PA is involved in palmitoylation, which consists on the post-translational attachment mainly between PA and the side-chain of cysteine, being a common process among integral and peripheral membrane proteins. Palmitoylation plays important roles in regulatory protein activity. The association of the protein with the palmitoyl moiety is reversible and facilitates protein–membrane interactions and subcellular trafficking of proteins (Legrand and Rioux, 2010; Blaskovic *et al*., 2013), which might be linked to membrane fluidity regulation, but also to toxicity responses since this process is pointed as a cell regulatory process that can be activated in response to extracellular stimuli (Escribá *et al*., 2007). Although further studies are required, given the increment of PA in the exposed organisms, it can be hypothesized that either this precursor is being requested for the synthesis of other FAs or this palmitoylation process is increasing in response to the metal stressors.

Regarding linoleic acid (18:2n-6, LA), the trend of response was different from the other FAs presented in figure 4. In fact, this fatty acid is described as the main precursor of arachidonic acid (20:4n-6, ARA). Most probably, LA was tangled in a biosynthetic pathway of conversion to longer-chain fatty acids, like ARA, possibly justifying its decrease with the metal exposure. Furthermore, despite being a common FA in marine organisms (Isay *et al*., 1984), ARA was not detected in *G. umbilicalis* control treatments. However, although in low amounts, this FA was identified in all metal contamination treatments. ARA is known to confer fluidity to cell membranes, as it has 4 points of unsaturation (Fukaya *et al*., 2007). It is also required in cell signalling and specifically as a substrate for synthesis of eicosanoids, which are critical in a very wide range of physiological processes in invertebrates, like spawning and hatching, egg production, mediating immunological responses to infections, and regulating neurophysiology, among other processes (Hurtado *et al*., 2009; Stanley-Samuelson *et al*., 1994). The ARA content in the snail under metal

exposure is presumably indicative that this FA is required for activation of eicosanoid synthesis through “arachidonic cascade” pathway.

Eicosanoids are also involved in the regulation of many cell and tissue responses, including inflammation and immunity responses. The main eicosanoids inflammatory mediators, prostaglandins and leukotrienes, are both generated from ARA via the cyclooxygenase, 5-lipoxygenase and cytochrome P450 pathways (Hsu *et al*., 2013). Inflammatory responses usually occur immediately after the harmful stimuli as a natural protective reaction of the cells and tissues, and this is when the concentration of ARA is expected to increase more expressively (Calder, 2015; Calder, 2010). This reflects the role of ARA as a precursor of the hormone-like eicosanoids and is thus involved in cell signalling associated with the tissue inflammation, immune response and adaptation to environmental condition. Analogous to mammals, molluscan hemolymph contains hemocytes, where ARA was demonstrated to play either a direct stimulatory role, or an indirect effect through ARA derived eicosanoid metabolits (Delaporte *et al*., 2006). Hemocytes are phagocytic and appear to attack invading pathogens via release of ROS. Furthermore, hemocytes participate in the transport of metals to the organs responsible for the accumulation and detoxification of the xenobiotics (Marigómez *et al.*, 2002; Pirie *et al.*, 1984, Rebelo et al, 2013). Therefore, the presence of ARA in the snails’ body under metal exposure may reflect detoxification, excretion, and immune system activation. The results here presented are in accordance with a study of Ramirez and Gimenez (2002), where the authors demonstrated a relation between Cd exposure and modifications in the immune-inflammatory function through changes in cellular lipid metabolism and fatty acid composition due to redox balance impairments. Given the importance of ARA and the fact that it can be an early indicator of immune response, the levels of ARA detected in this 168h exposure might indicate that this FA and specific associated pathways, as referred above, could be monitored during and after the exposure in related future targeted toxicological studies.

Eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic (22:6n-3, DHA) acids have been reported in literature with a normal tendency to decrease with increasing contamination levels, often associated to peroxidation or decreasing membrane permeability (Filimonova *et al.*, 2016; Signa *et al.*, 2015). Here, however, they were shown to increase in metal

contaminated groups (Fig. 4). These particular FAs contributed to the observed increase of *n3* content in the metal treatments. EPA and DHA have also been proven and reported in literature to be potent anti-inflammatory agents (e.g. Calder, 2015), and also to modulate the activity of membrane-associated proteins such as ion channels and ion transporters, as well as other physical properties of biological membranes, including membrane organization, ion permeability, elasticity and microdomain formation (Bruno *et al*., 2007). Since no significant lipid peroxidation was observed in this study for the tested metals' concentrations, the increment in these metabolically important n3 fatty acids might confirm the occurrence of these physiological or compensatory adjustments in lipid metabolism caused by contaminant exposure (Fokina *et al*., 2013). It can thus be hypothesized that EPA and DHA were being selectively retained or biosynthesized in *G. umbilicalis* tissues (as previously discussed) in a common response to the metals tested here, either for homeoviscous adaptation or as anti-inflammatory agents.

The *cis*-11,14,17-eicosatrienoic acid (20:3*n*-3, ETE) is usually detected in the phospholipids of animal tissues, but rarely represents more than 1% of total FAs (Das, 2011). This was also evident in this study for the control treatment in *G. umbilicalis* where total content for this FA was no more than 0.45% ±v0.62 (Table 1). However, in metal exposed organisms, this FA was found in average concentrations above 1%, where the differences were significant for at least one concentration of each metal (Fig. 4). ETE is one of the most active essential FA involved in the regulation of FA elongation/desaturation reactions that convert dietary C-18 fatty acids to C-20 eicosanoid precursors (Holman, 1986).

The present study demonstrates that metal exposure influences the FA profile, with mechanisms that may act as potent inducers of fatty acid modulation in *G. umbilicalis*. Although the responses at the FA profile level were more evident for Hg, Ni and Cd induced similar response trends in the snails, suggesting therefore a common effect to metal contamination. In particular, PA, ETE, ARA, EPA, and DHA seem to be the best indicators of *G. umbilicalis* responses to the array of metals used (Cd, Hg, and Ni), which can potentially be used as biomarkers for metal contamination in coastal environments.

Although the present research used high exposure concentrations aiming to develop and validate a prospect FA tool, one should note that the lowest concentrations tested, with

non-negligible effects for several endpoints, were 0.17 mg Cd L^{-1}, 0.02 mg Hg L^{-1}, and 0,26 mg Ni L^{-1}. Despite the apparent high concentrations, they are in same order of magnitude and sometimes lower than those reported in studies for Cd and Ni environmental presence, where Cd concentrations of 50 and up to 450 µg L^{-1} have been found in some heavily polluted estuaries harbors and ports (Chester, 1990; Vásquez-Sauceda *et al.*, 2012), while values up to 2 mg of nickel L^{-1} were found in water near industrial sites (Eisler, 1998). Additionally, here, 7d exposures were performed with the detailed impacts demonstrated, while in real scenarios these exposures may last longer and with other chemical/biotic/abiotic interactions that can enhance these effects and should be further addressed concerning this tool deployment.

4. Conclusions

The commonly used lipid related endpoints in contamination effect assessment, like LPO and total lipid content, did not show clear responses to the array of metals in the conditions tested. However, under same conditions, the wider functional approach tool addressing the fatty acid profile, showed a suitable sensitivity in assessing adverse effects induced by contaminant/pollutant exposure in molluscs, constituting a promising tool to understand the mechanisms of toxic action in coastal environments, using the widespread snail *G. umbilicalis*. In particular, five fatty acids (palmitic, eicosatrienoic, arachidonic, eicosapentaenoic, and docosahexaenoic acids), with an high functional link to membrane organization (homeoviscous adaptation) and immune responses, demonstrated to be especially good indicators of *G. umbilicalis* responses to the array of metals used.

The bonding between toxicology with lipidomics can likely lead to the emergence of sub-disciplines such as “lipidomic toxicology”, where the development of excellent analytical methods dealing with complex mixtures, coupled with sub-cellular fractioning, will be a major contributing factor to the progress in marine lipids chemistry to be used as prospect tools in ERA of coastal environments.

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Supplementary data

Table S1 - Fatty acid composition found in *Gibbula umbilicalis* exposed to cadmium, mercury and nickel for 168h, plus control. Data are expressed as percentage (mean ± standard deviation). SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

			Cd (mg L^{-1})		Hg (mg L^{-1})		Ni (mg L^{-1})	
FA		CT	0.17	1.87	0.02	0.27	0.26	2.79
SFA	C 12:0	4.00±4.04	1.77±2.59	2.59±2.55	1.87±2.86	1.37±2.89	2.79±3.59	2.90±3.25
	C 13:0	0.03±0.02	0.01±0.00	0.00±0.00	0.04±0.00	0.03±0.00	0.00±0.00	0.00±0.00
	C 14:0	3.33±1.92	2.21±0.78	2.08±1.06	2.71±0.87	2.73±1.08	3.01±1.20	3.16±1.75
	C 15:0	0.23±0.08	0.29±0.07	0.26±0.03	0.37±0.09	0.40±0.01	0.38±0.11	0.32±0.06
	C 16:0	28.07±2.53	36.52±3.45	33.44±3.72	35.14±3.43	29.92±5.53	33.93±5.58	33.77±3.31
	C 17:0	0.20±0.07	0.22±0.07	0.24±0.07	0.26±0.08	0.25±0.12	0.25±0.09	0.22±0.07
	C 18:0	28.52±5.65	27.40±1.99	27.09±5.32	28.32±4.32	21.91±9.82	28.61±6.06	26.18±4.04
	C 20:0	0.02±0.01	0.03±0.03	0.01±0.00	0.02±0.00	0.02±0.00	0.03±0.00	0.02±0.00
	C 22:0	0.04±0.06	0.03±0.03	0.01±0.00	0.05±0.01	0.01±0.00	0.04±0.00	0.18±0.00
	C 24:0	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.02±0.00	0.01±0.01	0.01±0.00
∑SFA		64.40±7.57	68.39±1.18	65.08±7.22	68.68±3.81	56.61±14.61	68.97±5.41	66.63±3.97
MUFA	C 15:1	0.08±0.04	0.11±0.03	0.11±0.01	0.13±0.05	0.13±0.08	0.13±0.06	0.13±0.05
	C 14:1	0.10±0.05	0.09±0.02	0.09±0.00	0.14±0.02	0.14±0.07	0.12±0.03	0.12±0.07
	C 16:1 n5	0.38±0.31	0.52±0.13	0.49±0.05	0.57±0.25	0.47±0.31	0.54±0.24	0.54±0.18
	C 16:1 n7	0.79±0.67	0.73±0.72	1.20±0.43	1.44±1.25	2.69±1.88	1.54±1.57	1.64±1.37
	C 16:1 n9	0.10±0.02	0.12±0.02	0.09±0.01	0.11±0.01	0.11±0.03	0.11±0.04	0.13±0.04
	C 17:1	0.07±0.04	0.07±0.02	0.06±00.01	0.06±0.04	0.19±0.12	0.07±0.03	0.10±0.01
	C 18:1 n7	0.79±0.74	1.13±0.50	1.27±0.28	1.41±1.01	1.67±1.16	1.46±1.31	1.61±1.08
	C 18:1 n9	12.92±8.81	7.68±2.37	7.96±1.17	6.99±0.86	13.70±9.67	6.43±1.44	7.65±2.98
	C 20:1 n9	0.12±0.09	0.06±0.02	0.09±0.05	0.09±0.04	0.31±0.36	0.07±0.03	0.10±0.04
	C 22:1 n9	0.06±0.05	0.10±0.04	0.10±0.02	0.08±0.08	0.12±0.11	0.11±0.05	0.12±0.04
	C 24:1 n9	0.03±0.03	0.03±0.01	0.02±0.01	0.02±0.01	0.04±0.04	0.02±0.02	0.02±0.00
∑MUFA		15.50±8.50	10.78±2.80	11.67±3.28	11.28±2.92	19.75±11.39	10.82±3.93	12.33±3.14
PUFA	C 16:2 n4	0.05±0.05	0.07±0.09	0.10±0.05	0.12±0.14	0.31±0.24	0.18±0.07	0.22±0.22
	C 16:2 n7	0.45±0.31	0.74±0.06	0.67±0.05	0.75±0.14	0.63±0.18	0.76±0.16	0.66±0.09
	C 18:2 n6	6.05±4.06	4.93±1.79	6.66±2.20	3.28±0.35	3.94±0.88	3.42±1.12	3.88±1.69
	C 16:3 n4	0.01±0.00	0.00±0.00	0.00±0.00	0.03±0.01	0.03±0.01	0.02±0.01	0.00±0.00
	C 18:3 n6	0.08±0.12	0.12±0.17	0.15±0.06	0.09±0.07	0.37±0.34	0.20±0.23	0.23±0.18
	C 18:3 n3	1.02±1.41	0.70±0.47	0.96±0.18	0.64±0.30	0.83±0.50	0.56±0.40	0.76±0.36
	C 18:4 n1	0.28±0.09	0.27±0.15	0.43±0.10	0.46±0.17	0.37±0.25	0.53±0.26	0.57±0.24
	C 18:4 n3	0.04±0.05	0.03±0.02	0.08±0.03	0.12±0.09	0.46±0.43	0.13±0.13	0.10±0.13
	C 20:2 n6	1.45±1.55	1.19±0.91	1.38±0.40	1.57±1.57	1.09±0.57	1.95±1.96	1.95±2.27
	C 20:3 n3	0.45±0.65	1.65±0.39	1.64±0.11	1.50±0.61	1.25±0.95	1.51±1.15	1.81±0.70
	C 20:3 n6	0.11±0.09	0.13±0.05	0.14±0.03	0.17±0.09	0.18±0.16	0.14±0.10	0.16±0.11
	C 20:4 n3	0.04±0.07	0.05±0.02	0.06±0.02	0.09±0.08	0.34±0.10	0.14±0.10	0.15±0.09

	C 20:4 n6	0.00±0.00	0.02±0.00	0.03±0.01	0.04±0.04	0.10±0.06	0.05±0.02	0.08±0.06
	C 20:5 n3	1.04±1.17	2.43±0.89	1.72±0.22	1.33±0.53	3.88±2.90	1.44±0.36	1.66±0.84
	C 21:5 n3	0.09±0.12	0.06±0.03	0.03±0.01	0.04±0.01	0.13±0.10	0.05±0.01	0.04±0.04
	C 22:2 n6	0.13±0.06	0.13±0.06	0.16±0.02	0.14±0.11	0.11±0.03	0.13±0.02	0.14±0.09
	C 22:3 n6	0.18±0.18	0.30±0.09	0.28±0.02	0.28±0.10	0.24±0.14	0.32±0.06	0.36±0.17
	C 22:5 n3	0.95±1.03	1.89±0658	1.89±0.65	1.33±0.52	1.51±0.74	1.49±0.34	1.61±0.70
	C 22:6 n3	0.20±0.15	0.43±0.31	0.86±0.31	0.24±0.09	1.66±2.04	0.25±0.09	0.58±0.62
∑PUFA		13.03±1.79	15.33±1.99	16.68±5.53	12.33±2.02	17.30±5.61	13.54±2.39	14.89±0.78
Unsat./Sat		0.46±0.08	0.38±0.07	0.45±0.18	0.35±0.09	0.76±0.52	0.36±0.11	0.41±0.09
n3		3.76±1.00	7.13±1.44	6.58±1.11	5.14±1.96	9.47±4.88	5.28±3.23	6.52±2.68
n6		8.24±1.61	7.03±2.10	8.97±5.09	5.74±1.53	6.13±0.56	5.94±2.64	6.88±2.39
n7		2.03±0.59	2.59±1.22	3.03±1.65	3.59±2.23	4.99±2.92	3.77±2.92	1.34±0.57
n9		13.23±3.33	7.95±2.40	8.23±2.86	7.30±0.85	14.27±10.15	6.73±1.48	7.98±2.98
n3/n6		0.57±0.20	1.12±0.46	0.88±0.36	0.97±0.39	1.52±0.75	1.57±2.45	1.13±0.67
n		7	7	6	7	6	7	6

Chapter III

Linking cholinesterase inhibition with behavioural alterations in the sea snail *Gibbula umbilicalis*: effects of the organophosphate pesticide chlorpyrifos

Chapter III – Linking cholinesterase inhibition with behavioural alterations in the sea snail *Gibbula umbilicalis*: effects of the organophosphate pesticide chlorpyrifos [1]

Abstract

Inhibition of acetylcholinesterase (AChE) activity has been widely used to assess the exposure and effects of anticholinergic environmental contaminants in several species. The aim of this study was to investigate if sublethal concentrations of the organophosphorous pesticide chlorpyrifos (CPF), a well-known AChE inhibitor, would also affect cholinesterases (ChE) in *Gibbula umbilicalis* and if this inhibition would result in an alteration of their behaviour, in an attempt to link the effects observed at the cellular level with effects at higher levels of ecological relevance. The biochemical properties of ChEs in this species were first characterized through the assessment of different enzymatic forms present in the sea snail, using different substrates and selective inhibitors. The results suggest that *G. umbilicalis* possess ChEs with characteristics of typical AChE, which should be the main form present. Additionally, *in vitro* and *in vivo* effects of CPF on AChE activity were investigated, along with effects on snails' behaviour: the ability of the snails to move/turn after exposure to the ontaminant (flipping test). As expected, CPF inhibited AChE activity both *in vitro* and *in vivo* conditions. Moreover, the link between AChE activity inhibition and adverse effects on behavioural changes was established: AChE inhibition was positively correlated with the flipping test, indicating a mechanistic relationship between the two endpoints determined in *in vivo* exposures.

This study highlights the importance of linking biochemical endpoints such as AChE activity with higher level endpoints like behavioural alterations, increasing the ecological relevance of the effects observed.

Keywords: Acetylcholinesterase activity, Behaviour, Cholinesterases characterization, Ecotoxicology, Marine Snails

[1] Silva, C. O., Novais, S. C., Alves, L. M., Soares, A. M., Barata, C., & Lemos, M. F. Published in Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, doi.org/10.1016/j.cbpc.2019.108570

1. Introduction

Human activities introduce a myriad of contaminants in the environment on a daily basis, and pesticides, being widely used in agriculture, are some of the most frequently found (Özkara et al., 2016). Although they are usually applied on land, pesticides frequently end up affecting larger areas, with residual concentrations being found in coastal and estuarine environments due to natural processes like runoff, often affecting non-target organisms (Damalas and Eleftherohorinos 2011; Readman *et al*., 1992).

Organophosphorus (OPs) compounds are still among the most used pesticides in developing countries, being generally accepted as the most effective means for protecting crops against insects (Eto 1974). These compounds are extremely toxic, being able to easily permeate cells and severely modify the neurological responses of organisms (Cao *et al*., 1999; Saunders *et al*., 2012). Chlorpyrifos [CPF; O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate], the active ingredient in the commercial formulation Dursban®, is still one of the most sold OP in the world having many urban and agricultural crop pest control uses (Lemus and Abdelghani, 2000).

Chlorpyrifos acts by inhibiting cholinesterases (ChE) activities and in particular acetylcholinesterase (AChE), an enzyme involved in the termination of nervous impulse transmission by catalyzing the hydrolysis of the neurotransmitter acetylcholine, and therefore playing an essential role in the nervous system coordination (Fournier *et al*., 1992). The inhibition of this enzyme results in a continuous binding of acetylcholine to the receptor, leading to an overstimulation of this cholinergic receptors, which may result in alterations in behaviour, potentially reducing the organisms' ability to move, eat, and reproduce (Azevedo-Pereira 2011). The assessment of this enzyme activity thus confers a high level of ecological relevance potential due to its link with higher levels of biological organization (Dhadialla *et al*., 1998; Khalil *et al*. 2013; Lemos *et al*., 2010; Sandahl *et al*.,

2005; Yen *et al*.,2011). *Gibbula umbilicalis* (Costa, 1778) is a small marine gastropod with an extensive geographical distribution, inhabiting the upper intertidal zone on rocky shores. This species is easy to identify, collect, maintain in laboratory and possess a convenient size, making them suitable organisms for ecotoxicological assays (Cabecinhas *et al*., 2015).

The main aim of this study was to assess if the inhibition of ChE in *G. umbilicalis,* by sublethal concentrations of CPF, would affect its behaviour, in an attempt to link the effects observed at the cellular level with effects at higher levels of ecological relevance. As a first part of this work, a characterization of the ChE present in *G. umbilicalis* was performed, as there was no data available on this matter and some studies point to the existence of differences among species of molluscs (Talesa *et al*., 1993; Mora *et al*., 1999). Cholinesterases are a family of enzymes, traditionally divided in two classes (in vertebrates) based on their properties and functions: acetylcholinesterases (AChEs), generically designated as true cholinesterases, are involved in the regulation of neurotransmission and neuromuscular functioning; and pseudocholinesterases such as butyrylcholinesterases (BChEs) and propionylcholinesterases (PChEs). These ChE forms may be distinguished functionally both kinetically and pharmacologically: AChE has a high affinity for acetylthiocholine (ATCh), being very sensitive to eserine and selectively inhibited by BW284C51 while relatively insensitive to iso-OMPA. Pseudocholinesterases have higher affinity for propionylthiocholine (PTCh) or butyrylthiocholine (BTCh), being sensitive to eserine and selectively inhibited by iso-OMPA, while relatively insensitive to BW284C51 (Eto 1974). The characterization of ChE is important because different ChE may respond differently to anticholinesterase agents such that the measurement of this enzyme activity in a given species using one substrate instead of the other can lead to misinterpretation of results in ecotoxicological studies (Alves 2015).

After ChE characterization, inhibition of ChE activity by the CPF metabolite (chlorpyrifos-oxon – CPO) was validated for this species in an *in vitro* setup. The last part of the work included *in vivo* exposures, where effects of CPF were addressed both on ChE activities and behaviour alterations.

2. Materials and methods

2.1. Test organisms

Gibbula umbilicalis (Costa, 1778), of similar size (10 ± 1mm) were hand-collected from Carreiro de Joannes, a rocky beach in Peniche, central Portugal, with unknown sources of chemical contamination. The organisms were acclimated in the laboratory, for 7 days prior to each experiment, in aquaria with natural seawater at 20 ±1 °C, with a 16h: 8 h (light:dark) photoperiod. During this period, they were fed *ad libitum* with the green macroalgae *Ulva lactuca* (Linnaeus, 1753). Prior to testing, organisms were kept fasting for 24 h.

2.2. Characterization of cholinesterases activity

To characterize *G. umbilicalis* ChE, a total of 6 replicates were used, each containing a pool of 3 organisms. The snails were sacrificed on ice and then homogenized in 15 mL potassium phosphate buffer (0.1 M, pH 7.2). The tissue homogenate of each sample was centrifuged for 3 min at 3000g (4 ºC) and total protein concentration in the supernatant quantified according to the Bradford (1976) method, adapted to microplate, using bovine γ-globuline as a standard. Samples were kept at −80 °C until further analysis.

Enzymatic activities were determined according to Ellman *et al*. (1961), using previously diluted samples to a final protein concentration of 0.8 mg L^{-1}, as described in Alves *et al*. (2015). In these assays, 250 µL of the reaction solution [30 ml potassium-phosphate buffer (0.1 M, pH=7.2), 1 mL of reagent 5,5'-dithiobis-(2-nitrobenzoic acid) 10 mM (DTNB) and 200 µL of substrate] were added to 50 µL of the diluted sample. The absorbance was measured at 414 nm (25 ºC) during 5 min. All spectrofotometric measurements were performed in triplicates using a microplate reader Synergy H1 Hybrid Multi-Mode (BioTek® Instruments, Vermont, USA).

2.2.1. Substrate affinity

The substrate preferences were investigated by determining the enzyme activity at 12 increasing concentrations (from 0.01 to 20.48 mM) of the substrates acetylthiocholine iodide (ATCh), propionylthiocholine iodide (PTCh), and S-butyrylthiocholine iodide (BTCh) (Sigma–Aldrich, USA). Cholinesterases activity was determined as described in section 2.2, with 200 µL of each substrate concentration being dissolved in the reaction buffer. For each substrate concentration, blanks were prepared using potassium phosphate buffer (0.1 M, pH 7.2) instead of sample.

2.2.2. Specific inhibitions

Eserine hemisulfate, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284c51) and tetra-(monoisopropyl)pyrophosphortetramide (iso-OMPA) (Sigma–Aldrich, USA) were used as selective inhibitors of total ChEs, AChE, and BChE, respectively. ChE activities were measured as described above, using 200 µL of ATCh 0.075 M as substrate (0.4 mM final assay concentration), and testing 6 increasing concentrations of eserine hemisulphate (from 0.781 to 800 µM), BW284C51 (from 0.781 to 800 µM), and iso-OMPA (from 0.016 to 16 mM), which were dissolved in the reaction buffer. The choice for these final assay concentrations were based on ChEs kinetic knowledge for other species. For each inhibitor concentration, blank reactions were made using the same volume of potassium phosphate buffer instead of the sample. Control reactions without the inhibitors in the reaction buffer were also performed. The percentage of inhibition was calculated in relation to control, assuming ChE activity in control as 100% (0% inhibition).

2.3. In *vitro* effects of chlorpyrifos on cholinesterases activity

The organophosphate metabolite chlorpyrifos-oxon (CPO) was used to test for the *in vitro* inhibition of ChE (Greyhound Chromatography, UK). Stock solutions of CPO were prepared in ethanol to obtain the final tested concentrations ranging from 0.0365 to 2400 nM. The *in vitro* effect of the pesticide metabolite was evaluated using the method described for selective inhibitors (see Section 2.2.2). The percentage of inhibition was calculated in relation to control, assuming ChE activity in control as 100% (0% inhibition). An extra

solvent control was performed using the same solvent concentration as in the maximum tested CPF concentration.

2.4. *In vivo* effects of chlorpyrifos on cholinesterases activity and behaviour

To address *in vivo* effects of CPF on *G. umbilicalis*, the commercial formulation Dursban® was used, having chlorpyrifos as active ingredient (23.5% of a.i.). Firstly, acute effects of this pesticide were assessed to determine lethal concentrations. Based on this information, two sublethal assays were then performed to evaluate: 1) effects on ChE activity; and 2) effects on behaviour (flipping test).

2.4.1. Exposure conditions

For acute assays, sea snails were exposed for 96 h to ten dilutions of the formulation (prepared in filtered seawater), according to the following concentrations of the active ingredient: 0.05, 0.07, 0.10, 0.14, 0.19, 0.26, 0.37, 0.51, 0.72, and 1.00 mg a.i.L^{-1}. Five replicates per treatment were used, including a control treatment with filtered seawater only, and mortality was the endpoint assessed after 96 h.

Based on the acute exposures, half of the LC_{10} was used as the highest concentration for the 96h sublethal bioassays, which were thus performed using the following range of pesticide concentrations: 7.95, 14.7, 27.18, 50.26, 92.95, and 171.9 μg of a.i. L^{-1}. Ten and eight replicates per treatment were used for ChE activity and behavioural parameters, respectively, including a control treatment with filtered seawater only.

Tests were conducted in a climatic room, at 20 ± 1°C, with a 16 h:8 h (light:dark) photoperiod, and experimental replicates consisted of 60 mL glass flasks, with one organism each, covered with a plastic mesh to prevent organism to escape and to assure constant submersion. During exposure, no food was added, and media was renewed every 24 h to prevent from excreta saturation and any potential volatilization.

2.4.2. Cholinesterases activity

At the end of exposure (96 h), organisms were sacrificed, and after shell removal with the aid of a vise, they were weighed and stored at -80°C until ChE activity measurement. ChE was measured as described in section 2.2, using ATCh 0.075 M as substrate.

2.4.3. Snail behaviour - flipping test

After exposing the organisms for 96 h to the different CPF concentrations, a flipping test was performed using the method previously described by Cabecinhas *et al*. (2015). Briefly, the snails were withdrawn from the glass flasks, placed into 6-well plastic microplates with clean filtrated seawater (1 snail per well) and were intentionally left with the foot up. The time that each snail took to flip back to an upright position was recorded and considered the behaviour endpoint.

2.5. Statistical analysis

For the ChEs characterization and to assess enzyme affinity to each substrate, the following kinetic parameters were estimated by fitting experimental data to Michaelis–Menten equation: maximal velocity (V_{max}), Michaelis-Menten constant (Km) and their ratio (Vmax/Km), which indicates the catalytic efficiency of the enzyme.

In vitro and in *vivo* inhibition concentration values (IC_{50}) for CPO and CPF, respectively, were calculated using a nonlinear four parameter logistic curve. Differences between treatments in ChE activity with the specific inhibitors, as well as from the *in vitro*, *in vivo* and behavioural parameter were analysed using one-way analysis of variance (ANOVA). When the criteria of normality and equality were not satisfied, the non-parametric Kruskal-Wallis test was used. Normality and homoscedasticity were checked by Kolmogorov-Smirnov and Levene tests, respectively. To discriminate differences relatively to the control group, either Dunnett's or Dunn's multiple comparisons tests were used as *post hoc* analyses. All statistical analyses were performed on the software Sigmaplot for Windows, version 12 (Systat Software Inc., California, USA).

To estimate lethal concentration values in the acute test (LC_X), as well as median effect concentration (EC_{50}) for the behavioral endpoint, probit and logit regression models were fitted, using SPSS 25.0 for Windows (IBM Corp., 2017).

3. Results and discussion

3.1. Characterization of cholinesterases activity

Enzymatic substrate affinity was tested in the marine snail using three different substrates (Fig. 1A). The substrate with higher hydrolysis rate was ATCh (maximum ChE activity of 161.4 nmol/min/mg protein), followed by PTCh (99.2 nmol/min/mg protein) and BTCh (4.4 nmol/min/mg protein). Looking at the parameters from the Michaelis-Menten equation (Table 1), the higher ratio Vmax/Km obtained for ATCh (meaning greater catalytic efficiencies) further confirmed the preference for this substrate, followed by PTCh and BTCh.

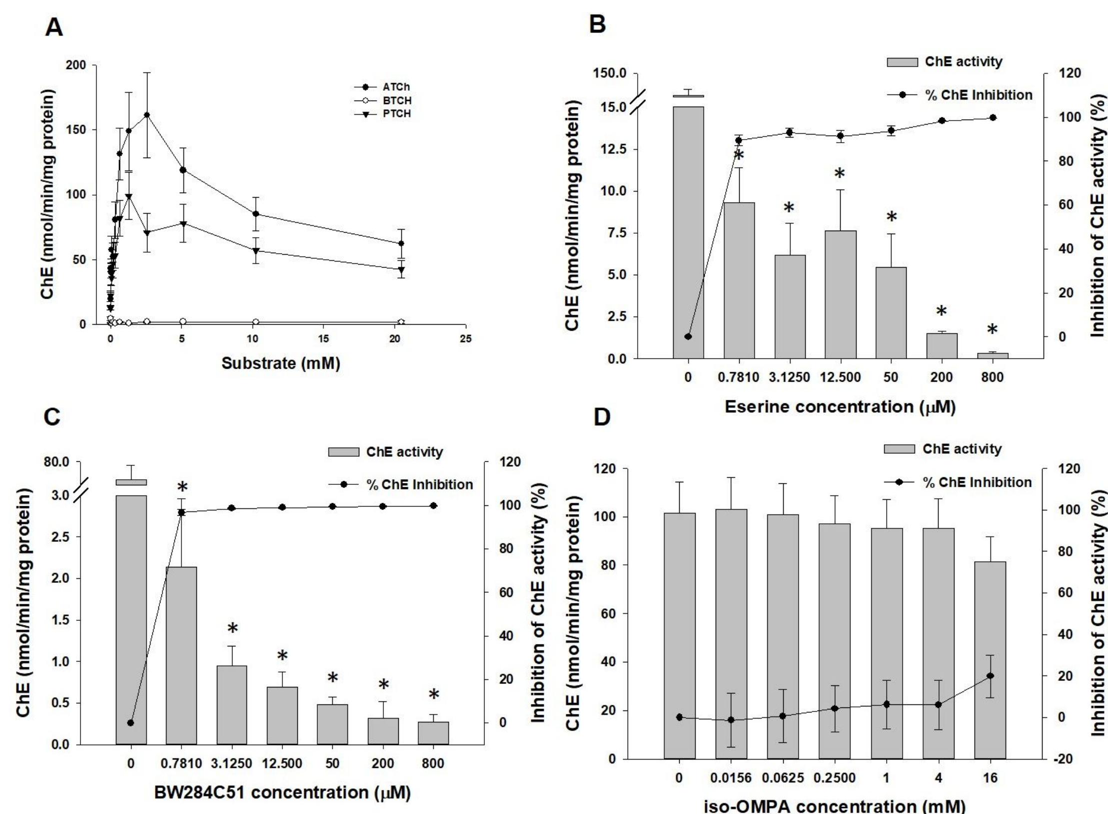

Figure 1 – Characterization of cholinesterases (ChE) activity in *Gibbula umbilicalis* in terms of substrate affinity (A) and effects of the specific inhibitors: B) eserine, C) BW284C51, and D) iso-OMPA. Activity is expressed as mean values ± SD. ATCh = Acetylthiocholine iodide. BTCh = S-

Butyrylthiocholine iodide. PTCh = Propionylthiocholine iodide. Asterisk indicate a significant difference from the control (*p* < 0.05).

Table I - Values of the Michaelis-Menten: constant (K_m), maximal velocity (V_{max}) and the catalytic efficiency of ChE (ratio V_{max}/ K_m) for the three substrates tested. Values of the Michaelis–Menten equation are expressed as the mean ± SE.

Substrate	K_m (mM)	V_{max} (nmol/min/mg protein)	V_{max}/K_m
ATCh	0.19 ± 0.038	168.69 ± 8.19	851.11
PTCh	0.13 ± 0.03	97.75 ± 6.16	739.99
BTCh	1.59e-11 ± 0.00	1.61 ± 0.17	1.01e-11

ATCh = Acetylthiocholine iodide; BTCh = S-Butyrylthiocholine iodide; PTCh = Propionylthiocholine iodide.

Additionally, a decrease on ChE activity with excess of substrate could be observed with ATCh and PTCh for concentrations higher than 2.56 and 1.28 mM, respectively (Fig. 1A). This inhibition by excess of substrate is a characteristic of true AChE (Toutant, 1989) and previously reported in gastropods species such as the sea snails *Monodonta lineata* and *Nucella lapillus* (Cunha *et al.*, 2007), or the freshwater snails *Biomphalaia glabrata* (Kristoff *et al*. 2006) and *Valvata piscinalis* (Gagnaire *et al*. 2008).

The enzymatic inhibition by excess of substrate, along with substrate preference for ATCh (higher hydrolysis rates and greater catalytic efficiencies), suggests a higher presence of AChE in these organisms, which is also supported by the results on the specific inhibitors detailed below (Fig. 1C, D).

The incubation with eserine showed significant and almost complete inhibition of ChE activity along the tested concentrations, with a significant inhibition of 89% observed already at the lowest concentration tested (ANOVA $F_{6,35}$=224.179, Dunnett's p<0.001; Fig. 1B). This is a clear indication that the enzymatic activity measured was mainly due to cholinesterases and not to other non-specific esterases. Likewise, incubation with BW284C51, a specific inhibitor of AChE, also significantly inhibited ChE activity at all tested concentrations (Kruskal-Wallis H_6=37.471, Dunnett's p<0.001), with a complete inhibition of ChE activity (100%) at the highest concentration (800 μM; Fig. 1C). Regarding iso-OMPA, the specific inhibitor of BChE, no significant effects were observed with the concentrations

tested (Kruskal-Wallis H_6=11.229, *p*=0.082; Fig. 1D), although there was a 20% inhibition at the maximum concentration (16mM).

The high sensitivity of these ChEs to BW284C51 and the preference for the substrate ATCh, suggests that *G. umbilicalis* possess ChEs with characteristics of typical AChE, which should be the main form present (Kozlovskaya *et al*., 1993). For this reason, all further results are referred to as AChE activity.

Several studies in invertebrates showed the occurrence of only one or more ChE types with complex kinetic characteristics. Specifically concerning the class Gastropoda, there is a very species-specific variability in the types of ChEs present. For instance, Kristoff *et al*. (2006) determined AChE to be the main ChE present in *Biomphalaria glabrata,* a freshwater snail. On the other hand, the freshwater gastropod *Potamopyrgus antipodarum* possesses two isoforms of ChE, one with mixed properties of AChE and PChE, and another minor isoform corresponding to a BChE (Gagnaire *et al*., 2008). In this same study by Gagnaire *et al*. (2008), the authors also determined that *V. piscinalis* seems to possess only one isoform displaying typical properties of AChE. In the marine gastropods *M. lineata* and *N. lapillus*, the ChE present in the soluble fraction of foot tissue homogenates could not be classified as true AChE or PChE since they showed properties typical of both enzymes. These very species-specific types of ChE in gastropods increase the relevance of the present ChE characterization in G. umbilicalis for future assessments of pollutant effects on this species.

3.2. *In vitro* effects of chlorpyrifos on acetylcholinesterases activity

In the *in vitro* assay of chlorpyrifos-oxon effects on AChE activity, no statistical differences were observed between the solvent control and control (Student t-test t_{10} = 0.329, *p* = 0.749; Fig. 2). All statistical comparisons were performed against solvent control.

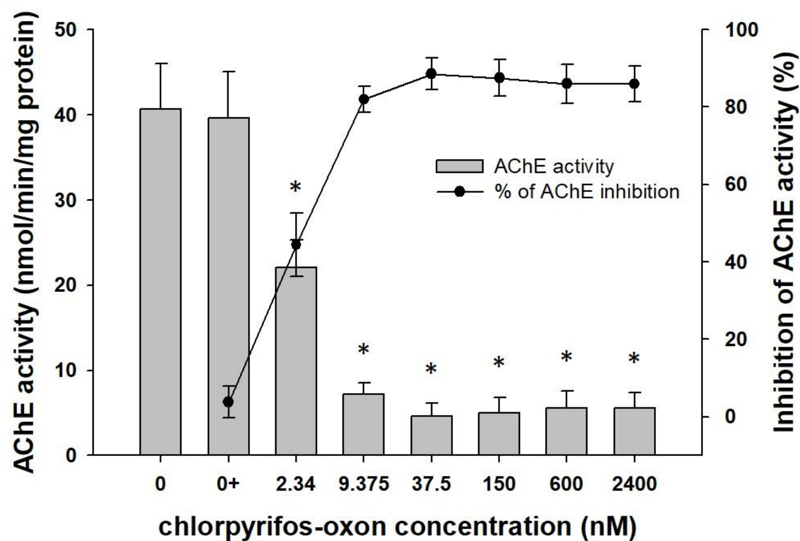

Figure 2 - Acetylcholinesterase (AChE) activity and percentage of activity inhibition (expressed as mean values ± SD) in *Gibbula umbilicalis* exposed *in vitro* to chlorpyrifos-oxon, using acetylthiocholine iodide (ATCh, 0.4 mM) as substrate. Asterisk indicate a significant difference from the solvent control (0+; Dunnett's, $p < 0.05$).

A dose-dependent inhibition of AChE activity with chlorpyrifos-oxon was observed over the range 2.34-37.5 nM (ANOVA $F_{7,40}$=139.149, Dunnett's p<0.001) with around 50% inhibition with respect to solvent control at the lowest concentrations (2.34 nM) and over 88% inhibition at 37.5 nM (Fig. 2). A median inhibitory concentration (IC_{50} ± SE) of CPO *in vitro* was estimated to be 2.29 ± 0.17 nM.

This chlorpyrifos oxon IC_{50} value is within the same range of the one reported by Xuereb *et al*. (2007) in the shrimp *Gammarus pulex* (0.99 nM) but much lower than the ones for the bivalves *M. galloprovincialis* and *Corbicula fluminea* with an IC_{50} of 0.62±0.04 µM and 6.15±0.73 µM, respectively (Mora *et al*. 1999). Although information concerning gastropods is scarce, these results point for a higher sensitivity of *G. umbilicalis* to the neuronal effects of this pesticide in comparison to bivalves.

3.3. *In vivo* effects of chlorpyrifos

3.3.1. Lethal effects

After the 96 h exposure, the obtained CPF LC_{50} [95% confidence Interval] for this snail was 0.33 [0.24-0.44] mg a.i. L^{-1} and the LC_{10} was 0.26 [0.09-0.31] mg a.i. L^{-1}. No mortality was found in the controls.

In the literature we can find a 96 h LC_{50} of 1.5 µg L^{-1} in the shrimp *Palaemonetes pugio* (Odenkirchen and Eisler 1988), which is lower than the 96 h LC_{50} of 0.247 mg L^{-1} for the bivalve *Donax faba* (JanakiDevi *et al*. 2013) and the 24 h LC_{50} of 3.19 mg L^{-1} (diferent time) for the brine shrimp *Artemia salina* (Varó *et al*. 2002), which are closer to the present results.

Larvae of other marine invertebrates have also showed to be more sensitive to this compound, for instance the ones of spider crab *Maja squinado*, showing a 24 h and 48 h LC_{50} of 22.5 and 0.79 µg L^{-1}, respectively, and of sea urchin *Paracentrotus serratu*s, showing a 24-h and 48-h LC_{50} of 0.35 µg L^{-1} and 0.22 µg L^{-1}, respectively (Bellas *et al*. 2005).

3.3.2. Effects on AChE activity and behaviour

The *in vivo* exposure to a chlorpyrifos-based formulation showed that this pesticide was capable of exerting significant effects both on AChE activity and flipping behaviour (Fig. 3). During the 96 h of experiment, no mortality was observed for *G. umbilicalis*, neither in controls, nor in treatments.

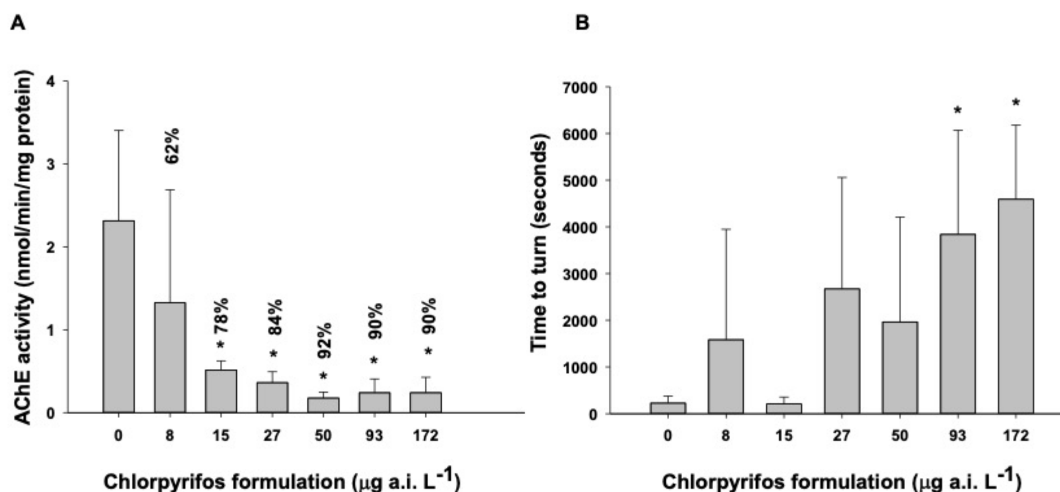

Figure 3 - Acetylcholinesterase (AChE) activity (A) and flipping (B) behavioural tests in *Gibbula umbilicalis* exposed *in vivo* to a chlorpyrifos-based formulation (µg a.i. L^{-1}). Values expressed as mean values ± SD. Asterisk indicate a significant difference from the control (p < 0.05). The percentage of AChE inhibition in relation to control is presented above each concentration bar in (A).

Basal AChE activity of non-exposed *G. umbilicalis* was 2.31 ± 0.34 nmol/min/mg protein (Fig.3A). Similar activity values have been obtained in *A. salina* (2.65 ± 0.15 nmol/min/mg protein) and *Artemia parthenogenetica* (3.69 ± 0.17 nmol/min/mg protein) (Varó *et al*., 2002). Higher values have been reported for other gastropods. For example, in the gastropod *Hexaple trunculus* an activity of 58.79 ± 8.71 nmol/min/mg protein was measured in the digestive gland and 33.71 ± 5.35 in the muscle (Roméo *et al*. 2006), and in *M. lineata* and *N. lapillus* values of 110.6 ± 29.7 and 31.8 ± 11.9 mol/min/mg protein were reported, respectively (Cunha *et al*. 2007). However, in this latter study different forms of ChEs were possibly being measured, and not exclusively AChE, since the characterization showed mixed properties between AChE and pseudocholinesterases.

Overall, the results showed that AChE activity of the sea snail decreased with increasing CPF concentrations, being significantly inhibited by concentrations equal or higher than 15 µg a.i. L^{-1} (Kruskal-Wallis H_6=51.320, Dunn's p<0.05; Fig. 3A). The chlorpyrifos concentration responsible for the inhibition of the 50% of AChE activity (IC_{50}) was estimated in 5.11 ± 1.84 µg a.i. L^{-1}. However, although the first concentration (8 µg a.i. L^{-1}) induced 62% inhibition in AChE activity, statistical differences to control were only detected after 15 µg a.i. L^{-1}.

Strong AChE inhibitions (equal or higher than 90%) were found for concentrations higher than 50 µg a.i. L^{-1}. AChE inhibition in *G. umbilicalis* is in agreement with ChE activity observed in previous CPF exposure studies using other test species. For instance, acute testing with the oysters *Crassostrea corteziensis* showed inhibitory effects at 80 and 160 µg a.i. L^{-1} (Benitez-Trinidad *et al*., 2014), while the effects with the clam *Donax faba* (JanakiDevi *et al*., 2013) were seen with increasing concentrations, for a 96 h period, in the range 79 - 1265 µg CPF L^{-1}. From the described data, it is clear that CPF is also a potent inhibitor of AChE in *G. umbilicalis*, which can result neurotoxicity impacts mediated by cholinergic synapses.

Indeed, the flipping speed, a proxy for behaviour, showed significant differences after exposure to the higher concentrations of CPF tested (ANOVA $F_{6,48}$=6.314, Dunnett's p<0.05; Fig.3B). The increase in snails turnover time (the time needed for a snail to right itself after being turned onto its back) was evident at 93 µg a.i. L^{-1} (p<0.003) and 172 µg a.i. L^{-1} (p<0.001) of CPF exposures. In this assay, the snails that flip faster are interpreted as being in better condition (Cabecinhas *et al*., 2015), so it can be concluded that in the present study the behaviour of these organisms was affected by the increasing CPF concentrations. A slowed righting ability has been previously demonstrated in gastropods under other stressful circumstances (Cabecinhas *et al*., 2015; Fong *et al*., 2017; Fong and Hoy 2012; Fong and Molnar 2013; Ford *et al*., 2018). The EC_{50} [95% confidence interval] for flipping test was estimated as 45.64 [23.84 ± 100.7] µg a.i. L^{-1}. When comparing concentrations of effect for these two *in vivo* exposure endpoints, it is possible to deduce that AChE was a more sensitive parameter than behaviour alterations measured through flipping test. These results are in accordance with the general knowledge that effects at a lower biological level precede and should be detectable earlier and at lower concentrations than effects at higher levels of organization (Lemos *et al*., 2010)

A correlation analysis between both measured endpoints also showed that AChE activity is significantly and negatively correlated with the snails' flipping ability (Pearson correlation, r^2 = -0.376, p = 0.0046). Given the present results, it can be hypothesised that the altered flipping behaviour of snails exposed to CPF may have been caused by the accumulation of the neurotransmitter acetylcholine in the synaptic junctions, which interferes with

coordination between the nervous and muscular junctions (neurotoxicity). Previous studies have already shown the linkage between AChE inhibition with effects at higher levels of biological organization. Cabecinhas *et al*., (2015), for instance, also reported a negative correlation between AChE inhibition and flipping behaviour using this same marine snail species exposed to the metal mercury. Relationships between AChE inhibition and alteration in locomotor behaviour were reported in vertebrates exposed to pesticides, such as the juvenile coho salmon (*Oncorhynchus kisutch*) exposed to CPF (Sandahl *et al*., 2005) and *Oncorhynchus mykiss* exposed to diazinon and malathion (OPs) (Beauvais *et al*., 2000). Cooper and Bidwell (2006) observed a reduced capacity of *Corbicula fulminea* exposed to CPF to burrow into the substrate in parallel to AchE inhibition. Xuereb *et al*., (2007) also reported relations between AChE inhibition and changes in feeding and locomotor behaviours in amphipods *Gammarus fossarum* exposed to CPF. This mechanistic link is particularly straightforward in OP pesticides, which have a specific mode of action (AChE inhibition). Chlorpyrifos can disrupt the structure of the enzyme by attacking the active serine hydroxyl group of AChE, increasing acetylcholine levels. This can also subsequently lead to the increase of catecholamines, which are involved in glicogenolysis and glycogen synthesis, thus interfering in energy metabolism of nerve cells, and ultimately in behaviour (Üner *et al*., 2006).

By affecting normal behaviour, inhibition of AChE activities can result in alterating the chances to survive. For example, it can be expected that changes in *G. umbilicalis* capacity to turn over, lead to increased drift and disruption in their escape reaction to escape from predators, feed, or even react in highly hydrodynamic areas, as observed in other studies (DeWhatley and Alexander, 2018; Ford *et al*., 2018). These disruptions at the individual level can ultimately impact responses at the population, community, and the ecosystem-levels.

4. Conclusions

In summary, results indicate that the main ChE form present in the marine snail *G. umbilicalis* is AChE. This is important knowledge for the successful application of this biomarker in future environmental biomonitoring surveys using this gastropod. Also, CPF as an organophosphate pesticide widely used for agricultural purposes and pest control, led to severe *in vivo* and *in vitro* AChE inhibition in this sea snail. Moreover, *in vivo* enzymatic inhibition was observed in the same pesticide treatments that induced alterations in the organisms' behavioural capacity to turn to their normal position, reflecting a negative correlation between both endpoints. This study provides the basis to interpret AChE inhibition in *G. umbilicalis* as a predictive endpoint of effects that might occur at higher ecologically relevant levels, such as behaviour, making it a sensitive representation of the organism's neuro-physiological responses to environmental stressors.

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Chapter IV

Impacts of the invasive seaweed *Asparagopsis armata* exudate on rockpool invertebrates

Chapter IV – Impacts of the invasive seaweed *Asparagopsis armata* exudate on rockpool invertebrates [1]

Abstract

The marine red algae *Asparagopsis armata* is an invasive species which competitive advantage arises through the production and release of large amounts of toxic compounds to the surrounding invaded area, reducing the abundance of native species. The main objective of this study was to evaluate the effects of this invasive seaweed on marine invertebrates by exposing the shrimp *Palaemon elegans* and the marine snail *Gibbula umbilicalis* to the exudate of this macroalgae. The seaweed was collected and placed in tanks, for 12 hours, in the dark in a 1:10 ratio. Afterwards the containing its secondary metabolites was collected for media for further testing. Lethal and sublethal effects of *A.armata* were investigated. Biochemical biomarkers responses associated with energy metabolism (lactate dehydrogenase, LDH; electron transport system activity, ETS; content in lipids, proteins and carbohydrates) were analyzed. The biomarker responses showed invertebrates' physiological status impairment after exposure to low concentrations of this algae exudate. Highest concentrations of exudate significantly increased lipid content in both organisms. In the shrimp, protein content, ETS, and LDH were also significantly increased. On the contrary, these parameters were significantly decreased in *G. umbilicalis.* A behavioural impairment was also observed in *G. umbilicalis* exposed to *A. armata* exudate, with reduction in feeding consumption. These results represent an important step in the research of natural toxic exudates released to the environment and prospective effects of this seaweed in invaded communities under increasing global change scenarios.

Keywords: Behaviour, Ecotoxicology, Invasive species, *Gibbula umbilicalis*, *Palaemon elegans*; Tidal pools

[1] Silva, C.O.; Novais, S.C.; Soares, A.M.; Barata, C.; Lemos, M.F. Preprints 2020, 2020080236 (doi: 10.20944/preprints202008.0236.v1)

1. Introduction

Overall rapid globalization, and increasing trends of trade and travel, have been accelerating marine biological invasions, by transporting species to areas outside their native range. These non-indigenous species (NIS) may then be considered invasive, once they establish, spread rapidly, and proliferate and dominate the new habitat without the direct support of humans (Richardson *et al*., 2011). *Asparagopsis armata* Harvey, 1855 (Bonnemaisoniales, Rhodophyta) is a red seaweed, native to Western Australia, and nowadays distributed throughout Europe in the Atlantic and Mediterranean basin, where it is highly invasive (Otero *et al*., 2013). This seaweed possesses chemical defence mechanisms that are nuclear to their invasiveness, based on the synthesis and storage of an array of secondary metabolites, which include over 100 halogenated compounds such as haloforms, haloacids, and haloketones (McConnell & Fenical, 1977). These halogenated volatile hydrocarbons containing one to four carbons are known antifeedant and cytotoxic compounds, among others (Pinteus *et al*., 2015; Zubia *et al*., 2009), and the pungent aroma of these algae is attributed 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, stored in vacuoles within gland cells (Burreson *et al*., 1976). These compounds potent biological effects are capable of inducing significant changes in terms of native community composition (Paul *et al*., 2006a) favoring *A. armata* in a given niche. Due to the enclosed environment during low-tide, rocky pools are ought to be sensitive sites to the increase of released compounds by retained *A. armata*, which may ultimately present adverse effects for other organisms such as other seaweed, vertebrates, and invertebrates, leading to severe consequences for coastal ecosystems. To investigate potential ecological impairments caused by these compounds, key role species in the structure and functioning of costal ecosystems were used: marine invertebrates such as the shrimp *Palaemon elegans* and the gastropod *Gibbula umbilicalis*, which inhabit the upper intertidal zone on rocky shores where *A. armata* is often found attached to the substrate, or unattached (drifting), and therefore releasing its chemical exudates. In this work, the assessment of *A. armata* exudate effects over these marine invertebrates was

performed addressing survival and sublethal effects through behavioural and biochemical responses.

Several behavioural parameters have been chosen as indicators of invertebrate health status. Flipping and feeding activity have been shown to be sensitive tools to assess the impact of contaminants at concentrations far below lethal levels (Cabecinhas *et al*., 2015; Silva *et al*., 2019; Dell'Omo, 2002; Fong & Molnar, 2013). Impairment of behavioural features such as the capacity to turnover/flipping due to contaminants, in the case of gastropods, may have direct effects on the capability of animals to escape from predators but also indirect effects such as disturbances in fitness and reproduction success due to alterations in feeding and thus energy metabolism. Enzymes involved in energy production have been frequently used as biomarkers to assess the effects of stressors, since exposed organisms usually need additional energy to maintain physiological/biochemical functions (Aderemi *et al*., 2018; Kühnhold *et al*., 2017; Silva *et al*., 2016). Thus, biomarkers such as total content in energy reserves, Lactate Dehydrogenase (LDH) and Electron Transport System (ETS) activities may provide valuable information on the physiological status of the studied organisms. Therefore, the purpose of this research was to address the effects of *A. armata* exudated secondary metabolites on the survival, behaviour, and energetic metabolism of two marine invertebrates inhabiting rock pools (*G. umbilicalis* and *P.elegans*), giving further ecotoxicological insight on this seaweed invasive strategy.

2. Material and methods

2.1. Test organisms

The shrimp *Palaemon elegans* and the sea snail *Gibbula umbilicalis* were collected from Carreiro de Joannes, a rocky beach in Peniche, central Portugal (39°21'18.0"N, 9°23'40.6"W), with no known sources of chemical contamination. The organisms were maintained during 7 d in the laboratory in natural seawater at 20 ± 1 °C, with a 16 h:8 h (light:dark) photoperiod in aerated aquaria. Shrimps were fed with frozen mussel and snails fed with *Ulva lactuca* until used in experiments. Prior to testing, organisms were kept fasting for 24 h.

2.2. *Asparagopsis armata* collection and preparation of exudates

Asparagopsis armata was collected in Berlenga Island, Peniche, Portugal (39°25'03.0"N, 9°30'23.6"W), a marine protected area, by SCUBA. In the lab, after cleaned and sorted, four aquaria with 5 kg of *A. armata* and 50 L of natural filtered seawater (through 0.45 µm cellulose acetate membrane filters) were left in the dark at 20 °C. After 12 h, the algae was removed, the water from the different aquaria was pooled and sieved for bigger particles, followed by a filtration through a 0.45 µm cellulose acetate membrane filter. This exudate was then kept in PET bottles at -20 ºC until further use. This, due to the obvious quantification constrains, constitutes the stock solution for all experiments and the 100% concentration.

2.3. Exposure setup

All experiments were conducted in a climatic room at 20 ± 1 °C, with a 16 h:8 h (light:dark), and experimental replicates consisted of glass vials with 60 mL and 750 mL exudate solution (or seawater in controls) for snails and shrimps, respectively, with one organism each. Flasks were covered with a plastic mesh to prevent organism to escape and to assure constant submersion. Exudate solutions were renewed every 24 h to avoid excreta accumulation and possible loss of volatile compounds. Exudate concentrations are presented as % of the exudate produced as described in 2.2.

2.3.1. Survival

After a range finding test, sea snails were exposed to increasing concentrations ranging from 1 to 15% of exudate (1; 1.57; 2.47; 3.87; 6.08; 9.55; and 15%), and shrimp from 4 to 10% of exudate (4; 4.66; 5.43; 6.32; 7.37; 8.58; and 10%). Exposures lasted 96 h and mortality was recorded daily. During exposures, no food was added. Eight and five replicates per treatment were used for sea snails and shrimps respectively, including a control treatment with filtered seawater only.

2.3.2. Sublethal exposure for biomarker analysis

Information on the lethal effects was used to establish maximum concentrations and conditions for each independent sublethal test, using half the LC_{10} as the highest concentrations tested. Sea snails were exposed to increasing concentrations of exudate ranging from 0.04 to 0.87% (0; 0.04; 0.07; 0.14; 0.25; 0.47; and 0.87%), and shrimp were exposed from 0.11 to 2.46% of exudate (0; 0.11; 0.21; 0.39; 0.72; 1.33; and 2.46%). Exposures lasted 168 h and 16 replicates per treatment were used for snails and 8 for shrimps, including a control treatment with filtered seawater only. At the end of the exposure period, snail's shell was broken with a vise, and soft tissues removed with forceps, weighed and kept on ice for operculum removal. Shrimps were sacrificed by decapitation and dissected. The abdominal muscle was rapidly isolated on ice and weighed. Tissues were maintained at -80 °C until further analysis.

2.3.3. Sublethal exposure for behavioural endpoints

Feeding activity

For the feeding activity assay, concentrations of the exudate were the same as used for the biomarkers exposure, using 8 replicates per treatment for both invertebrates and exposed for 96 h. Organisms were fed with discs of *Ulva lactuca* with c.a. 10 cm^2 previously dried at 60 °C for 48 h, weighed and re-hydrated just before adding to the medium (one disc per replicate). At the end of the 96 h exudate-exposed feeding test, the discs were rinsed in clean water, dried again, weighed, and the feeding was assessed by subtracting the algae final weight to its initial dry mass (mg).

Snail Flipping test

Flipping test represents the ability for the snail to move/turn when intentionally placed with the foot up (Cabecinhas *et al*., 2015). After the feeding assay exposure (96 h), snails were moved to 6-well plastic microplates with clean filtrated seawater (1 snail per well) and then completely inverted with the foot up. After being gently touched with a blunt

wooden stick, in order to have all organisms inside their shell, the righting time was recorded as a behavioural proxy (Cabecinhas *et al.*, 2015).

2.4. Biomarkers analysis

2.4.1. Tissue preparation

Snails were processed as pools of two individually exposed organisms, with each pool being considered as one biological replicate for the biomarker analysis (N=8). For shrimps, the muscle tissue of each organism was processed individually and considered as one biological replicate (N=8). The replicate tissues of each invertebrate species were homogenized in potassium phosphate buffer (0.1 M, pH 7.4) at a proportion of 1:12 for *G. umbilicalis* and 1:10 (m:v) for *P. elegans*. The homogenate was then separated into different microtubes for the analysis of total protein, carbohydrate and lipid content. The remaining homogenate was separated into two fractions centrifuged respectively at 1000 g for 5min (4 ºC) for ETS measurement and at 3000g for 5 min (4 °C) for LDH measurement. All aliquots were stored at -80 °C until further analysis.

2.4.2. Energy reserves

Carbohydrate, lipid and total protein contents were measured according to the approaches outlined by De Coen and Janssen (1997, 2003). The total carbohydrate content was determined in a reaction with phenol 5% and H_2SO_4 (95–97%), using glucose as standard and measuring absorbance at 490 nm (De Coen & Janssen, 1997). Lipid content was determined according to Bligh and Dyer (1959), using tripalmitin as standard and measuring absorbance at 400 nm. The total protein content was determined using the Bradford method (1976), with bovine serum albumin as standard, measuring absorbance at 600 nm. Following De Coen and Janssen (1997, 2003), all energy reserves were transformed into their energetic equivalents (39.5 kJ g^{-1} lipid, 24 kJ g^{-1} protein, 17.5 kJ g^{-1} glycogen).

2.4.3. Energy metabolism related enzymes

Electron transport system (ETS) activity was determined following the method described by De Coen & Janssen (1997). The ETS activity was measured spectrophotometrically by adding NADPH solution and INT (*p* iodo-nitro-tetrazolium) to the sample and absorbance was read at 490 for 3 minutes. The oxygen consumption was then calculated using the stoichiometric relationship: 2 µmol of formazan formed = 1 µmol of oxygen consumed. The oxygen consumption rate was then converted into the energetic equivalent of 484kJ/mol O_2 for average carbohydrate, lipid, and protein consumption combinations (Gnaiger, 1983). The activity of LDH was measured following Vassault (1983) with adaptations of Diamantino *et al*. (2001). The process is based on the efficiency of LDH to convert pyruvate to lactate, in the presence of NADH, which results in NADH oxidation and consequent decrease in absorbance. The absorbance was read at 340 nm for 5 min. A molar extinction coefficient of 6.3×10^3 M cm^{-1} was used, and results were expressed as nmol min^{-1} mg $protein^{-1}$.

2.5. Statistical analysis

Significant differences between each treatment for biomarker analyses and behavioural parameters were studied using one-way analysis of variance (ANOVA) and differences to control were addressed by Dunnett's post hoc test. Normality was checked by Shapiro-Wilk test and homoscedasticity by Levene test. In case of non-normally distributed data, the Kruskal–Wallis test was applied followed by Dunn's post hoc test. Statistical analyses for biochemical and behaviour endpoints were performed with the software SigmaPlot (Systat Software, San Jose, CA) and LCs and the correspondent 95% confidence intervals and global fitting were done on GraphPad Prism version 7 for Mac (GraphPad software, San Diego, CA).

3. Results

3.1. Survival

Acute toxicity tests revealed that *A. armata* exudate affects both species, with *P. elegans* being more tolerant than *G. umbilicalis* with significantly higher Lc_{50} ($F_{2,100}$=53.03, p<0.001) (Fig.1). *Gibbula umbilicalis* has a 96 h LC_{50} [95% CI] of 2.79% [1.66-4.69] and *P. elegans* a 96 h LC_{50} [95% CI] of 5.04% [4.84-5.25].

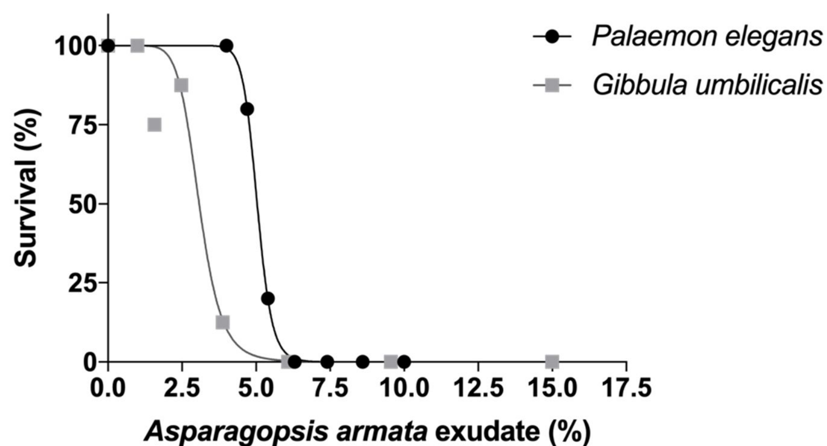

Figure 1 - Survival rate of two marine invertebrates after 96 h exposure to *Asparagopsis armata* exudate. Black circle: *Palaemon elegans*; Gray square: *Gibbula umbilicalis*.

3.2. Behavioural responses - Feeding activity and Flipping test

Feeding activity was affected in *G. umbilicalis* ($F_{6,49}$=5.304, p<0.001; Fig. 2A) when exposed to *A. armata* exudate at 0.07% (Dunnett's p=0.022), 0.47% (Dunnett's p<0.001), and 0.87% (Dunnett's p<0.001). No significant differences were found in *P. elegans* feeding activity ($F_{6,36}$=0.178, p=0.981; Fig. 2B).

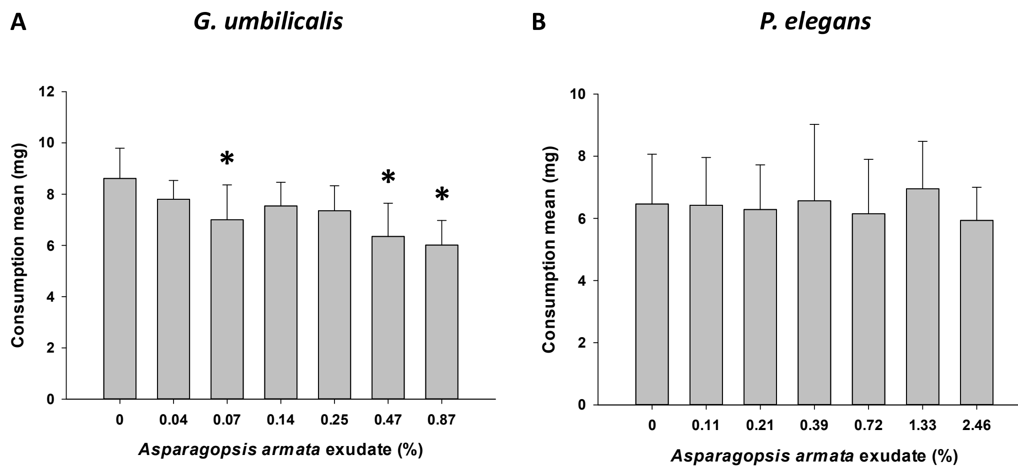

Figure 2 – Feeding activity behaviour of *Gibbula umbilicalis* (A) and *Palaemon elegans* (B) exposed to *Asparagopsis armata* exudate for 96 h. Results are expressed as mean values ± SE; Asterisks indicate significant differences to the control treatment (0%).

No significant differences were observed for flipping behaviour between control and experimental treatments.

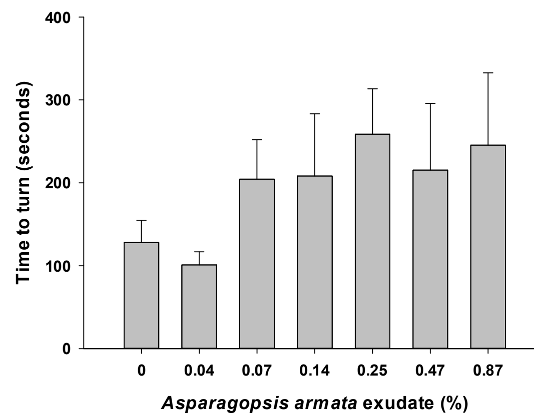

Figure 3 – Flipping behaviour of *Gibbula umbilicalis* exposed to *Asparagopsis armata* exudate for 96 h. Results are expressed as mean values ± SE.

3.3. Energy metabolism related biomarkers

Regarding *G. umbilicalis* exposure to *A. armata* exudate, no significant differences were observed in the carbohydrate and protein contents (Fig. 4a,c). However, there was an increase in accumulation of reserve lipids ($F_{6,47=}$8.099; p<0.001; Fig.4b) in 0.07% (Dunnett's p=0.031) and 0.87% (Dunnett's p<0.001) exudate exposures. ETS activity showed a significant decrease at 0.14% treatment (H_6=19.784, Dunn's p=0.003; Figure 4d) while LDH was also significantly inhibited ($F_{6,44}$=4.041, p=0.003; Fig. 4e) at 0.14% (Dunnett's p=0.002) and 0.87% (Dunnett's p=0.022).

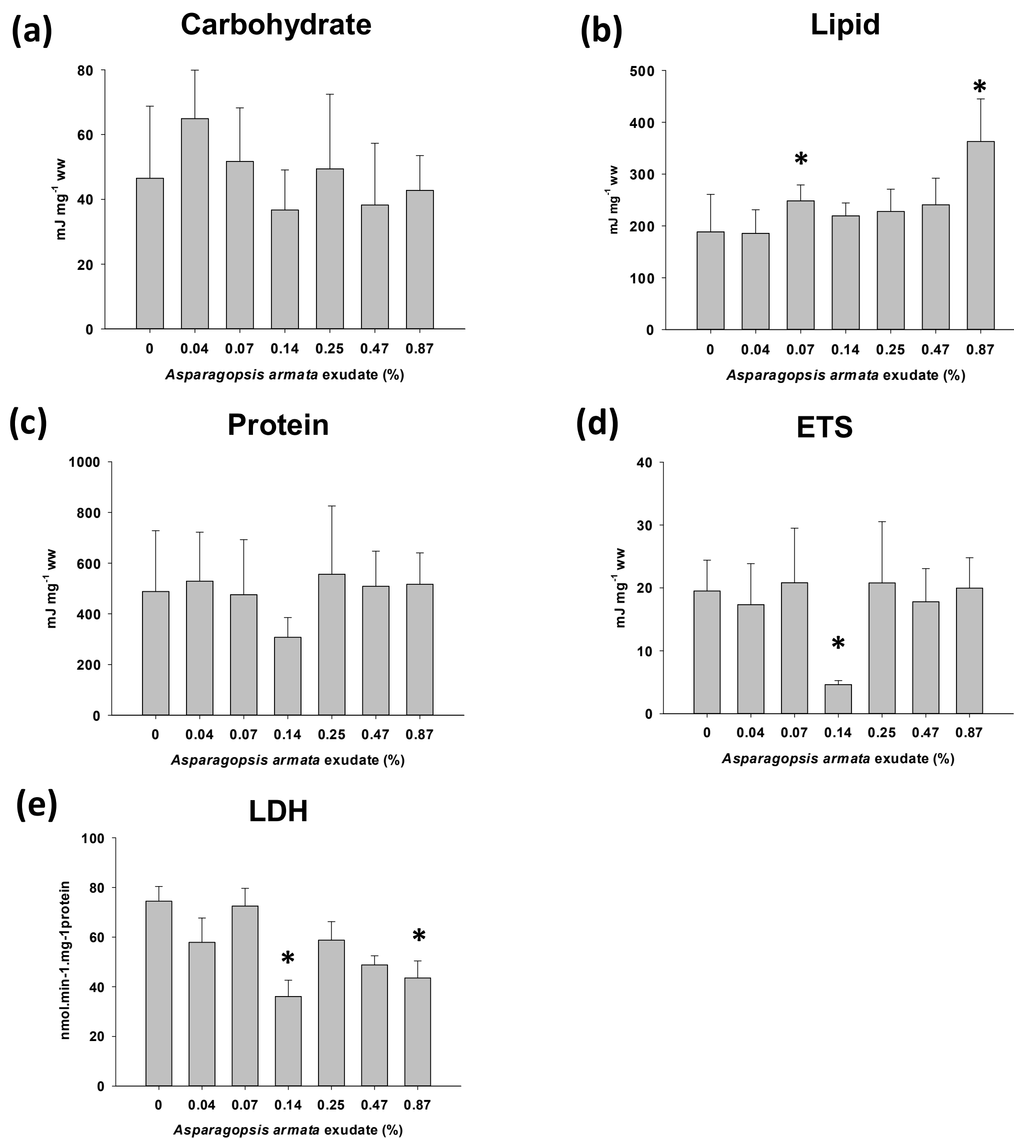

Figure 4 – Energy related parameters in *Gibbula umbilicalis* when exposed to *Asparagopsis armata* exudate for 168 h: (a) total carbohydrate content; (b) total lipid content; (c) total protein content; (d) electron transport system (ETS) activity; (e) lactate dehydrogenase (LDH) activity. Results are expressed as mean values ± SE; * Significant differences from the control (Dunnett's or Dunn's, $p<0.05$).

As for the energy reserves measured in the muscle tissue of *P. elegans*, no effects were observed for carbohydrates ($H_6=9.431$, $p=0.151$) after exudate exposure (Fig. 5a) but there was an increase in total lipids ($F_{6,44}=5.580$, $p<0.001$; Fig. 5b) at the highest tested

concentration (Dunnett's *p*=0.020). Mean protein levels were also significantly increased at all concentrations ($F_{(6,38)}$=32.667, *p*<0.001; Fig. 5c) except at the lowest 0.11% (Dunnett's *p*=0.272). ETS followed the same pattern as protein accumulation, with an increase in activity for concentrations equal or higher than 0.21% of exudate ($F_{6,45}$=5.757, *p*<0.001; Fig. 5d). LDH, on the other hand, only showed an increment in activity ($F_{6,46}$=3.106, *p*=0.012; Fig. 5e) at the intermediary concentrations 0.21% (Dunnett's *p*=0.009) and 0.39% (Dunnett's *p*=0.014) and 0.72% (Dunnett's *p*=0.036).

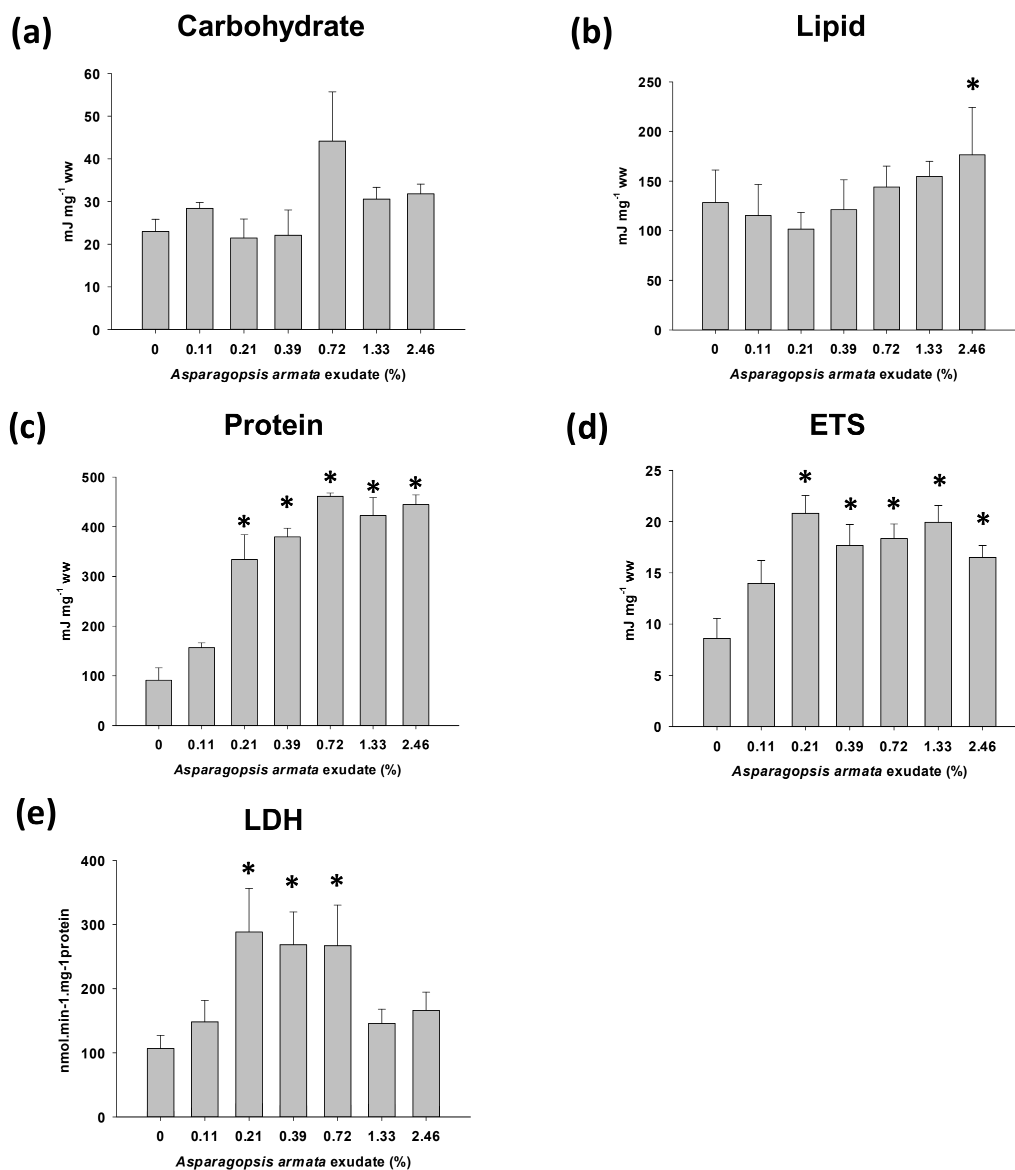

Figure 5 - Energy related parameters in *Palaemon elegans* when exposed to *Asparagopsis armata* exudate for 168 h: (a) total carbohydrate content; (b) total lipid content; (c) total protein content; (d) electron transport system (ETS) activity; (e) lactate dehydrogenase (LDH) activity. Results are expressed as mean values ± SE; * Significant differences from the control (Dunnett's or Dunn's, $p<0.05$).

4. Discussion and conclusions

There are some studies addressing the toxicity of seaweeds and its detrimental effects to invertebrates, but very few address toxicity of seaweed secondary metabolites, such as in the study of the effects of *Ulva* sp. exudate on the gastropods *Littorina littorea* and *L. obtusata* (Peckol & Putnam, 2017). The present study shows the potential of *A. armata* exudates to affect marine invertebrates such as *G. umbilicalis* and *P. elegans*. The acute tests demonstrated that the exudate could induce mortalities at very high dilution from the initial seaweed exudate, with a 96 h LC_{50} of 2.93% for *G. umbilicalis* and of 5.05% for *P. elegan*s. The reasons for such low *A. armata* exudate tolerance in both invertebrates is of some concern due to the well documented importance of these species to the functioning of rocky shore communities as principal microalgal consumers of seagrass biofilm (Orth & Van Montfrans 1984).

The comparison between 96 h dose-response curves revealed that *P. elegans* were more tolerant than *G. umbilicalis* with significantly higher LC_{50}. In fact, the high sensitivity of *G. umbilicalis* to this exudate is less obvious, given the well-documented tolerance of this species to extreme environmental conditions (Southward, 1958). Additionally, *G. umbilicalis* have been found to be relatively tolerant to other contaminants (organophosphate pesticides and metals) (Cabecinhas *et al*., 2015; Silva *et al*., 2017; Silva *et al*., 2019).

Alteration of normal behavioural patterns, such as feeding, caused by exposure to contaminants may pose serious risks to the success of the community species that co-inhabit in intertidal rockpools, like shrimps and snails such as the ones tested here. Here, feeding activity was impaired by the seaweed exudate at sublethal concentrations, which agrees with the literature showing that behavioural endpoints are sensitive tools to evaluate sub-lethal effects of contaminants (Amiard-Triquet *et al*., 2012). In the literature,

there are few examples of feeding inhibition by macroalgae compounds. Sea urchin (*Lytechinus variegatus*) feeding was inhibited with caulerpenyne, an oxygenated sesquiterpene extracted from *Caulerpa prolifera,* and cymopol, a monoterpene-bromohydroquinone from *Cymopolia barbata,* both green invasive macroalgae (McConnell *et al*., 1982). Other studies evidenced that halogenated monoterpenes isolated from the red algae *Plocamium lepitophyllum* inhibit food consumption by sea-urchin and gastropods (Sakata *et al*., 1991) and *A. armata* dichloromethane extracts have also revealed feeding deterrence (Paul *et al*., 2006b). Feeding is generally one of the first responses to environmental perturbations and its inhibition can cause a reduction in an organism's energy assimilation resulting in a reduction in resource allocation to growth, reproduction, and survival (McLoughlin *et al*., 2000; Sokolova *et al*., 2012). In this work, the exudate derived from *A. armata* deterred the feeding of the marine gastropod *G. umbilicalis*, which is potentially due to the chemical defence characteristics of *A. armata*, documented in the literature as possessing compounds, mostly halogenated, with the primary function to deter herbivory (Borell *et al*., 2004; Paul *et al*., 2006b). Often, feeding inhibition derives from movement reduction due to toxic exposure, and consequent less capacity to find food and to process it (Cabecinhas *et al*., 2015). In sum, behavioural impairments, in general, reveal disturbances that may be associated with differences in energy uptake and allocation, which may have important ecological consequences. The secondary metabolites (i.e. allelochemicals) produced by *A. armata* act as chemical defences against competitors and predators (Hay & Fenical, 1988; Paul & Ritson-Williams, 2008), however, few studies have examined the effects of macroalgal allelochemicals on both biochemical and behavioural responses, with most research focused on their defensive functions against herbivory. In this work, it was observed that *A. armata* exudate not only interfere with the feeding behaviour of one of the species but also induce changes in several energy metabolism related biochemical parameters of both invertebrates.

Regarding effects on *G. umbilicalis*, *A. armata* induced a significant increase in total lipids, along with decreased activities of LDH and ETS. The primary source of energy are the carbohydrates followed by lipids and then proteins (Ayuningtias, 2011), and their mobilization is often used to counter toxic stress. Notwithstanding, in this case, the high

lipid content may be related to the fact that the invertebrates maintain the energy reserves as they alter their behaviour, decreasing their activity, and thus with less expenditure. In fact, the sea snail has become less active, as seen in the feeding at higher concentrations of exudate. This may ultimately lead to an energetic shift with a lesser energy expenditure, despite the probable higher energetic demands for detoxification mechanisms. This trend has been reported in the literature for other compounds as is the case of the study of Verslycke and co-authors (2004) with mysids, where a decrease in feeding was observed along with increasing lipids levels after exposure to chlorpyrifos.

LDH is an important glycolytic enzyme in biological systems and its activity is an indication of increased energy demand to cope with exposure to toxicant challenge (Wu & Lam, 1997; Diamantino *et al*., 2001). The significant decrease in LDH verified in the higher exudate concentrations, indicative of a reduction in anaerobic capacity, may reflect systemic toxicity impairing the organisms to fight the toxic stress, which might later on have further severe lethal consequences – knowing the last concentration is almost lethal range. This decrease in general cellular metabolism is also in line with ETS results, where the decrease in the aerobic capacity is also suggested by the significant reduction of ETS in marine snails exposed to the medium concentration of 0.14%. This non-monotonic response may derive from the complexity in the media and differentiated mechanisms of action of different individual compounds and their different concentrations in the mixture at a given exudate dilution, which constitutes an extra challenge to the interpretation of results of such nature.

In exposed *Palaemon elegans* there was an increase in lipids and proteins along with the increase of ETS and LDH activities. The increase in lipids was similar as discussed previously for the sea snails, but for P. *elegans*, the exudate also exerted a significant increase of protein content. This increase may also reflect an induction in protein synthesis for detoxification processes and other defence mechanisms (Smolders *et al*., 2003). This is also supported by the metabolic reactions assessed that, contrary to the sea snails, indicate an enhancement of the cellular metabolism, with LDH and ETS activities being increased at the same concentrations. This indicates that the organisms are spending energy both anaerobically and aerobically to fight stress caused by *A. armata* exudate. This pattern

indicates that the amount of energy available for growth, molting, reproduction and other biological functions might be compromised (Sokolova *et al*., 2012).

These results indicate that organisms have different energy requirements to deal with the stress caused by the macroalgae exudate. This comparative analysis may provide important insights into the heterogeneous effects of the *A. armata* exudate, driven by species-specific metabolic susceptibility patterns.

The present work was performed using exudates which, to obtain naturally, demand that the seaweed is placed in seawater at a given ratio and defined conditions, which will mostly represent what is exudated by *A. armata* in the nature. Ratios of 1:10 and over are commonly found in rock pools, which may remain enclosed from minutes to several hours during a tidal period. Other factors which may induce additional stress and compound release may influence more toxicity such as the case of temperature or hydrodynamics (Gschwend *et al*., 1985). Also, the exudates were prepared in the dark, and the production of volatile halogenated organic compounds (VHOCs) production rate tend to decrease under dark conditions (Bondu *et al*., 2008). Despite the difficulties to benchmark this stressor preparation and impacts with a real case scenario, the very high toxicity of this seaweed might not even reflect the worst case scenario of exposure to exudates from this macroalgae, especially in bloom events, in summer, in tide pools, where a body of water separates from the sea for hours during a tidal cycle, where seaweed concentrations are high and water dilution is little.

The present study is an important step in the research of natural toxic exudates released into the environment and the mechanisms on how they can affect the surrounding organisms and their mode of action in the invaded ecosystems.

To the present knowledge, this represents the first work to study the exudate *per se* and its impacts in costal invertebrates. Although, as stated, this exudate contains a myriad of compounds, its toxicity is attributed mainly to the halogenated compounds (secondary metabolites) produced and stored in vacuoles within *A. armata*'s gland cells (Burreson *et al*., 1976). Additionally to the results and toxic effects seen here at high dilutions of the prepared exudate, to better understand the real impact on coastal environments, and

specially in more exposed tidal pools, further studies should be made to understand the concentrations found and its variation, considering seaweed density and other biotic and abiotic factors to better address this seaweed chemical defences true impact in coastal environments. These impacts may probably be more extent compared to the ones here found due to the referred increased stress and conditions that may lead to an increased production of secondary metabolites and also densities that can be much higher, especially when considering seasonal seaweed stranding, and all other factors affecting these organisms in a high stress burden ecosystem.

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Chapter V

Asparagopsis armata exudate cocktail: the quest for the mechanisms of toxic action of an invasive seaweed on marine invertebrates

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Chapter V - *Asparagopsis armata* exudate cocktail: the quest for the mechanisms of toxic action of an invasive seaweed on marine invertebrates[1]

Abstract

The red seaweed *Asparagopsis armata* exhibits a strong invasive behavior and is included in the list of the "Worst invasive alien species threatening biodiversity in Europe". This seaweed has been shown to produce a large diversity of halogenated compounds with potent biological effects, deeply affecting rockpool species. Therefore, the present study aimed to investigate the biochemical responses to sublethal concentrations of *Asparagopsis armata* exudate on two coastal organisms, a marine snail, *Gibbulla umbilicalis* and the rockpool shrimp *Palaemon elegans*. Antioxidant defences superoxide dismutase (SOD) and glutathione-S-transferase (GST), oxidative damage lipid peroxidation (LPO) and DNA damage, the neuronal parameter acetylcholinesterase (AChE), as well as the fatty acid profile were evaluated. Results revealed different metabolic responses between species, indicating that *A. armata* exudate affected the organisms through different pathways. Despite previous studies indicating that the exudate effected *G. umbilicalis*' survival and behaviour, this does not seem to result from oxidative stress or addressed neurotoxicity. On the other hand, for *P. elegans*, an inhibition of AChE and the decrease of antioxidant capacity concomitant with the increase of LPO, suggests neurotoxicity and oxidative stress as mechanisms of exudate toxicity for this species. For fatty acids, there were different profile changes between species, also more pronounced for *P. elegans* with a general increase in PUFA with exudate exposure, which commonly means a defence mechanism protecting from membrane disruption. Nonetheless, the omega-3 PUFAs ARA and DPA were increased in both invertebrates, indicating a common mechanism regulation of inflammation and immunity responses to this stress. This work provides further insight into the mechanisms of invertebrate response and tolerance to an expanding coastal environmental stress as is the marine invader *A. armata.*

Keywords: Biomarkers, Fatty acid profile, Halogenated compounds, Oxidative stress, Red macroalgae, Secondary metabolites,

[1] Silva, C. O., Simões, T.; Félix, R.; Soares, A.; Barata, C.; Novais, S.; Lemos, M. Preprints 2020, 2020100133 (doi: 10.20944/preprints202010.0133.v1)

1. Introduction

More than 3800 halogenated compounds are known to exist (Gribble, 2003), and many are known to be present in the environment, having both biogenic and anthropogenic sources. The largest source of biogenic organohalogens are seaweeds, sponges, corals, tunicates and bacteria (Gribble, 2003). Seaweeds produce an array of organohalogens, which exhibit important and vital ecological roles as defence compounds (Amsler, 2008).

Asparagopsis armata, a species of the family Bonnemaisoniaceae is known to form specialized cells, known as vesicle or gland cells, which are sources of these halogenated products including halomethanes, haloalkanes, haloacids and haloketones (McConnell & Fenical 1977), reported to have potent biological effects to protect themselves from attacks by herbivores and pathogens (Paul *et al.*, 2006b & c).

The eutrophication and the occurrence of algal blooms may result in negative ecological consequences to the aquatic ecosystem. Algal blooms of some seaweeds, such as *A. armata*, can be retained in the rockpools and release high concentrations of halogenated compounds, which can be harmful and compromise inhabiting biota (Silva *et al*., 2020). Limited information is available on effects of macroalgae exudated secondary metabolites in aquatic environments. However, some negative effects of these compounds on aquatic ecosystem can be found in the literature (Paul at al., 2006a, Silva *et al*., 2020). Besides suppressing the growth of other algae (Harlin *et al*., 1987), macroalgae exudates can affect the development and grazing of invertebrates (Paul *et al*., 2006b) and even vertebrates (Nelson *et al*., 2003).

In this study, *Gibbula umbilicalis* (da Costa 1778) and *Palaemon elegans* (Rathke 1837), abundant species on rocky shores and having wide geographical distributions (Gaudèncio

& Guerra 1986), were used as testing model species to assess the impacts of *A. armata* on coastal communities through a biomarker mechanistic approach.

Once these invertebrates are exposed to pollutants such as *A. armata* exudate, these compounds go through biotransformation reactions, stimulating the production of reactive oxygen species (ROS) which can damage cellular macromolecules (Livingstone, 2003) in the form of lipid peroxidation (LPO) and DNA strand breaks. Key antioxidant enzymes that protect cells against ROS include superoxide dismutase (SOD), representing the primary defence against oxygen toxicity, being responsible for the transformation of $O2^{\bullet-}$ into H_2O_2 (Stegeman *et al*., 1992). Glutathione-S-transferase (GST) plays a role in the second phase of the detoxification process, where it facilitates the excretion of xenobiotics (Boutet *et al*., 2004). Environmental stressors may also promote neurotoxicity. Acetylcholinesterase, involved in the synaptic transmission of nerve impulse through the hydrolysis of neurotransmitter acetylcholine into choline and acetate, is known to be inhibited by environmental stressors such as pesticides (Barata *et al*., 2004; Monteiro *et al*., 2019; Silva *et al*., 2019).

Fatty acid profile (FAP) has also been used as a biochemical response to pollutant exposure (Filimonova *et al*., 2016; Silva *et al*., 2017; Silva *et al*., 2018). Fatty acids (FAs) are among the main constituents of the cell membrane and are involved in a wide range of biological pathways, from the production and permeability of cell membrane to lipids main components, while also being signalling mediators and used as fuel in all metabolic systems (Neves *et al*., 2015).

This biomarker approach allows for a mode-of-action assessment of *Asparagopsis armata* exudates impact in organisms and eventual repercussions in higher levels of biological organization, such as population or even community levels (Lemos *et al*., 2010).

This work aimed to evaluate biochemical responses of the common species *Palaemon elegans* and *Gibbula umbilicalis* after exposure to impactful concentrations of *Asparagopsis armata* exudate, by assessing oxidative damage (lipid peroxidation and DNA damage), antioxidant and detoxification enzymes (superoxide dismutase and glutathione S-transferase), neuronal activity (acetylcholinesterase) and fatty acid profile changes.

2. Material and methods

2.1. Test organisms

The collection of the shrimps *Palaemon elegans* and *Gibbula umbilicalis* was performed in an intertidal rocky shore (Carreiro de Joannes), in Peniche, central Portugal (39°21'18.0"N, 9°23'40.6"W). Their maintenance in laboratory was carried out with temperature kept at 20 ± 1 °C, with a photoperiod of 16 h: 8 h (light:dark), and constant aeration. During this period, every two days the organisms were fed *ad libitum* with small fragments of mussels for the shrimps and *Ulva lactuca* for snails.

2.2. Experimental setup

Asparagopsis armata collection, preparation of exudates, and experimental design followed previous work by Silva *et al*. (2020). Briefly, after collected in Berlenga Island (Peniche), by SCUBA, algae was cleaned and 5kg of *A. armata* was placed in aquaria containing 50L of filtered seawater for 12h in the dark at 20 °C. Then, algae was removed and the water was filtered and kept at -20 ºC until further use (exudate). As in Silva *et al*., (2020), this produced exudate constitutes the stock solution for all experiments, and experimental concentrations are presented as % of the exudate produced.

After the acclimation period (7 days), the organisms were randomly transferred to glass sampling flaks, and the concentrations of exudate used were: 0; 0.04; 0.07; 0.14; 0.25; 0.47; 0.87% for sea snail, and 0; 0.11; 0.21; 0.39; 0.72; 1.33; 2.46% for shrimp [highest concentration for both ranges based on half the LC_{10}; Silva *et al*., (2020)]. Media was obtained by adding corresponding exudate percentages to natural seawater (v/v). Exposures lasted for 168h and 16 and 8 replicates were used per treatment for sea snails and shrimps, respectively. At the end of the exposure period, organisms were sacrificed and kept at -80 °C until further analysis.

2.3. Biochemical analysis

2.3.1. Tissue preparation

Test organisms were sacrificed, and soft tissues were removed and dissected on ice. Pools of two snails (each pool considered as one biological replicate) were homogenized in a proportion of 1:12 (m:v) of potassium phosphate buffer (0.1 M, pH 7.4). The homogenate was then divided into 3 microtubes and kept at -80 ºC until further analysis of DNA damage, FAP and lipid peroxidation (LPO). For the latter, sampling microtubes contained BHT (2.6-dieter-butyl-4-metylphenol) 4% in methanol to prevent tissue oxidation. The rest of the homogenate was separated into two microtubes that followed different centrifugations: 1) centrifuged for 5 min at 3000g (4 °C) for the analysis of AChE activity on the resulting supernatant stored at -80 °C; 2) centrifuged for 20 min at 10000 g (4 °C) to obtain the post-mitochondrial supernatant (PMS), which was stored at -80 °C for posterior analysis of SOD and GST activities.

For the *P. elegans* samples, the homogenate was separated by different tissues into microtubes, following different centrifugations: eyes were homogenized on 300 µL of potassium phosphate buffer (0.1 M, pH 7.4) and centrifuged for 5 min at 3000g (4 °C) for the analysis of AChE activity; hepatopancreas was also homogenized in potassium phosphate buffer in a proportion 1:10 (w:v) and divided into different microtubes for the analysis of LPO, DNA damage and FAP; the remaining homogenate was centrifuged for 20 min at 10000 g (4 °C) to obtain the PMS for posterior analysis of SOD and GST activities. All aliquots were kept at -80 ºC until further analysis.

2.3.2. Antioxidant and detoxification defences

GST activity was determined by the Habig method (1974) adapted to microplate, following the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm for 3 min.. GST activity was calculated, using a molar extinction coefficient of 9.6×10^3 M cm^{-1}, and expressed in nmol min^{-1} mg^{-1} of protein.

SOD activity was measured according to McCord & Fridovich, (1969), adapted to microplate, using the xanthine/xanthine oxidase mediated reduction of cytochrome C. The decrease of the cytochrome C reduction was followed at 550 nm and SOD activity was expressed in U mg $^{-1}$ of protein using a SOD standard of 1.5 U ml^{-1}, where 1 U represents the amount of enzyme required to inhibit the rate of reduction of cytochrome c by 50%

2.3.3. Oxidative damage

The LPO levels were determined by measuring the content of thiobarbituric acid reactive substances (TBARS), following Ohkawa *et al*. (1979) and Bird & Draper (1984). After the reaction with TBA (2-thiobarbituric acid), absorbance was read at 535 nm and results were expressed in nmol TBARS g $^{-1}$ ww (wet weight), using a molar extinction coefficient of $1.56x10^5$ M cm^{-1}.

DNA damage (strand breaks) analysis was based in the DNA alkaline precipitation assay (Olive, 1988) with adaptations from de Lafontaine *et al*. (2000). After the precipitation of nucleoproteins and intact DNA, the DNA kept in the supernatant was linked with Hoesch dye (1 µg mL^{-1} bis-benzimide, Sigma-Aldrich), allowing the estimation of damage levels by fluorescence, using an excitation/emission wavelength of 360/460 nm. Results were expressed as µg g^{-1} ww of DNA using calf thymus DNA as standard to extrapolate DNA concentration.

2.3.4. Neuromuscular biomarker

The AChE activity was determined through the methodology proposed by Ellman *et al*. (1961) adapted to microplate (Guilhermino *et al*. 1996). The absorbance was read at 414 nm for 5 min, monitoring the formation of 5-thio-2-nitrobenzoate anion (TNB), which results from the reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) with thiocholine, a product of the acetylcholine substrate hydrolysis performed by AChE. Results are expressed in nmol min^{-1} mg^{-1} of protein using a molar extinction coefficient of $13.6x10^3$ M cm^{-1}.

2.3.5. Fatty acid profile

The methodologies for FA preparation and analysis were performed according to Silva *et al*., (2017), with minor modifications. Briefly, for the initial saponification step, 150 µL of 2M KOH (diluted in 67% ethanol; v/v) was added to 150µl of homogenate. Samples were then similarly kept at 80°C for 1 hour, cooled to room temperature, diluted with water, acidified (HCl) and FAs isolated with hexane.

To the FA fractions isolated from each sample after saponification, 1.5 mL of acetyl chloride:methanol (1:20 v/v) solution was added for the derivatization step, and samples were kept at 80 ºC for 1 hour. After adding, 1 mL of Mili-Q water and 1 mL of hexane for phase separation, the organic layer was recovered to clean GC vials and solvent was evaporated in a vacuum concentrator (SpeedvacTM) for 10 minutes. Samples were then resuspended in 50 uL of hexane and methylated nonadecanoic acid (50 uL; 10 mg mL^{-1}) was added as an internal standard to each sample, prior to gas chromatography analysis. Fatty acid methyl ester mixes (PUFA No1 from Marine source and PUFA No 3 from Menhaden oil) were used as external standards (Supelco, Bellefonte, Pa., U.S.A.). Operating conditions were as described by Silva *et al*., (2017). Theoretical correction factor (FCT) for FID detectors was applied in FA quantification, according to Xinghua Guo (2014).

2.4. Statistical analysis

All statistical analyses were run using R software (version 3.6.3) (R Core Team, 2020) in combination with user interface RStudio 1.2.5033 (RStudio Team, 2019). Boxplot graphs were prepared in Graphpad Prism version 7 for Mac (GraphPad Software, San Diego, CA). Both biomarker and fatty acid data were checked for normality with Shapiro-Wilk test ("shapiro.test" function), with most variables having p-value<0.05 (indicative of non-normal distribution). Thus, Kruskal–Wallis one-way analysis of variance test was performed ("kruskal.test" function) to determine significant differences between exudate treatments for biomarkers and for every fatty acid detected ($p < 0.05$). For significantly different variables, this was followed by a post-hoc Nemenyi-Test ("kwAllPairsNemenyiTest" function), package PMCMRplus (Pohlert, 2018), to find which specific treatments

significantly differed. For the metabolic shift analysis, each significantly different fatty acids' mean values was normalized to the respective mean of the control, and a Bray-Curtis dissimilarity matrix was computed ("vegdist" function), package vegan (Oksanen *et al.*, 2019), that was used in the production of heatmaps. To visualize the different levels of expression, a degree of colour was assigned where green represents the lowest amount of fatty acid, passing through black and ending in red, as a higher amount of fatty acid.

3. Results

3.1. Biochemical biomarkers

Measurements of antioxidant defences, oxidative damage, and neuromuscular biomarkers in gastropod (*G. umbilicalis*) and shrimp (*P. elegans*) are illustrated in Figure 1 and 2, respectively. In general, *A. armata* exudate induced biochemical alterations in both *G. umbilicalis* and *P. elegans* metabolism, yet with different trends of effects between species.

The antioxidant and detoxification enzymes evaluated revealed a significant decrease in GST activity in *G. umbilicalis* at 0.14 (C3; p=0.001) and 0.25% (C4; p=0.015) concentrations of *A. armata* exudate, but for SOD, no significant differences were found (Fig. 1a,b). Concerning the parameters addressing oxidative damage, a significant decrease was observed in peroxidation of lipids at lower exudate concentrations (0.04%), (C1; p=0.041) but with no effects for DNA-strand breaks being registered (Fig.1c,d). Neuromuscular parameter (AChE) detected significant higher activities at 0.14 (C3; p=0.0214) and 0.47% (C5; p=0.0246) of *A. armata* exudate (Fig.1e).

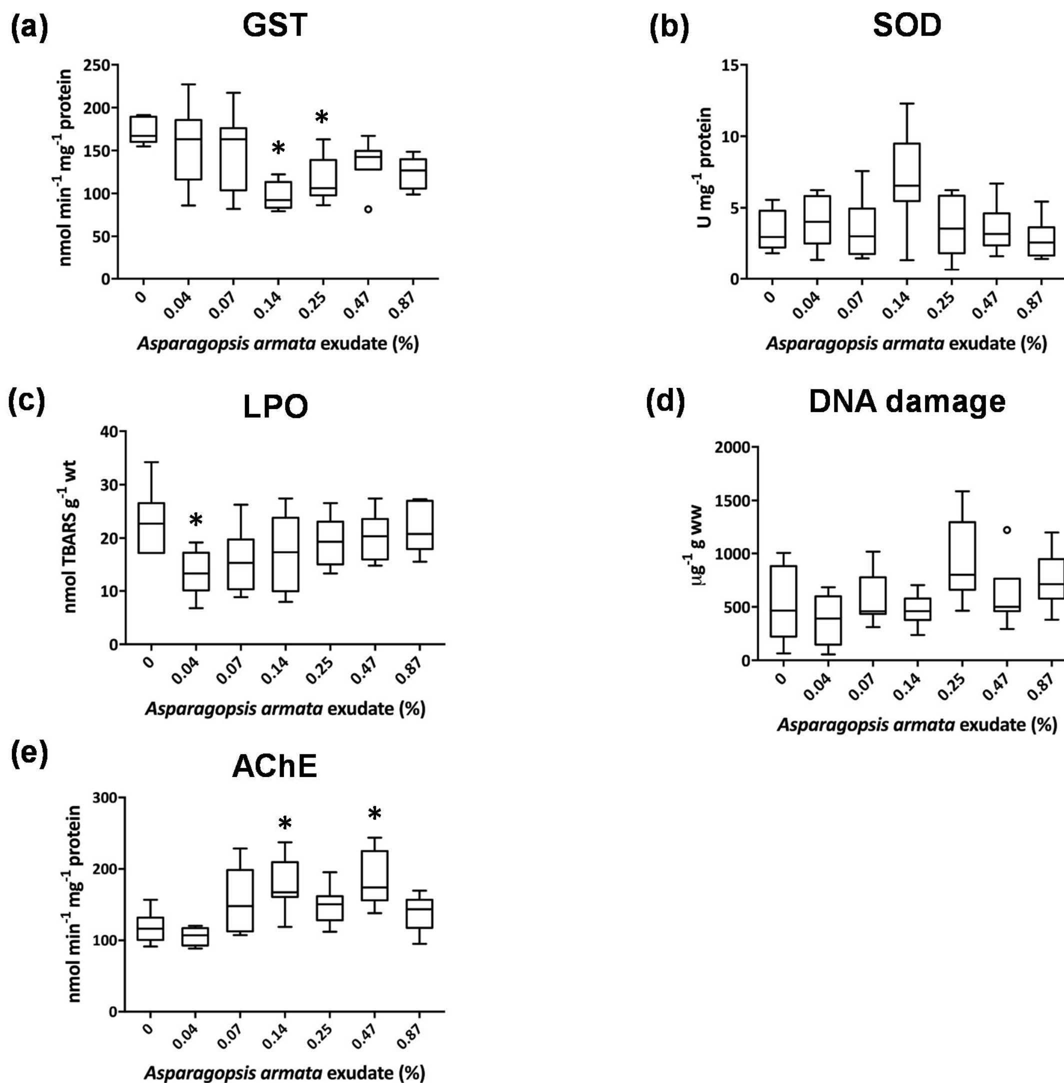

Figure 1 - Results of detoxification and antioxidant defences (a) and (b), oxidative damage (c) and (d), and neuromuscular (e) biomarkers in *Gibbula umbilicalis* when exposed to *Asparagopsis armata* exudate for 168 h. Results are shown in boxplot (i.e. the median, the first and the third quartiles, the non-outliers range and the outliers); * Significant differences from the control (Nemenyi, $p<0.05$).

The biochemical analysis to the shrimp *P. elegans*, revealed no significant differences detected for GST, but a decrease in SOD was observed at 0.11% (C1; $p=0.028$) of *A. armata* exudate concentration (Fig.2a,b). Regarding the oxidative damage, significant effects of the exudate were observed in LPO at the highest exudate concentration of exposure (C6; $p=0.0018$), and with a trend to increase through consecutive concentrations (Fig.2c). As

observed for *G. umbilicalis*, no damage was observed in DNA-strands for *P. elegans* (Fig. 2d). Also, an inhibition of the neuromuscular parameter AChE was identified at 0.21% of *A. armata* exudate (C2; *p*= 0.032) (Fig.2e).

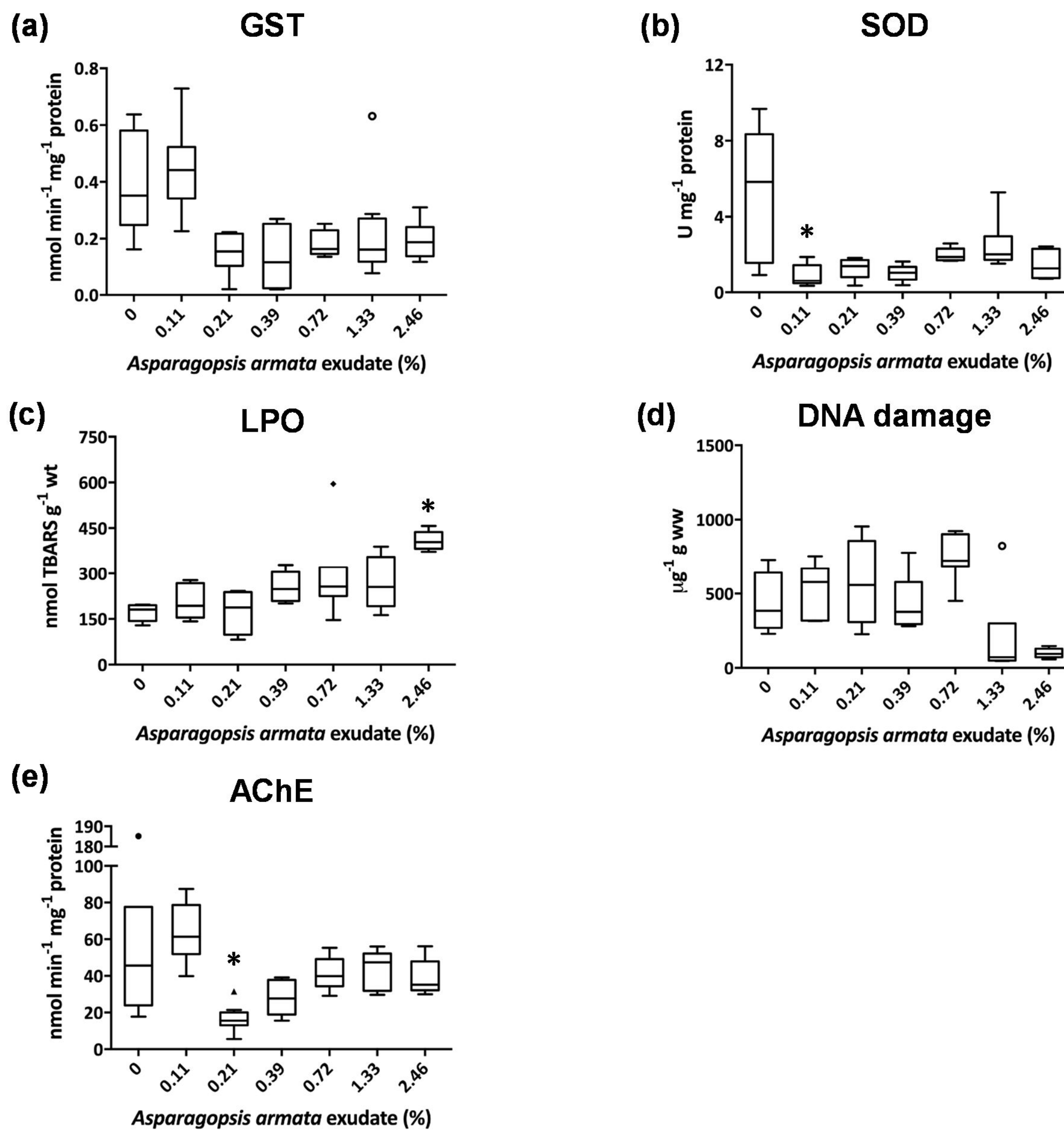

Figure 2 - Results of detoxification and antioxidant defences (a) and (b), oxidative damage (c) and (d), and neuromuscular (e) biomarkers in *Palaemon elegans* when exposed to *Asparagopsis armata* exudate exudate for 168 h. Results are shown in boxplot (i.e. the median, the first and the third quartiles, the non-outliers range and the outliers); * Significant differences from the control (Nemenyi, *p*<0.05).

3.2 Fatty acid profile

Globally, the tissues of the two invertebrates presented FAs ranging from capric acid (10:0) to nervonic acid (24:1 n9), in a total of 43 different FAs detected and identified throughout this study. The complete list of fatty acids found for the different treatments and for both species tested can be consulted in Tables S1 and S2 (supplementary data). After exposure to *A. armata* exudate, both invertebrates presented significant alterations in some particular FAs. Such carboxylic acids from both invertebrates that were considered metabolically important and presenting significant differences in at least one of the treatments (17 FA in total), were selected to be further discussed and are presented in Fig.3. To streamline the overall interpretation, these FAs were clustered according to similarities in their concentration levels across the different exudate treatments.

Heatmaps depicts thus an overall comparison of 13 significantly different fatty acids for *G. umbilicalis* (Fig. 3A) and 10 for *P. elegans* (Fig. 3B). It should be noted that there is a generalized shift in lipid metabolism between concentration 0.07% (C2) and 0.14% (C3) for *G. umbilicalis* and between concentrations 0.39% (C3) and 0.72% (C4) for *P. elegans,* where especially for *P. elegans*, more unsaturated FAs tend to increase and more saturated FAs tend to decrease. In *G. umbilicalis*, FAs were separated by two major groups. Vaccenic (18:1 n7; p=0.045), adrenic (AdA; 22:4 *n*6; p=0.01), docosapentaenoic (DPA; 22:5 *n*3; p=0.00), arachidonic (ARA; 20:4 *n*6; p=0.00) and hexadecenoic (16:1 n5; p=0.00) acids comprise the first cluster. When compared to control, there is an increase in metabolic levels of these FAs generally starting from 0.14% (C3) of exudate. The opposite was observed for the second cluster, composed by behenic acid (22:0, p=0.020), 22:3 *n*6 (p=0.007), myristoleic acid (14:1; p=0.024), dihomo-gamma-linolenic acid (DGLA; 20:3 *n*6; p=0.018), eicosadienoic acid (EDA; 20:2 *n*6; p=0.037), heneicosapentaenoic acid (HPA; 21:5 *n*3; p=0.032), eicosenoic acid (EA; 20:1 n9; p=0.031), and decanoic acid (10:0; p=0.008). Here, there is a general decrease in metabolic levels of these FAs and especially after the 0.14% concentration (C3). Exceptionally, *n*6 DGLA and eicosadienoic acids presented an increase in C5 (0.47%; p=0.018 and p=0.037, respectively), and 22:3 n6 also increase in C3 (0.14%; p=0.008).

For *P. elegans,* 3 main clusters were identified, in which the first one is composed by pentadecenoic acid (15:1), remaining invariable throughout treatments, but significantly higher for the higher concentration (C6; *p*=0.002).

In the second cluster, EA (20:1 n9; *p*=0.001) and tridecanoic acid (13:0; *p*=0.000) presented higher concentrations in control and first treatments, with further depletion in higher exudate concentrated treatments. However, the third cluster, which comprises mostly polyunsaturated FAs: DPA (22:5 *n*3; *p*=0.004), docosadienoic acid (22:2 *n*6; *p*=0.008), HPA (21:5 *n*3; *p*=0.003), 22:3 *n*6 (*p*=0.004), alpha-linoleic acid (ALA; 18:3 *n*3; *p*=0.022), ARA (20:4 *n*6; p=0.034) and decanoic acid (10:0; *p*=0.006), demonstrate an inverse behaviour to the previous cluster, by overall increased FA quantities with increasing concentrations of the exudate (from 0.72%, C4).

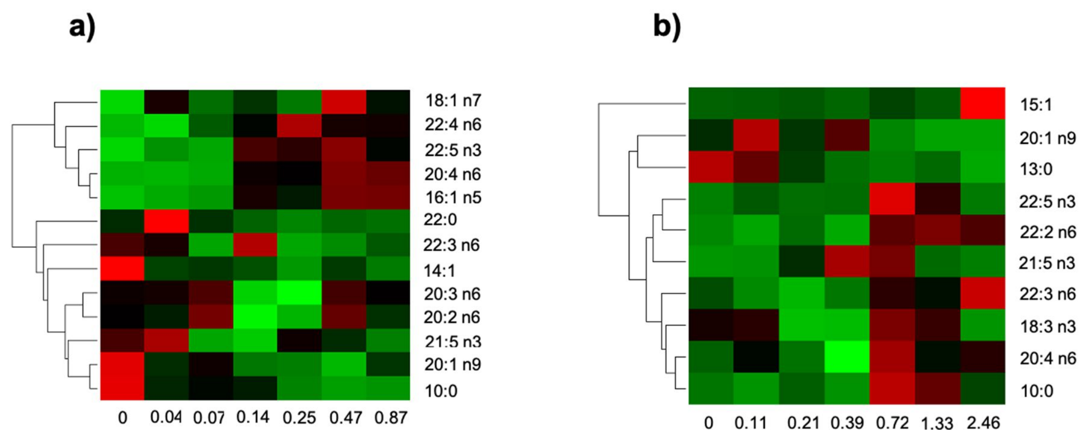

Figure 3 - Heatmap depicts an overall comparison of the significantly different fatty acids in *Gibbula umbilicalis* (**A**) and *Palaemon elegans* (**B**) on the basis of their sensitivity against different concentrations of *Asparagopsis armata* exudate. Rows are clustered using correlation distance and colour scaling was performed by row.

4. Discussion

The present study was designed to further address potential mechanisms of action involved in the previously observed effects on two coastal invertebrate species, the marine snail *Gibbula umbilicalis* and the shrimp *Palaemon elegans*, exposed to the exudate of *A. armata*

(Silva *et al*., 2020). This was performed by evaluating parameters including neuronal, detoxification and oxidative stress biochemical biomarkers and fatty acid composition. In the present study, *A. armata* exudate induced shifts in the studied endpoints, in both snails and shrimps, yet with different responses between species. The dissimilarities between biomarkers from both species suggest different metabolic processes being affected, the same way previous study (Silva *et al*., 2020) indicated different species' sensitivity and energetic impacts.

Red algae are a rich source of halogens, predominantly bromine and iodine (Paul, 2006a). In the case of *A. armata*, the major natural products known are numerous halogenated metabolites which possess a wide range of volatility and solubility (McConnell & Fenical, 1977). The overall toxicity of halogen-containing compounds seems to be derived from their abilities as alkylating agents (McConnell & Fenical, 1977) or by inducing ROS production (Dring, 2005). Also, the alkylating agents, such as haloacetones found in *A. armata*, are well-known enzyme inhibitors, able of cross-linking serine and histidine residues in various proteins (McConnell & Fenical, 1977).

Here, the activity of the enzyme GST on the marine snail was in fact significantly decreased in mid exudate concentrations. An inhibition of GST is often found as result of an increased level of produced ROS, which among other damage also inhibits enzymes, and has previously been reported for other marine snails (e.g., Cunha *et al*., 2007). In the present case, this inhibition may also be due to the aforementioned direct action of the exudate compounds on the enzyme (McConnell & Fenical, 1977), while some allelochemicals of plants are also known to act as GST inhibitors (Lee, 1991). Owing their toxicity to a myriad of secondary metabolites, including more than 100 halogenated compounds (McConnell & Fenical, 1977), exudate dilutions representing a cocktail of different compounds, present in different concentrations, are due to trigger differentiated mechanisms of action and thus non-monotonic dose-responses (Silva *et al*., 2020), a factor that increases the need for a less straightforward analysis of organism responses along concentrations.

Regarding oxidative stress parameters, for *G. umbilicalis*, the non-observed effects in antioxidant defence enzymes is concomitant with the non-observed damage in both DNA and lipids. In fact, LPO levels decreased with the snail exposure to lower concentrations of

the exudate. The rationale for this decrease, also seen in a vast number of other studies (e.g., Aderemi *et al*., 2018; Silva *et al*., 2016), is yet not clear, and entails further research and careful analysis.

Regarding the neuronal parameter, AChE is involved in the regulation of the transmission of nerve impulses, and contaminants such as chlorpyrifos has been demonstrated to inhibit AChE activity in *G. umbilicalis* (Silva *et al*., 2019). However, in this work, a significant induction of AChE was observed in *G. umbilicalis* exposed to 0.14 and 0.47% exudate concentrations. Reddy *et al*., 1990 also found an increase in the enzyme activity on crab *Barytelphusa guerini* after 4d of exposure to fluoride, a halogenated compound. The induction of AChE activity after macroalgae exudate exposure could be due to a phenomenon of overcompensation as proposed by Badiou *et al.*, (2008).

Regarding the shrimp *P. elegans*, and similar to *G. umbilicalis*, an over-production of ROS (Zhang *et al*., 2012) and/or exudate compounds' enzyme inhibitory action (McConnell & Fenical, 1977) might have resulted in the seen inhibition of SOD activity and the trend for a decreased level of GST. Further on, this inhibition might have led to the accumulation of ROS which in turn led to an increase of LPO. These results are also in agreement with the study of Box *et al*., (2009) where the invasive red macroalgae *Lophocladia lallemandii* induced the increase of MDA levels generated by lipid peroxidation, to the bivalve *Pinna nobilis*). Notwithstanding, and although alkylating agents are present in some *A. armata* extracts and have been proved to possess genotoxic properties (McConnell & Fenical, 1977), damage on DNA was not found in the present study.

In *P. elegans*, AChE was inhibited at 0.21% of exudate exposure. In literature, methanolic extracts from *Sargassum* sp. and *Gracilaria gracilis* showed to inhibit the fish *Nile tilapia* ChEs (Natarajan *et al*., 2009). Moreover, Custódio *et al*., 2016 has shown that extracts of *A. armata* had potent inhibitory capacity on AChE (58.4% at 10 mg mL^{-1}) of human cells. Typically, this enzyme inhibition is known as an early sign of behavioural impairments. Although no effects were seen for feeding activity after the same exudate exposure for 96h Silva *et al*., (2020), this enzyme inhibition may disclose potential higher-level behavioural effects in longer exposures, not addressed in the present study.

Additionally to the different species sensitivity and differentiated mechanisms of toxic response, one has to note that the choice of sub-lethal concentrations were made considering the survival effects (half the LC_{10} as top concentration) (Silva *et al*., 2020). This implied having an appreciably higher concentration range for the shrimp in this study, which may explain the more explicit oxidative and neurotoxic effects here seen.

Differentiated fatty acids profiles shifts due to exudate exposure were also observed between species. The obtained results demonstrated 13 and 10 differentiating metabolites in *G. umbilicalis* and *P. elegans*, respectively, indicating *A. armata* exudate altered fatty acid biosynthesis and metabolism in the organisms, although through a different way. Many studies have shown that pollution can change the composition of FAs from organisms in the aquatic environment (Fokina *et al*., 2013; Maazouzi *et al*., 2008; Perrat *et al*., 2013; Silva *et al*., 2017).

In a broad observation, and especially for *P.elegans*, an increase in PUFA could be observed, while saturated and monounsaturated FAs tended to decrease. The increase in PUFA content can be considered a defence mechanism, protecting the membranes from oxidation disruption (Fokina *et al*., 2013). For *G. umbilicalis*, an increase in the first cluster that includes ARA and DPA can be seen. Further, in the literature, an increase of these two PUFA was also found for the bivalve *Mizuhopecren yessoensis* after being exposed to cadmium (Chelomin & Belcheva, 1991). It is known that 22:5 *n*3 (DPA) can be retro-converted to eicosapentaenoic acid (EPA; 20:5 *n*3) and that it reacts with lipoxygenases to form distinctive oxylipins, such as the specialized pro-resolving mediators involved in the resolution of inflammation (Lipid Maps 2003). Also, omega-3 PUFAs like DPA serve as precursors of eicosanoids (prostaglandins, thromboxanes, leukotrienes, etc.), which have a wide range of physiological functions in immune system, inflammatory response, neural function, reproduction, and improve the organisms' adaptation to environmental stress (Calder, 2010). The increasing of ARA (20:4 n6) in both invertebrates is an indication that this FA was possibly required for activation of eicosanoid synthesis for the regulation of inflammation and immunity responses, triggered by the exudate (Delaporte *et al*., 2006). This increase may be an adaptive response of treated organisms to face the detrimental effects. This fact is in accordance with a study of Silva *et al*., 2017, where levels of ARA in

G. umbilicalis were observed to increase after metal exposure. Additionally, DGLA (20:3 n6) and EDA (20:2 n6), although clustered in a different group, presented increase, particularly at 0.47% (C5). These particular FAs are also involved in eicosanoid synthesis and DGLA is also desaturated to form ARA, thus explaining their increase.

In *P. elegans*, a metabolic shift between the third and fourth treatments was observed in terms of FA profiling, where a general increase in PUFA was denoted in 0.74% (C4) of *A. armata* exudate. The event may be attributed to an inflammatory response due to the increase of *n*3 and *n*6 FAs, as previously reported by Simopoulos (2002), and more evident for the shrimp than for the snail. These class of PUFA serve as potent anti-inflammatory and immunomodulatory agents. HPA (21:5 *n*3) is a stronger inhibitor of the conversion of α-linoleic acid and dihomo-γ-linolenic acid to ARA and inactivates prostaglandin H synthase as rapidly as do ARA (Larsen *et al*., 1997).

Although there is a trend to an increase in PUFA with increasing exudate concentrations in *P. elegans*, in the higher concentration (2.46%, C6) there is a decrease of *n*3 PUFA. This may be due to the loss of ability of *P. elegans* to cope with the stressor at higher concentrations, which might have led to the observed increase in lipid peroxidation levels. However, and most likely, the pro-oxidant effect of exudate altered membrane integrity and fluidity in the shrimp membrane cells, due to decreased adaptation and activity of the membrane-bound enzymes and pumps that block the membrane permeability. At the same concentration, despite the overall PUFA reduction, *n*6 and short chain FAs increased. This event is suggestive of a post-inflammatory response (Mirmonsef *et al*., 2012). This behaviour, where *n*-3 PUFA decrease and *n*-6 PUFA increase, was also previously reported in literature for *Venus verrucosa*, exposed to lead (Bejaoui *et al*., 2019), and for *Mytilus edulis* under cadmium and copper exposure (Fokina *et al*., 2013).

Comparing the responses in the two species we can highlight the fatty acids DPA and ARA with more similar responses, where there was an evident increase with the exposure. Overall, *A. armata* exudate exposure had an influence on membrane functioning by means of disturbing the membrane of the organisms, which could be significantly injurious to membrane-bound enzymes.

5. Conclusion

In the present study, potential neurotoxicity, oxidative stress mechanisms of action and fatty acid profile changes, behind the seen *A. armata* exudate impacts, were addressed in two rockpool invertebrates. In *P. elegans*, oxidative stress and neurotoxic effects were found as potential higher levels of biological toxicity drives, while for *G. umbilicalis* these routes do not seem to relate to seen impacts (i.e. feeding behaviour).

Fatty acids also revealed different metabolic responses between species, indicating that *A. armata* exudate may in fact be affecting the organisms through different pathways, despite the increase of PUFAs ARA and DPA, in both invertebrates, which points towards common inflammatory and immunity regulation response.

Given the paramount rate of expansion of this invasive species across aquatic environments, where different species are present, a greater body of research is necessary to investigate how *A. armata*, and even other potentially harmful algae blooms, impact rockpools and other environments and even how other global change drivers may act and even enhance this problem.

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Supplementary data

Table S1 - Fatty acid composition found in *Gibbula umbilicalis* exposed to *Asparagopsis armata* for 168h, plus control. Data are expressed as mg g^{-1} (mean ± standard error). SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

		Asparagopsis armata exudate (%)						
FA		0	0.04	0.07	0.14	0.25	0.47	0.87
SFA	C 10:0	0.09±0.01	0.06±0.01	0.06±0.01	0.06±0.01	0.04±0.01	0.05±0.01	0.03±0.00
	C 12:0	0.52±0.29	0.82±0.28	0.94±0.56	0.45±0.23	0.68±0.22	1.13±0.35	0.31±0.17
	C 13:0	0.08±0.02	0.11±0.03	0.30±0.12	0.23±0.07	0.22±0.06	0.07±0.02	0.07±0.02
	C 14:0	0.76±0.12	0.87±0.16	0.99±0.28	0.69±0.07	0.70±0.09	1.19±0.13	0.56±0.03
	C 15:0	0.07±0.01	0.06±0.01	0.09±0.03	0.08±0.00	0.07±0.01	0.11±0.02	0.06±0.01
	C 16:0	7.15±1.01	7.46±1.12	8.16±1.22	8.08±1.14	7.36±0.53	8.64±1.47	7.39±1.48
	C 17:0	0.04±0.02	0.03±0.01	0.02±0.01	0.03±0.01	0.03±0.01	0.04±0.02	0.04±0.01
	C 18:0	5.98±0.62	6.90±1.48	6.98±0.95	7.92±1.64	6.67±0.30	7.07±1.18	6.26±1.05
	C 20:0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	C 22:0	0.02±0.01	0.08±0.06	0.01±0.01	0.01±0.00	0.00±0.00	0.01±0.00	0.00±0.00
	C 24:0	0.11±0.02	0.10±0.03	0.10±0.05	0.07±0.02	0.14±0.06	0.08±0.03	0.04±0.01
∑SFA		14.69±1.64	16.38±2.53	17.67±2.87	17.54±2.61	15.77±0.55	18.30±2.51	14.72±2.41
MUFA	C 14:1	0.05±0.01	0.02±0.01	0.02±0.00	0.01±0.00	0.00±0.00	0.02±0.01	0.01±0.00
	C 15:1	0.01±0.00	0.00±0.00	0.01±0.00	0.01±0.01	0.01±0.00	0.02±0.01	0.01±0.00
	C 16:1 n5	0.03±0.01	0.04±0.01	0.04±0.01	0.08±0.01	0.07±0.01	0.14±0.04	0.10±0.02
	C 16:1 n7	0.25±0.13	0.14±0.03	0.13±0.03	0.22±0.08	0.15±0.02	0.32±0.12	0.12±0.02
	C 16:1 n9	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.04±0.03	0.01±0.00
	C 17:1	0.02±0.01	0.01±0.00	0.01±0.00	0.01±0.01	0.01±0.00	0.01±0.00	0.02±0.01
	C 18:1 n7	0.01±0.00	0.05±0.02	0.03±0.02	0.03±0.01	0.02±0.01	0.08±0.04	0.03±0.01
	C 18:1 n9	1.95±0.37	2.47±0.73	2.97±1.27	1.53±0.38	3.19±1.23	1.69±0.19	1.22±0.36
	C 20:1 n9	0.27±0.13	0.17±0.03	0.21±0.02	0.13±0.02	0.15±0.03	0.13±0.05	0.15±0.04
	C 22:1 n9	0.01±0.00	0.01±0.00	0.05±0.04	0.02±0.02	0.00±0.00	0.06±0.05	0.02±0.01

	C 24:1 n9	0.18±0.07	0.13±0.06	0.13±0.06	0.02±0.01	0.01±0.00	0.11±0.06	0.13±0.05
∑MUFA		2.79±0.45	3.04±0.79	3.60±1.35	2.08±0.40	3.62±1.24	2.63±0.35	1.80±0.42
PUFA	C 16:2 n4	0.10±0.02	0.12±0.03	0.12±0.02	0.14±0.03	0.10±0.02	0.13±0.03	0.16±0.05
	C 16:2 n7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	C 18:2 n6	0.69±0.14	0.76±0.12	0.92±0.20	0.56±0.07	0.69±0.22	0.87±0.19	0.64±0.09
	C 16:3 n4	0.09±0.08	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.00
	C 18:3 n6	0.01±0.00	0.03±0.01	0.04±0.03	0.010.00	0.03±0.01	0.08±0.05	0.02±0.01
	C 18:3 n3	0.05±0.01	0.15±0.06	0.10±0.04	0.10±0.04	0.07±0.02	0.17±0.04	0.06±0.02
	C 18:4 n1	0.02±0.00	0.01±0.00	0.04±0.02	0.02±0.00	0.03±0.02	0.02±0.01	0.01±0.00
	C 18:4 n3	0.04±0.03	0.30±0.18	0.09±0.01	0.26±0.16	0.19±0.06	0.14±0.03	0.10±0.02
	C 20:2 n6	0.26±0.10	0.27±0.18	0.48±0.27	0.06±0.02	0.12±0.05	0.34±0.08	0.21±0.06
	C 20:3 n3	0.02±0.01	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
	C 20:3 n6	0.12±0.01	0.13±0.02	0.13±0.02	0.08±0.01	0.07±0.01	0.15±0.03	0.10±0.01
	C 20:4 n3	0.09±0.07	0.07±0.05	0.05±0.02	0.09±0.08	0.21±0.08	0.04±0.02	0.07±0.05
	C 20:4 n6	0.20±0.04	0.20±0.02	0.24±0.06	0.47±0.06	0.46±0.03	0.77±0.14	0.55±0.09
	C 20:5 n3	0.38±0.10	0.53±0.10	0.38±0.08	0.64±0.05	0.57±0.06	1.00±0.28	0.65±0.24
	C 21:5 n3	0.00±0.00	0.01±0.00	0.00±0.00	0.00±0.00	0.01±0.00	0.01±0.00	0.00±0.00
	C 22:2 n6	0.02±0.01	0.01±0.00	0.02±0.01	0.01±0.01	0.01±0.00	0.04±0.02	0.02±0.01
	C 22:3 n6	0.02±0.01	0.02±0.01	0.00±0.00	0.03±0.01	0.00±0.00	0.00±0.00	0.01±0.00
	22:4w6	0.01±0.00	0.00±0.00	0.02±0.01	0.03±0.01	0.05±0.01	0.05±0.04	0.03±0.01
	C 22:5 n3	0.08±0.02	0.13±0.02	0.13±0.03	0.30±0.04	0.29±0.01	0.45±0.13	0.22±0.03
	22:5w6	0.01±0.00	0.03±0.01	0.02±0.01	0.01±0.00	0.02±0.01	0.03±0.02	0.02±0.01
	C 22:6 n3	0.22±0.07	0.48±0.14	0.25±0.11	0.40±0.05	0.36±0.11	0.64±0.23	0.74±0.38
∑PUFA		2.56±0.45	3.25±0.38	3.01±0.67	1.43±0.18	3.26±0.36	4.91±0.93	3.62±0.88
Unsat./Sat		0.36±0.02	0.42±0.05	0.35±0.04	0.33±0.04	0.44±0.07	0.41±0.03	0.39±0.06
n3		1.04±0.30	1.68±0.32	1.01±0.20	1.80±0.25	1.70±0.25	2.45±0.64	1.86±0.64
n6		1.34±0.27	1.45±0.30	1.88±0.55	1.26±0.07	1.46±0.25	2.33±0.32	1.59±0.23
n7		0.26±0.13	14.40±2.55	0.16±0.05	0.25±0.08	0.17±0.02	0.40±0.15	0.15±0.03
n9		2.41±0.40	0.58±0.14	3.37±1.30	1.71±0.39	3.35±1.23	2.03±0.26	1.53±0.39

n3/n6	0.85±0.20	1.48±0.37	0.68±0.18	1.46±0.24	1.32±0.26	1.00±0.20	1.07±0.22
n	7	6	6	7	7	6	6

Table S2 - Fatty acid composition found in *Palaemon elegans* exposed to *Asparagopsis armata* for 168h, plus control. Data are expressed as mg g^{-1} (mean ± standard error). SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

		Asparagopsis armata exudate (%)						
FA		0	0.11	0.21	0.39	0.72	1.33	2.46
SFA	C 10:0	0.06±0.02	0.05±0.02	0.04±0.01	0.04±0.01	0.11±0.02	0.06±0.01	0.07±0.01
	C 12:0	1.12±0.57	0.33±0.16	0.30±0.11	1.09±0.42	0.62±0.20	0.35±0.21	0.57±2.69
	C 13:0	0.59±0.22	0.27±0.07	0.08±0.02	0.08±0.03	0.07±0.02	0.07±0.01	0.04±0.01
	C 14:0	0.83±0.27	0.43±0.08	0.24±0.05	0.79±0.27	0.60±0.12	0.42±0.14	0.57±0.13
	C 15:0	0.05±0.01	0.04±0.01	0.03±0.00	0.05±0.01	0.06±0.01	0.04±0.01	0.08±0.03
	C 16:0	7.15±1.44	5.21±0.46	3.68±0.44	5.48±1.26	5.49±1.13	4.49±0.65	5.67±0.95
	C 17:0	0.03±0.00	0.02±0.00	0.01±0.00	0.02±0.01	0.03±0.01	0.02±0.00	0.03±0.01
	C 18:0	7.89±1.95	5.32±0.66	3.96±0.64	7.25±1.81	5.34±0.91	4.65±0.78	5.64±0.90
	C 20:0	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.02±0.02
	C 22:0	0.10±0.05	0.01±0.00	0.01±0.00	0.03±0.01	0.03±0.01	0.02±0.01	0.15±0.12
	C 24:0	0.01±0.00	0.07±0.03	0.04±0.02	0.01±0.00	0.01±0.00	0.01±0.01	0.13±0.08
∑SFA		11.26±2.48	11.69±1.23	8.35±1.02	14.82±3.59	12.35±2.13	10.11±1.70	11.26±2.48
MUFA	C 14:1	0.01±0.01	0.00±0.00	0.01±0.01	0.01±0.00	0.02±0.00	0.01±0.00	0.01±0.01
	C 15:1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.00	0.00±0.00	0.04±0.01
	C 16:1 n5	0.03±0.01	0.03±0.00	0.02±0.00	0.02±0.01	0.03±0.01	0.02±0.00	0.03±0.01
	C 16:1 n7	0.23±0.07	0.13±0.06	0.07±0.01	0.09±0.02	0.16±0.04	0.11±0.03	0.14±0.02
	C 16:1 n9	0.03±0.02	0.01±0.00	0.00±0.00	0.01±0.00	0.01±0.00	0.00±0.00	0.01±0.00
	C 17:1	0.01±0.01	0.01±0.00	0.01±0.00	0.01±0.00	0.02±0.00	0.01±0.00	0.02±0.00
	C 18:1 n7	0.05±0.02	0.03±0.01	0.01±0.01	0.01±0.00	0.04±0.01	0,02±0.00	0.04±0.01
	C 18:1 n9	2.84±1.45	1.48±0.21	0.51±0.17	1.37±0.41	1.77±0.49	1.16±0.24	0.01±0.00

	C 20:1 n9	0.02±0.01	0.02±0.01	0.00±0.00	0.01±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	C 22:1 n9	0.01±0.00	0.01±0.01	0.00±0.00	0.05±0.04	0.01±0.00	0.00±0.00	0.00±0.00
	C 24:1 n9	0.09±0.03	0.16±0.05	0.09±0.03	0.11±0.03	0.18±0.11	0.08±0.03	0.08±0.04
∑MUFA		3.31±1.54	1.88±0.26	0.73±0.18	1.70±0.44	2.25±0.59	1.42±0.26	2.64±0.74
PUFA	C 16:2 n4	0.15±0.04	0.11±0.01	0.08±0.01	0.13±0.04	0.14±0.03	0,10±0.02	0.13±0.02
	C 16:2 n7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0,00±0.00	0.00±0.00
	C 18:2 n6	0.88±0.27	0.48±0.05	0.22±0.03	0.67±0.30	1.30±0.64	0.38±0.08	0.43±0.10
	C 16:3 n4	0.03±0.01	0.02±0.00	0.02±0.00	0.03±0.01	0.02±0.00	0.01±0.00	0.02±0.00
	C 18:3 n6	0.02±0.01	0.01±0.01	0.01±0.01	0.01±0.01	0.02±0.01	0.03±0.01	0.11±0.08
	C 18:3 n3	0.07±0.02	0.05±0.01	0.01±0.00	0.03±0.02	0.09±0.03	0.05±0.02	0.03±0.01
	C 18:4 n1	0.15±0.04	0.12±0.13	0.08±0.01	0.10±0.02	0.20±0.04	0.12±0.01	0.16±0.02
	C 18:4 n3	0.26±0.18	0.17±0.08	0.10±0.04	0.17±0.10	0.09±0.03	0.12±0.05	0.19±0.06
	C 20:2 n6	0.40±0.29	0.08±0.02	0.08±0.05	0.20±0.10	0.20±0.06	0.07±0.04	0.08±0.04
	C 20:3 n3	0.02±0.01	0.01±0.01	0.01±0.00	0.01±0.00	0.02±0.00	0.01±0.00	0.03±0.02
	C 20:3 n6	0.07±0.07	0.07±0.03	0.05±0.01	0.08±0.02	0.07±0.01	0.05±0.01	0.07±0.01
	C 20:4 n3	0.02±0.01	0.13±0.27	0.05±0.02	0.02±0.02	0.02±0.01	0.07±0.05	0.15±0.07
	C 20:4 n6	0.19±0.05	0.14±0.07	0.06±0.02	0.05±0.02	0.21±0.03	0.10±0.02	0.14±0.03
	C 20:5 n3	0.68±0.23	0.59±0.34	0.32±0.09	0.30±0.08	0.83±0.17	0.40±0.09	0.58±0.15
	C 21:5 n3	0.01±0.00	0.00±0.00	0.01±0.00	0.02±0.01	0.01±0.00	0.00±0.00	0.00±0.00
	C 22:2 n6	0.03±0.01	0.01±0.01	0.02±0.01	0.01±0.00	0.09±0.03	0.07±0.02	0.06±0.02
	C 22:3 n6	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.01±0.00	0.00±0.00	0.01±0.01
	22:4w6	0.04±0.04	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	C 22:5 n3	0.03±0.00	0.05±0.04	0.02±0.01	0.04±0.02	0,13±0.03	0.06±0.01	0.03±0.01
	22:5w6	0.01±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.03±0.01	0.00±0.00	0.01±0.00
	C 22:6 n3	0.28±0.06	0.29±0.15	0.15±0.05	0.14±0.06	0.55±0.16	0.29±0.09	0.38±0.10
∑PUFA		3.21±0.79	2.23±0.32	1.21±0.14	1.94±0.51	3.85±0.80	1.83±0.37	3.60±0.96
Unsat./Sat		0.36±0.07	0.35±0.02	0.26±0.05	0.26±0.04	0.47±0.06	0.32±0.02	1.25±0.86
n3		1.38±0.36	1.29±0.27	0.67±0.14	0.75±0.17	1.75±0.33	1.02±0.28	2.50±0.93
n6		1.65±0.63	0.80±0.08	0.45±0.08	1.04±0.37	1.95±0.69	0.70±0.15	0.94±0.13

n7	0.28±0.08	0.15±0.03	0.09±0.02	0.10±0.03	0.21±0.04	0.13±0.03	0.18±0.03
n9	2.98±1.49	1.68±0.25	0.61±0.17	0.27±0.08	0.79±0.18	0.44±0.10	0.73±0.10
n3/n6	1.32±0.56	1.61±0.28	1.84±0.43	1.75±0.72	1.52±0.35	1.99±0.50	2.81±1.03
n	5	8	7	7	7	8	7

Chapter VI

The effects of the invasive seaweed *Asparagopsis armata* on native rock pool communities: evidences from experimental exclusion

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Chapter VI – The effects of the invasive seaweed *Asparagopsis armata* on native rock pool communities: evidences from experimental exclusion [1]

Abstract

Biological invasions represent a threat to ecosystems, through competition and habitat destruction, which may result in significant changes of the invaded community. *Asparagopsis armata* is a red macroalgae (Rodophyta) globally recognized as an invasive species. It is found from the intertidal to shallow subtidal areas, on rock or epiphytic, forming natural vegetation belts on exposed coasts. This study evaluated the variations on native intertidal seaweed and macroinvertebrate assemblages inhabiting rock pools with and without the presence of the invasive macroalgae *A. armata*. To achieve this, manipulation experiments on Atlantic (Portugal) rock pools were done. Three rock pools were maintained without *A. armata* by manual removal of macroalgae, and three others were not experimentally manipulated during the study period and *A. armata* was freely present. In this study the variations between different rock pools were assessed. Results showed different patterns in the macroalgae composition of assemblages but not for the macrobenthic communities. *Ellisolandia elongata* was the main algal species affected by the invasion of *A. armata*. Invaded pools tended to show less species richness, showing a more constant and conservative structure, with lower variation of its taxonomic composition than the pools not containing *A. armata*, where the variability between samples was always higher. Although the importance of the achieved results, further data based on observation of long-term series are needed, in order to understand the effects of the invader *A. armata* on native macroalgal assemblage.

Keywords: *Asparagopsis armata*, Biodiversity, Intertidal assemblages, Invasive exotics, Marine invasion, Non-indigenous species (NIS)

[1] Silva, C.O.; Lemos, M.; Gaspar, R.; Gonçalves, C.; Neto, J. Preprints 2020, 2020100089 (doi: 10.20944/preprints202010.0089.v1).

1. Introduction

The extent and significance of biological invasions in coastal marine ecosystems has become increasingly evident in recent years and has been recognized as one of the main causes of biodiversity loss and changes for native populations, community dynamics, and major ecosystem processes (Grosholz, 2002). Contrarily to the presence of an non-indigenous species (NIS) (also exotic species, introduced species, alien species, foreign species, or non-native species), which may be present in a community for long time without significantly affecting it, the increasing rate of biological invasions (by invasive exotics) is affecting ecosystems with strong ecological impacts on resident assemblages (Olabarria *et al*., 2009; Piazzi *et al*., 2001; Sánchez *et al*., 2005).

Displacement of native flora by invasive exotic macroalgal species has been largely reported as provoking changes of species' composition and their trophic food webs (Piazzi *et al*., 2001; Sánchez *et al*., 2005; Stæhr *et al*., 2000). Together with climate change, NIS macroalgae are becoming one of the most important threats to marine biodiversity (Stachowicz *et al*., 2002). The impacts associated with these introductions are typically expressed as community dominance through the monopolization of space and changing competitive relationships in native assemblage (Schaffelke & Hewitt, 2007).

Invasive exotics have been shown to alter benthic habitats and biotic communities, resulting in potential ecosystem impacts in a wide geographical range. Important examples are: *Sargassum muticum* in the Galician coast (northwestern Spain) (Gestoso *et al*., 2012; Olabarria *et al*., 2009), *Asparagopsis armata* Harvey 1855 in western Atlantic coast of the Iberian Peninsula (Rubal *et al*., 2018), *Codium fragile* and *Grateloupia turuturu* in New England, USA (Jones & Thornber, 2010), to name a few.

The rhodophyta seaweed *Asparagopsis armata* is native from Southern Australia and New Zealand (Horridge, 1951). In Europe, this macroalgae was introduced in the Atlantic and Mediterranean in the 1920s. The species is now widely distributed from the British Isles to Senegal, including the Azores, Canary, and Madeira Islands (Cacabelos *et al*., 2020), where it is considered an invasive exotic species. This seaweed is regarded as invasive because it spreads avidly in receiving habitats in short time, colonizing a wide area, displacing native

species and producing a significant change in terms of community composition (Chualáin *et al.*, 2004; Soler-Hurtado & Guerra García, 2011). *Asparagopsis armata* presents lateral basal branches with retrorse spines (harpoon-like branches), which become entangled among other marine organisms thus permitting thalli to sprawl loosely over large areas (Andreakis, 2006). It is found from the intertidal to shallow subtidal areas, on rock or epiphytic, forming compact vegetation belts on exposed coasts.

In this study, the main aim was to evaluate the effect of *A. armata* on intertidal seaweed and macroinvertebrate assemblages using a removal experiment in which the presence of this exotic species was manipulated. It is predicted that with the presence of *A. armata* in certain pools, the remaining assemblages would be distinct, in terms of composition and structure, from those found in rockpools where *A. armata* was experimentally removed.

2. Materials and methods

2.1. Study area

The study was carried out from February 2018 to December 2018 in Portinho da Areia Norte (WGS84: 39.369587, -9.377899) at the south part of the Peniche peninsula, central western coast of Portugal. It belongs to an exposed coast to high wave action, the national coastal water type A5, but very near of the transition southward to coastal water type A6 (Bettencourt *et al.*, 2004). In January 2018, 6 tide pools were randomly selected in the rocky intertidal area, with relatively similar size and invaded by *A. armata*. All pools were located at approximately the same tide level, which allow them to be isolated from the sea about the same time during a tidal cycle. The tidal rock pools position (semi-exposed sites) allowed seawater renewal every tidal cycle. Four hours was the duration time of rockpools, from formation to its complete disappearance. Two hours before and 2 hours after the low tide moment was the full period to the complete exposure.

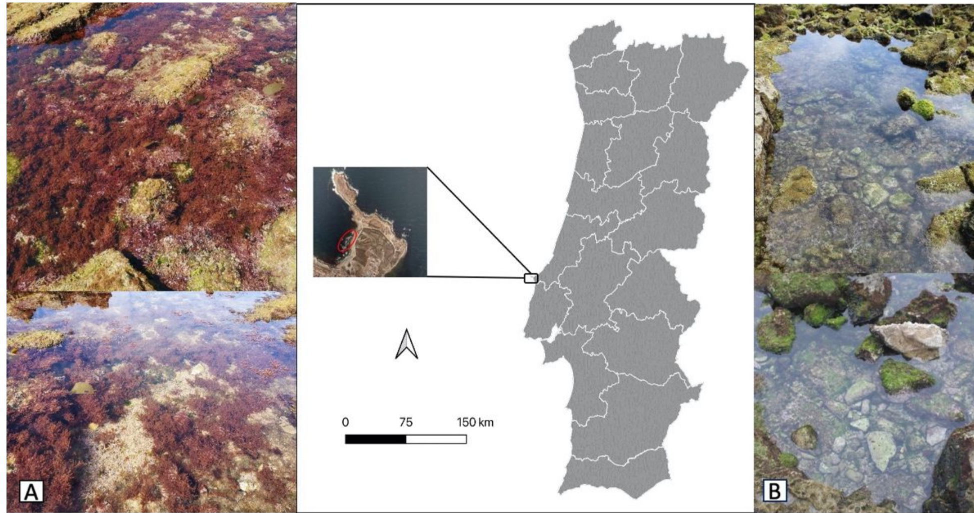

Figure 1 - Map of Peniche peninsula, western coast of Portugal, showing the location and photos of the sampling areas: **A** – Pools (A) with *A. armata*; **B** – Pools (C) without *A. armata*.

2.2. Field sampling and laboratory procedures

Sampling was performed during low-tide. Six intertidal rock pools (exposed during low tide) were used on experimental manipulations. Three rock pools were maintained without *A. armata* (C pools) by regular manual removal of macroalgae so that the effect of its absence could be assessed in the community. Three other rock pools were not experimentally manipulated during the study period and *A. armata* was freely present (A pools). The manipulation period lasted for 10 months and community samples were collected twice per season. The manipulation was maintained over the course of the experiment by periodically removing new *Asparagopsis* recruits, every 2-3 weeks.

When rockpools were formed, physical and chemical parameters were measured for Initial water pool conditions. The water temperature (°C), conductivity (µS cm^{-1}), oxidation-reduction potential (ORP) (mV), salinity, dissolved oxygen (DO) (%), and pH parameters were registered in situ (YSI Professional Plus handheld multiparameter probe). This procedure was repeated before the rockpools disappear, in all rockpools, for the assessment of final conditions. While the rockpools were formed, biological samples were collected inside each selected pool by removing the inner material, using a paint scraper, from a 0.1 × 0.1 m sampling square, randomly placed on the rocky surface, and immediately sorted for taxonomic identification and abundance quantification of biological material.

In all cases, sampling consisted of complete removal of all algae and macroinvertebrates present in randomly chosen plots. In the laboratory, each sample was washed in tap water. Water was then sieved (mesh size: 0.5 mm) to retain the macrofaunal invertebrates and macroalgae were separated. Macroalgae were identified to species, dried to constant weight (60 °C, 48 h), and weighed to the nearest 0.01 g. Macroalgae biomass was determined as dry weight (DW). Macroinvertebrates were preserved in formalin (4%) and inked with rose Bengal for later counting and identification, to the lowest possible taxonomic level (usually species).

2.3. Statistical analyses

2.3.1. Physical-chemical parameters analysis

The environmental parameters (temperature, conductivity, oxidation-reduction potential, salinity, dissolved oxygen and pH) were used to ordinate the sampling pools by performing principal coordinate (PCO) analyses. Euclidean similarity measure was used in the calculation of similarity matrices, after square root transformation, followed by normalization. Then, to see differences between samples, average for factor "pool*season" was selected.

2.3.2. Macroalgae data analysis

Macroalgae biomass was converted to dry-weight per unit (g DW m^{-2}). Bray Curtis similarity measure was used in the calculation of similarity matrices, after fourth root transformation of data.

The statistical significance of variance was tested using 9999 permutations of residuals under a reduced model, with a significance level of α-level of 0.05. PERMANOVA was applied including two fixed factors, 'Pools' (two levels: A - with *A. armata* and C - without *A. armata*) and 'Season' (nested in four levels: Wi (winter), Sp (spring), Su (summer) and Au (autumn)). *Asparagopsis armata* was not included in this analysis in order to identify effects on native diversity. To test whether differences of assemblages between pools were

due to different multivariate dispersion between groups rather than in the location of centroids, the PERMDISP procedure was done.

To identify the taxa which contribute mostly to the communities' structural variation between sites, Similarity Percentage Analysis (SIMPER) was applied. Dissimilarities between groups were assessed using two-way crossed designs with factors 'Pools' and 'Season' (as for PERMANOVA), with a 95% cut off for macroalgae. Principal Coordinate Analysis (PCO) was used as an ordination method to visualize patterns in data. Vectors based on Pearson correlations (greater than 0.5 to target variables with high correlations) were used.

2.3.3. Macrofauna data analysis

Abundance data of invertebrates was converted to density (ind. m^{-2}). The Bray-Curtis similarity measure was used in the calculation of similarity matrices, after the fourth root transformation of data (to reduce natural species dominance). PERMANOVA was performed to test differences between pools and stations, followed by pair-wise tests. The statistical significance of variance components was tested using 9999 permutations of residuals under a reduced model, with an a priori chosen significance level of $\alpha = 0.05$. The Similarity Percentages- species contributions (SIMPER) analysis was used to determine which macrofauna species contributed most for the dissimilarity between pools and stations. Dissimilarities between groups were assessed using two-way crossed designs with factors 'Pools' and 'Season' (as for PERMANOVA), with an 85% cut off for macroinvertebrate. Principal Coordinate Analysis (PCO) was used as an ordination method to visualize patterns in data. Vectors based on Pearson correlations (greater than 0.5 to target variables with high correlations) were used.

The diversity of macrobenthic fauna was assessed by different ecological indices: 1) Margalef richness index (d) (Margalef, 1968); 2) Shannon-Wiener diversity index ($\log_e$); 3) Pielou evenness index (J') (Pielou, 1969); and 4) Simpson domination index ($1-\lambda$) (Simpson, 1949), using the following algorithms:

(1) $d=(S-1)/\log(N)$;

(2) $H'=\Sigma pi\log(pi)$;

(3) $J'=H'/\log(S)$

(4) $1-\lambda=1-\Sigma(Ni*(Ni-1)/(N*(N-1))$

Where S is the number of species, N is the total number of individuals, pi is the proportion of abundance of species.

All multivariate analyses were carried out with PERMANOVA+ for PRIMER software (PRIMER v6 & PERMANOVA+ v1, PRIMER-E Ltd.).

Two-way analysis of variance (ANOVA) was applied to ecological indices**.** Considered factors were: 1) Pools, with two levels; and 2) Season with four levels, using SigmaPlot software for Windows, version 12.0.

3. Results

3.1. Environmental data

The physico-chemical parameters exhibited no significant differences between the initial and final rockpools conditions. Although, at the end of emersion period, the environmental variables were markedly different between seasons (*P*(perm)=0.0001) (Table I).

Table I - Summary of PERMANOVA of physico-chemical parameters from the sampling pools after it was created (Initial) and before it was flooded (Final).

Source of variation	Initial				Final			
	df	**MS**	**Pseudo-F**	**P(perm)**	**df**	**MS**	**Pseudo-F**	**P(perm)**
Pools	1	3.891	0.667	0.582	1	1.058	0.234	0.9636
Seasons	3	9.480	1.626	0.090	3	28.037	6.213	**0.0001**
Pools x Seasons	3	4.977	0.853	0.544	3	2.303	0.510	0.9496

The PCO analysis indicated that the first two axes explained a total of 80.3% and 82.2% variation for the initial pool conditions and final pool conditions, respectively Figure 2 A and B). In the Initial conditions, the parameters strongly correlated with the first axis (PCO1; $r > \pm 0.80$) were temperature (r = 0.80) and conductivity (r = - 0.91). And DO (%) (r = 0.95) and ORP (mV) (r = -0.86), with PCO2. In the Final conditions, pH showed the strongest correlations (r = 0.97) with PCO1 and conductivity (r = 0.83) with PCO2. Before the emersion (A), there is no clear separations between seasons. After being isolated from the sea during low tide, the parameters showed more consistency, and different pools “A” and “C” showed similar pattern per season.

The selected rockpools presented different volumes and depth, which could affect assemblage composition and bias the final results, but no physico-chemical variability existed between pool A and C initial conditions.

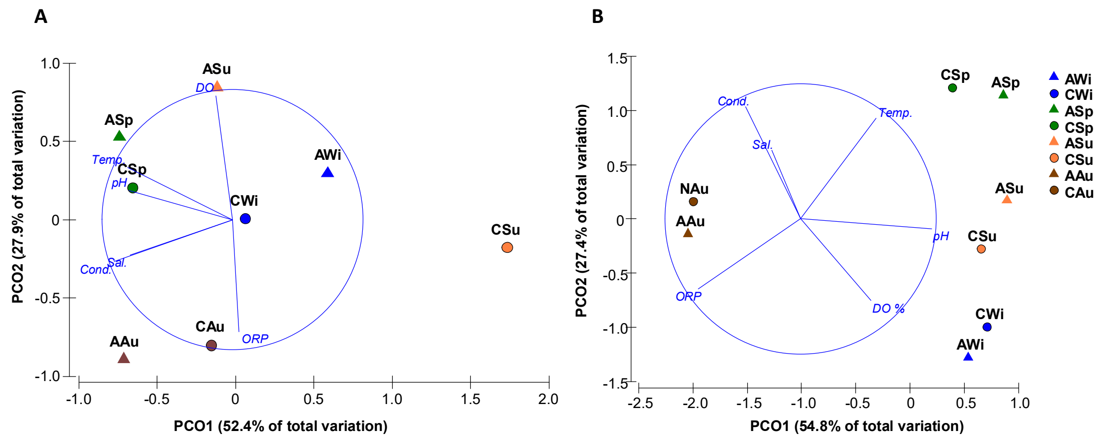

Figure 2 - Principal Coordinates Ordination (PCO) of the physico-chemical parameters: (**A**) Initial pool conditions; (**B**) Final pool conditions. Vectors are the raw Pearson correlations of variables with the PCO axes. Key: upward triangles = Pool "A"; circles = Pool "C"; Wi = winter; Sp = spring; Su = summer; Au = autumn.

3.2. Assemblage composition

In the studied assemblages, 50 macroalgal taxa were identified plus *Asparagopsis armata* and its tetrasphorophyte phase (*Falkenbergia rufolanosa*); in total: 37 Rhodophyta, 7 Chlorophyta and 8 Ochrophyta (Appendix A). In both pool A and pool C there were 48 and 44 macroalgae taxa, respectively. The group Rhodophyta showed higher species richness (34 and 30 taxa for pool A and pool C, respectively). Chlorophyta and Phaeophyceae had the same number of taxa (7) in pool C and pool A. Pools C and A from summer season obtained the highest mean number of species (15.4±0.66 and 15.4±0.93, respectively), whereas pool C in winter obtained the lowest number (6.25±0.74) (Fig.1, A). Mean biomass highest value was found for pool C in winter (9.13±2.84 g DW m^{-2}), when *Asparagopsis armata* mean biomass was 1.95±0.65 g DW m^{-2} while the lowest value (0.17±0.07 g DW m^{-2}) was found for pool A in summer, with 0.70±0.33 g DW m^{-2} of *A. armata* biomass (Fig. 3).

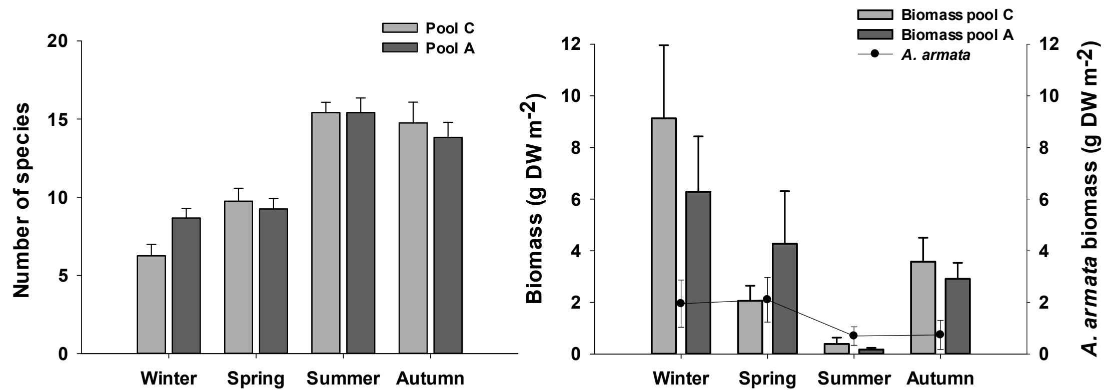

Figure 3 – Mean number of species (± SE) (A) and macroalgae mean biomass (g DW m^{-2} ± SE) with *Asparagopsis armata biomass* mean (SE) (B) per rockpool for all seasons.

PERMANOVA (Table II) revealed statistically significant differences in macroalgae biomass between pools (*P*(perm)=0.0192), between seasons (*P*(perm)=0.0001) and also the interaction Pools*Seasons (*P* (perm)=0.0046. But such patterns were not consistent in time. The pairwise on the "pools" revealed only significant differences between spring and summer season (*P*(perm)=0.0025 and 0.0257), respectively. The lack of significant differences on the PERMDISP analysis indicated that the dispersion of samples did not provide a significant contribution (F = 1.4, *p* = 0.3) to the differences detected by PERMANOVA, indicating that there is only a location effect.

Table II - Summary of PERMANOVA analyses

Source	df	MS	Pseudo-F	P(perm)
Stations	1	3636	2.5185	**0.0192**
Seasons	3	14861	10.294	**0.0001**
Stations x Seasons	3	2967.9	2.0558	**0.0046**
Groups		**t**		**P(perm)**
Winter A ≠ C		1.198		0.2208
Spring A ≠ C		1.9789		**0.0025**
Summer A ≠ C		1.4354		**0.0257**
Autumn A ≠ C		0.9784		0.4438

SIMPER analysis identified taxa that contributed for the differentiation between invaded (Pools A) and non-invaded (Pools C) rockpools, with 60.2% dissimilarity between groups.

The top three species at the *A. armata* invaded sites (Pool A) that most contributed to this dissimilarity percentage (Table III) were *Vertebrata thuyoides/Vertebrata fruticulosa* (5.91%), *Ulva* spp. (5.86%) and *Lithophyllum incrustans* (5.09%). At the pools without the invasive macroalgae (Pool C), the species that most contributed to this dissimilarity were *Ellisolandia elongata* (21.73%), *Mesophyllum lichenoides* (4.12%), and *Jania rubens* (3.41%). Some species were equally abundant along all sampling areas (as *Gastrocolonium ovatum* and *Amphiroa* spp.)

Table III - Contribution of individual taxa to the average Bray-Curtis dissimilarity in macroalgae assemblage (Pool C, without *Asparagopsis armata*; and Pool A, with *A. armata*).

Taxa	Pool C	Pool A	Average Dissimilarity	Contribution %
	Average abundance			
Ellisolandia elongata	**1.79**	1.26	13.08	21.73
Vertebrata thuyoides/Vertebrata fruticulosa	0.39	**0.42**	3.56	5.91
Ulva spp.	0.32	**0.54**	3.53	5.86
Lithophyllum incrustans	0.23	**0.24**	3.06	5.09
Mesophyllum lichenoides	**0.22**	0.18	2.48	4.12
Chondracanthus acicularis	0.19	**0.30**	2.27	3.77
Jania rubens	**0.19**	0.14	2.05	3.41
Ulva clathrata	0.16	**0.17**	1.91	3.18
Ceramium spp./*Gayliella* spp.	0.25	**0.30**	1.69	2.80
Champia parvula	0.18	**0.21**	1.63	2.71
Osmundea pinnatifida/Laurencia pyramidalis	0.12	**0.15**	1.51	2.52
Crouania attenuata	0.11	**0.14**	1.47	2.45
Cladophora spp.	**0.11**	0.10	1.39	2.30
Polysiphonia spp. *or Other Rhodomelaceae*	0.10	**0.11**	1.32	2.19
Peyssonelia spp.	**0.11**	0.06	1.27	2.11
Caulacanthus ustulatus	**0.10**	0.08	1.25	2.07
Gastrocolonium ovatum	0.08	0.08	1.11	1.84
Amphiroa spp.	0.07	0.07	1.05	1.74

Regarding the macroinvertebrates, in both pool types, five groups contributed to about 90% of the total density. They were Gastropoda, Malacostraca, Bivalvia, Polyplacophora, and Polychaeta, with the higher species richness belonging to Gastropoda (17 and 12 taxa for pool C and A, respectively) (Appendix B). The macroinvertebrates highest mean number of species was registered in pool C in autumn (13.83±1.23) and the lowest values were recorded in pool C in winter (6.73±0.93) (Fig. 4, A). Regarding the mean density, the highest value was found for pool C in autumn (1743.3 ± 285.3 ind m^{-2}), when *A. armata* biomass was 0.74 ± 0.40 g DW m^{-2} (nearly the lowest), while lower values were found for pool C in winter (251.8 ± 37.8) when *A. armata* biomass was registered with values of 1.95 ± 0.65 g DW m^{-2} (nearly the highest) (Fig. 4, B).

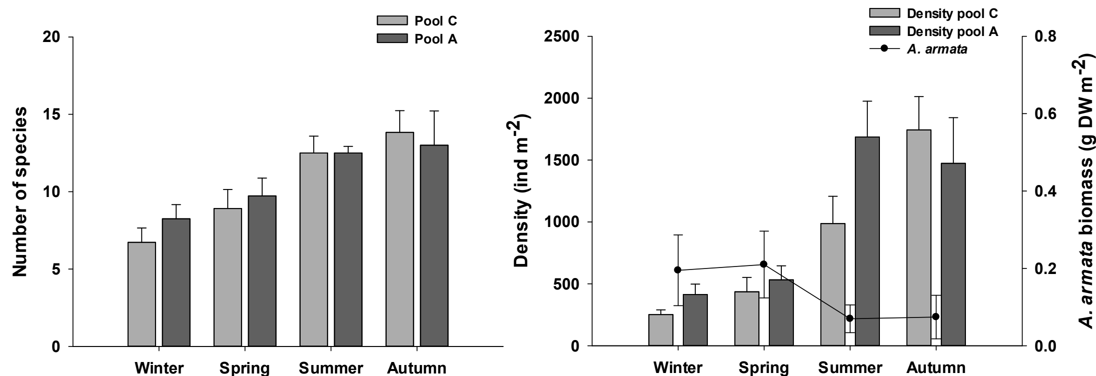

Figure 4 – Mean number of species (SE) (**A**) and macroinvertebrate mean density (ind m^{-2} ± SE) with *Asparagopsis armata biomass* mean (SE) (**B**) per rockpool for all seasons.

Univariate analyses did not detect significant differences between the two pools for the diversity of benthic macrofauna or for S, H' or N, except for factor 'season' concerning the number of taxa (S), the total number of individuals (N) and for the Margalef index (Table IV; Fig. 5, A–D).

Table IV- Results of ANOVAs testing for differences in the total number of taxa (S), total number of individuals (N), Shannon's diversity index (H'), Margalef richness (d), Pielou eveness (J) and Simpson domination (1-D). Significant differences are indicated in bold.

Source of variation	df	S			N			H’		
		MS	F	P	MS	F	P	MS	F	P
Pools	1	2.22	0.18	0.68	0.19	1.99	0.16	0.007	0.04	0.85
Season	3	123.35	9.83	**0.001**	2.10	22.53	**0.001**	0.30	1.4	0.24
Pools*Season	3	4.13	0.33	0.804	0.10	1.07	0.37	0.09	0.41	0.75
Residual	61	12.55			0.09			0.21		
	68	16.93			0.18			0.20		
Source of variation	**df**	**d**			**J**			**1-λ**		
		MS	F	P	MS	F	P	MS	F	P
Pools	1	0.005	0.02	0.88	0.00 1	0.05	0.81	0.005	0.18	0.68
Season	3	1.13	4.84	**0.004**	0.04	2.61	0.06	0.02	0.78	0.51
Pools*Season	3	0.08	0.35	0.79	00.0 05	0.27	0.85	0.006	0.23	0.88
Residual	61	0.23			0.02			0.03		
	68	0.26			0.02			0.03		

In general, Margalef index (Fig. 5A) presented higher values in Autumn, pool C with 1.72±0.2 and pool A with 1.66±0.3, and lower values in winter, where pool A got higher values (1.22±0.1) this time. Values for Pielou (Fig. 5B) were higher in winter and spring, and Simpson (Fig. 5D) were very similar around all year. Shannon index showed a similar pattern to Simpson index, with values ranging from 1.55 in winter and 1.70 in spring for pool A and to 1.36 in winter and 1.8 in spring for pool C.

For all indices there were no significant differences between pools (p>0.05) and the interaction pools*season (p>0.05).

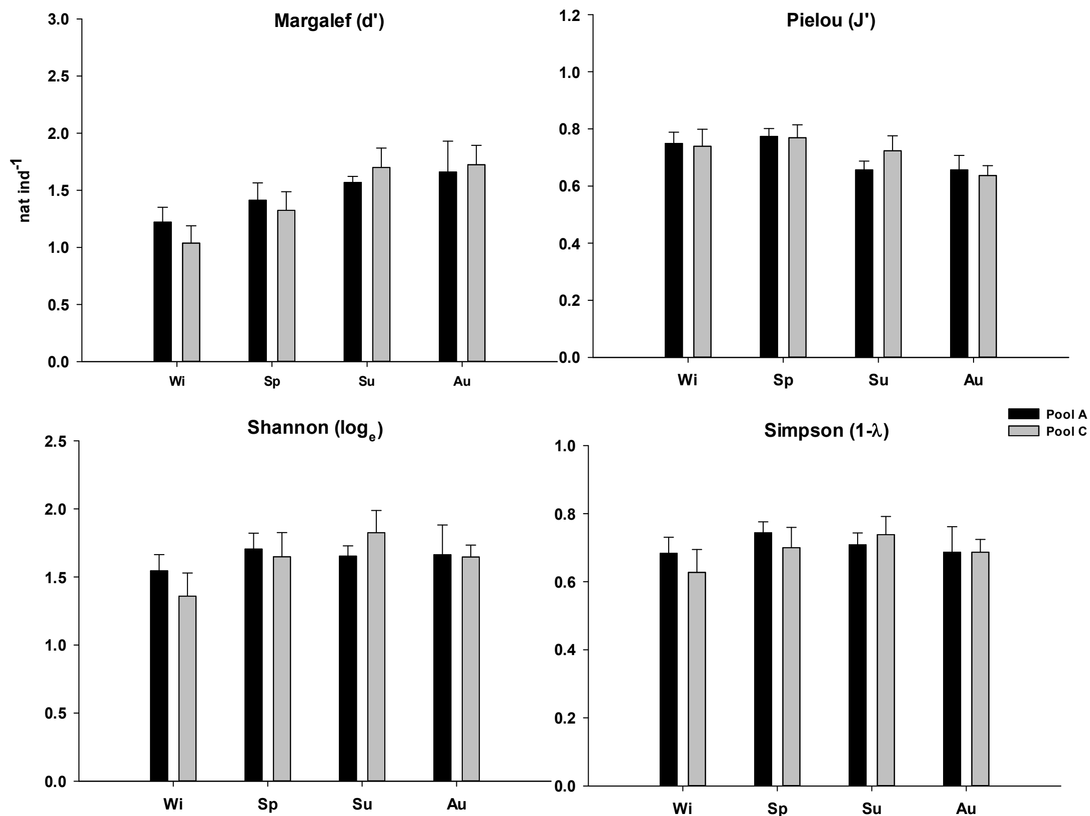

Fig. 5 - Variation of Margalef (**A**), Pielou (**B**), Shannon (**C**), and Simpson (**D**) indices per pool within seasons (Wi, Winter; Sp, Spring; Au, Autumn; Su, Summer).

Concerning macroinvertebrates density, PERMANOVA did not detect any significant differences between control and *A. armata* invaded rock pools (*P*(perm)=0.5095), only a significant temporal variability was confirmed for factor 'Season' (Table V). However, macroinvertebrates density in summer was slightly higher in the invaded pools (A) (1686.7 ind. m^{-2} ± 311.3) than in the non-invaded pools (C). In Autumn, the pools C, without the invasive macroalgae, presented higher macroinvertebrate densities (as already mentioned) than pools A for the same season (1473.3 ind. m^{-2} ± 319.7) (Fig. 4).

Table V - Summary of PERMANOVA analyses

Source	df	MS	Pseudo-F	P(perm)
Pool	1	1331.5	0.93382	0.5095
Season	3	6842.7	4.7988	**0.0001**
Pool x Season	3	12674	0.88883	0.6316

The SIMPER analysis showed 55.5% dissimilarity in mean abundance composition between pools C, without invasive *A. armata*, and pools A, with *A. armata* (Table VI). For the pools A, the top three dominant taxa that most contributed to this dissimilarity percentage were *Skeneopsis planorbis* (8.02%), *Rissoa parva* (6.73%), and *Melarhaphe neritoides* (6.46%) (Table 5). For pools C, where *A. armata* was experimentally removed, the species that most contributed to dissimilarity were *Amphipholis squamata* (6.33%), Polychaeta (5.17%), and *Mytilus sp.* (2.43%). Although most species contributing to differences between pools with and without *A. armata* were mainly present at the former ones, Polyplacophora, Polychaeta, and Ophiuroidea showed higher abundances in pools C, without the invasive species.

Table VI - Contribution of individual taxa to the average Bray-Curtis dissimilarity in macroinvertebrate assemblage (Pool C, without *Asparagopsis armata*; and Pool A, with *A. armata*).

Taxa	Pool C	Pool A	Average Dissimilarity	Contribution %
	Average abundance			
Skeneopsis planorbis	2.62	**3.05**	4.45	8.02
Rissoa parva	1.53	**1.87**	3.74	6.73
Melarhaphe neritoides	1.52	**1.68**	3.59	6.46
Amphipholis squamata	**3.18**	3.1	3.51	6.33
Bittium reticulatum	1.65	**1.75**	3.16	5.69
Amphipoda	1.51	**1.88**	2.89	5.2
Polychaeta	**1.92**	1.65	2.87	5.17
Dynamene magnitorata	1.26	**1.62**	2.8	5.04
Cymodoce truncata	1	**1.1**	2.69	4.84
Parvicardium scriptum	0.68	**1.07**	2.44	4.39
Gibbula sp.	0.66	**1.07**	2.36	4.25
Musculus costulatus	0.57	**1**	2.18	3.93
Gibbula pennanti	0.56	**0.59**	2	3.6
Mytilus sp.	**0.54**	0.42	1.35	2.43
Lepidochitona sp.	**0.46**	0.33	1.34	2.42
Gibbula umbilicais	**0.45**	0.31	1.31	2.37

A Principal Coordinates Analysis (PCO) based on the Bray-Curtis resemblance matrix was applied to visualize differences and similarities between samples. The PCO for the

macroalgae biomass and macrofauna density, in general, did not show clear differences between the studied pools and seasons. For macroalgae it provided a tenuous distinction between pools and in some cases between seasons of the same pool (pools in spring and in autumn, Fig. 6A). The first two principal component axis explain 32.7% and 19.1% of the samples variability, for macroalgae biomass (A) and invertebrate's density (B) respectively. For both macroalgal assemblages, six taxa showed a moderate to strong correlation with the first two PCO axes (Pearson's coefficient >0.5, Fig. 6A). The macroalgae taxa with higher value of correlation (>0.5) were related to Pool A, in autumn season, while macrofauna taxa with higher correlation are distributed between pools from spring and autumn season. For this assemblages, 5 taxa were the ones influencing more the relative distribution of different pools along seasons (Fig. 6B).

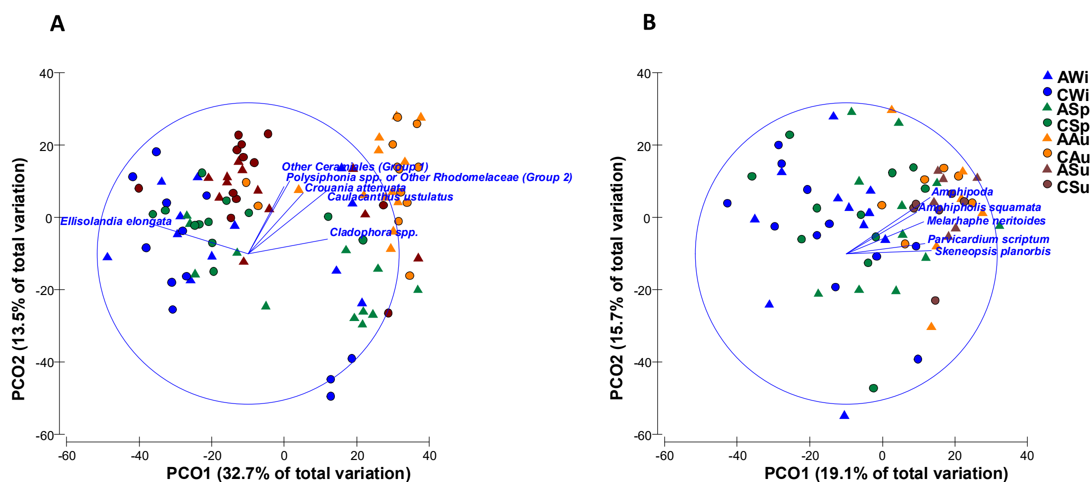

Figure 6 - Principal Coordinate analysis (PCO) plot based on macroalgae biomass (**A**) and macrofauna density (**B**) per pool for all seasons with the representation of the species with vectors longer than 0.5. A and C = pool A and pool C. Wi, Sp, Au and Su =Winter, Spring, August and Summer.

4. Discussion

This study aims to evaluate the variation observed on intertidal seaweed and benthic macroinvertebrate assemblages inhabiting rock pools with and without the presence of the invasive macroalgae *A. armata.*

Several studies have been made using invasive exotic species and assessing the induced changes in native macroalgal assemblages (De Leij *et al*., 2017; Olabarria *et al*., 2009; Sánchez *et al*., 2005). Nevertheless, to the present knowledge, in the literature, there are no studies showing the impact of *A. armata* on macroalgae and macroinvertebrate community assemblages. In the present study, *A. armata* had a significant impact on the native intertidal algal assemblage but not so evident over macroinvertebrate assemblage. Results indicated that physico-chemical parameters, did not have a strong impact on different pools, with most of the variation occurring between seasons. As many invasive macrophytes, *A. armata* exhibits seasonal development patterns having therefore a temporal variation in biomass (Klein & Verlaque, 2009).

In this study, during 2018, *A. armata* reached the peak biomass score in spring, which accumulated and started to decompose in the rock polls. A sharp biomass fall was observed in the summer suggesting spring as an important growing period for the species. Although similar seasonal patterns in biomass of *A. armata* have been recorded in the Azores (NE Atlantic archipelago) (Neto, 2000), this should also be verified in other *A. armata* populations. Also, supporting the growing pattern observed in the study, this algae may disappear from the high intertidal zone during summer, being found mainly in lower abundances in the low intertidal zone or in the shallow subtidal during this period of the year (Fa *et al*., 2000). The capacity of this species, nonetheless, to disperse with water currents, attached to floating objects, suggests that shoreline shape and local currents may play an important role in determining its distribution at the coast (Archambault & Bourget, 1999).

Asparagopsis armata produces very high amounts of secondary metabolites including volatile components as phenolic compounds (Carpenter *et al*., 2000), which toxicity to invertebrates, and seaweeds has often been associated to the invasive process (Paul *et al*., 2006b, Silva *et al*., 2020). These phenolic compounds from seaweeds have also shown to vary throughout the year (Ragan & Jensen, 1978), while the release of defensive compounds may also be altered by several abiotic factors such as temperature, light, and nutrient availability (Greff *et al.*, 2017).

In the present work, the effect of *A. armata* removal on the macroalgal assemblage was detectable in spring and summer seasons. During summer, with lower *A. armata* biomass, a higher algal richness is noted. This result agrees with the concept known as "biotic resistance" proposed by Elton (1958) that states that diverse communities should be less susceptible to invasion because of a more complete utilization of resources.

Considering the strong seasonal changes in individual biomass of *A. armata*, faunal shifts are expected to be especially relevant during the growth season (Spring). Episodes of exponential growth can happen followed by sudden drops of abundance like the congeneric species *Asparagopsis taxiformis* (Navarro-Barranco *et al*., 2018). Consequently, macrofauna resilience would be conditioned by their response to high variability in *A. armata* biomass. In spring, where *A. armata* registered higher biomass, in the invaded pools a higher biomass of native macroalgae was registered. In fact, increased light intensity, daylight duration and temperature during the spring and summer stimulates the growth of many native sub-canopy algal (Vye *et al*., 2018). This fact might increase competition for primary resources with *A. armata.* Also, pools with *A. armata* tended to present lower species richness, which may contribute to increased variability within invaded rockpools in the intertidal zone (Loreau *et al*., 2001). High densities of *A. armata* inhabiting the tide pools could be responsible for a decrease in the macroalgae abundance through competitive interactions. These hypotheses are not mutually exclusive and experimental evidence from this study and others (Britton-Simmons, 2004; Stæhr *et al*., 2000. may explain the observed field patterns. The experimental exclusion of *A. armata* from the studied rockpools, and the difference found on macroalgae assemblages from pools with and without this invasive species may also be an important indication about the recovery potential of these marine ecosystems. Native macroalgal assemblages found in pools C (without *A. armata*), were more diverse then from pools A, foreseeing the importance of management actions on habitat recovery.

Ellisolandia elongata contributed substantially to the total dissimilarity between different pools. This species decreased its biomass when *A. armata* was present. This is sustained by previous studies by Guerra-García *et al*. (2012) that reports *E. elongata* as the main algal

species affected by the presence of *A. armata*, while it is also known to dominate algal assemblages by producing massive coverage (Streftaris & Zenetos, 2006).

Concerning macrofauna, no significant differences were detected by PERMANOVA, showing that both rock pools type did not differ in the abundance of macrofauna species. PCO results (Fig. 6B) did not portray differences among different pools and only the factor season was significant in the PERMANOVA test. Furthermore, the structure of the benthic assemblage tends to be different between the different pools. There was a shift between the season summer to autumn where the higher number of macroinvertebrates started to decrease in the invaded pools and the number of species in pools without *A. armata* started to increase. There is a tendency towards increased variability of assemblage structure and towards decreased species richness in the presence of invasive species. Further work following the trajectory of macroalgal and macrofauna communities over a longer duration would increase information to assess the full community dynamics with and after *A. armata* removal.

However, there is a tendency of Gastropoda, Bivalvia, and Crustacea to preferer invaded pools and Polyplacophora, Polychaeta, and Ophiuroidea to prefer the native macroalgal assemblage pools. Many small and relatively sedentary herbivores such as polychaetes may preferentially feed on defended seaweeds in order to reduce their susceptibility to natural enemies (Monteiro *et al*., 2009). Crustaceans are the dominant epifaunal group on *A. armata*, representing more than 50% of the total fauna, as also reported by Pacios *et al*. (2011).

Due to their nature, grazers play an important role in structuring macroalgal assemblages, but secondary metabolites (terpenes, phenolic compounds, etc.) of *A. armata* ought to affect the palatability, or acceptability to herbivores (Paul *et al*., 2006b, Silva *et al*., 2020). Many of the gastropod species are microherbivores that feed on the biofilm established on the macrophyte fronds. In fact, lower densities of bacteria are found on the surface of the alga with halogenated compounds like those found in *A. armata* (Paul *et al*., 2006a). For that, a major presence of gastropoda in invaded pools is not expected. Despite Zwerschke *et al*. (2016) stating that NIS can play an important role in maintaining biodiversity in

human-altered environments, the stated presence of relatively high levels of phenolic compounds in *A. armata* tend to result in lower grazing losses and organism diversity.

These results are similar to previous studies that have compared faunal assemblages with other invasive species to those associated with other native canopy species. Most of these studies considered that the introduction of invasive macroalgae have not produced substantial modifications in the composition of faunal assemblages (e.g., loss of diversity) suggesting a weak impact in native faunal diversity. Thomsen *et al*. (2009) stated that there was no evidence for severe effects of invasive macroalgae on fauna density and assemblages. Other studies, such as Cacabelos *et al*. (2010), stated that epifaunal assemblages associated with the native *Laminaria ochroleuca* and with the invasive algal species *Sargassum muticum* differed, but only for epifaunal organisms, since the number of taxa and diversity did not clearly differ between the two algae. Viejo (1999) also demonstrated that the composition of the epifaunal assemblages associated to the invasive *Sargassum muticum* and the local *Cystoseira nodicaulis* were very similar.

However, Navarro-Barranco *et al*. (2018) showed differences in macrofauna composition between native *Halopteris scoparia* and the invasive species *Asparagopsis taxiformis*, using presence/absence data. Species richness, abundance and diversity of peracarids were significantly lower in invaded assemblages than in *H. scoparia* ones.

The general low abundance of *A. armata* at this intertidal level and its pseudoperennial character (high cover percentages are only recorded during a few months of the year) (Arenas & Fernández 2000) appear to or may limit competitive processes with native species. Due to the great differences in morphology and size of the two phases of *A. armata* (the diploid tetraesporophyte is known as '*Falkenbergia'*) their ability to harbour mollusc assemblages can differ in macrofauna composition, which further studies would certainly benefit current knowledge.

Nevertheless, macrofauna density in native and invaded rockpools can vary depending on past diet, physiological condition of algae, and other local factors, such as grazing pressure or competition (Cacabelos *et al*., 2010). These factors may influence the upper and/or lower limits of distribution of individual species similarly on rocky shores (Reichert, 2008).

The effects of the native and the invasive species on macrofaunal assemblages at larger spatial and temporal scales are yet to be determined, but may include effects on spatial patterns in β-diversity and detrital food webs, and the population dynamics of species associated with macrophytes at some or all stages of their life-history.
Nevertheless, the here seen trend, may point to a progressive replacement of *E. elongata* by *A. armata* that may directly or indirectly have considerable impacts on the ecology of rocky intertidal zone.

5. Conclusions

Results suggest that there was limited effect of *Asparagopsis armata* on community assemblages, due to short time follow-up, but the spreading of this species ought to drive to a homogenisation of communities (lower differences between sampling stations). Nevertheless, the constant increase in the algal biomass, even with seasonal changes, and its presence along the intertidal suggests that the effect will be greater in the future, in an equilibrium situation. Even the protection provided by Marine Protected Areas (MPAs) is very limited to prevent the establishment and spread of the most abundant invasive macroalgae (Blanco *et al*., 2020; Cacabelos *et al*., 2020). Predicting the impacts of invasive species on food webs and communities is one of the biggest challenges facing ecologists. Nevertheless, ecological management may have critical importance in the control of biological invasions, specifically by reducing the ecological pressure over native populations. On the other hand, this management may be reinforced when approached in a perspective of "transforming threats into opportunities", using the high *A. armata* biomass as a natural resource with economic values and opening a new window of opportunities for sustainable production.

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Supplementary data

Table S1 – List of macroalgae taxa found on Portinho de Areia Norte, Peniche

		C				S			
Group	Taxon	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
Chlorophyta									
	Chaetomorpha aerea			x	x			x	x
	Cladophora spp.		x	x	x	x	x	x	x
	Codium spp. (erect)				x		x		x
	Codium adhaerens				x	x			
	Ulva clathrata		x	x	x	x		x	x
	Ulva spp. (Leaf-like forms)	x	x	x	x	x	x	x	x
	Ulva spp. (Tubular - (ex Enteromorpha spp.) like forms			x				x	
Heterokontophyta (Phaeophyceae)									
	Cladostephus spongiosus							x	
	Colpomenia peregrina		x	x	x			x	x
	Cystoseira tamariscifolia			x					
	Dictyota dichotoma				x			x	
	Ectocarpales/Sphacelaria spp.			x	x			x	x
	Halopteris filicina			x	x			x	
	Halopteris scoparia	x	x	x	x		x		x
	Sargassum vulgare		x	x					x
Rhodophyta									
	Amphiroa spp.	x		x	x	x	x		x

	Anotrichium furcellatum/Bornetia secundiflora/Griffithsia spp.			x	x				
	Apoglossum ruscifolium/Hypoglossum hypoglossoides			x					
	Acrosorium ciliolatum/ cryptopleura ramosa	x	x	x		x	x	x	
	Asparagopsis armata	x	x	x	x		x	x	x
	Caulacanthus ustulatus			x	x			x	x
	Ceramium spp./Gayliella spp.	x	x	x	x	x	x	x	x
	Champia parvula	x	x	x	x	x	x	x	x
	Chondracanthus acicularis	x	x	x	x	x	x	x	x
	Chondracanthus teedei				x				x
	Chondria spp.		x	x	x		x	x	x
	Crouania attenuata	x	x	x	x		x	x	x
	Dasya spp.			x	x				
	Ellisolandia elongata	x	x	x	x	x	x	x	x
	Falkenbergia rufolanosa				x				
	G1[a]			x	x			x	x
	G2[b]	x		x	x	x	x	x	x
	G3[c]			x	x			x	x
	Gastroclonium ovatum	x		x	x	x	x	x	x
	Gelidium corneum/Gelidium spinosum				x			x	x
	Gelidium pulchellum			x			x	x	
	Gelidium pusillum	x	x			x		x	x
	Gymnogongrus griffithsiae			x					
	Jania rubens	x	x	x	x	x	x	x	x
	Liagora viscida							x	
	Lithophyllum incrustans	x	x	x	x	x	x	x	x
	Lomentaria orcadensis				x				

	Mesophyllum lichenoides	x	x	x	x	x	x	x	x
	Osmundea pinnatifida/Laurencia pyramidalis	x	x		x	x	x	x	x
	Peyssonnelia spp.	x			x	x	x	x	x
	Plocamium cartilagineum		x	x	x			x	x
	Porphyra spp			x	x				
	Pterosiphonia complanata			x	x		x	x	x
	Sphaerococcus coronopifolius			x				x	
	symphiocladia marchanthioides			x			x		
	Vertebrata thuyoides/Vertebrata fruticulosa	x	x	x	x	x	x	x	x
	Xiphosiphonia ardreana/Xiphosiphonia pennata	x		x			x	x	
	[a] *Group 1: Other Ceramiales (e.g. Aglaothamnion spp., Antithamnion spp., Callithamnion spp., Compsothamnion thuyoides, Pleonosporium borreri)*								
	[b] *Group 2: Polysiphonia spp. or Other Rhodomelaceae (e.g. Herposiphonia tenella, Melanothamnus collabens; other Vertebrata spp., Leptosiphonia schousboei, Lophosiphonia reptabunda)*								
	[c] *Group 3: Other Delesseriaceae (e.g. Erythroglossum laciniatum, Nithophyllum punctactum)*								

Table S2 - List of macroinvertebrate taxa found on Portinho de Areia Norte, Peniche

		C				S			
Group	Taxon	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
Polychaeta									
	Polychaeta	x	x	x	x	x	x	x	x
	Sabellaria alveolata	x							
Amphipoda									
	Amphipoda	x	x	x	x	x	x	x	x
Decapoda									
	Carcinus maenas				x	x	x		x
	Crangon crangon					x			
Isopoda									
	Dynamene magnitorata	x	x	x	x	x	x	x	x
	Cymodoce truncata	x	x	x	x	x	x		x
	Anthura gracilis	x	x		x				x
	Dynamene bidentata	x			x				
	Idotea granulosa								x
	Idotea chelipes	x				x			
Tanaidacea									
	Tanais dulongii	x	x	x		x	x	x	x
	Apseudes latreillii							x	
Insecta									
	Chironomidae		x	x			x	x	
Bivalvia									
	Musculus discors	x			x	x			
	Musculus costulatus	x	x	x	x	x	x	x	x

	Parvicardium scriptum	x	x	x	x		x	x	x
	Mytilus sp.		x	x	x	x	x	x	x
	Irus irus		x		x				x
	Modiolula phaseolina		x		x			x	x
	Pseudopythina macandrewi		x		x			x	
Gastropoda									
	Skeneopsis planorbis	x	x	x	x	x	x	x	x
	Gibbula pennanti	x	x			x	x		x
	Bittium reticulatum	x	x	x	x	x	x	x	x
	Rissoa parva	x	x	x	x	x	x	x	x
	Melarhaphe neritoides	x	x	x	x	x	x	x	x
	Gibbula sp.	x	x	x	x	x	x	x	x
	Patella sp.	x			x	x	x		
	Gibbula umbilicalis	x	x	x		x	x	x	x
	Calliostoma zizyphium	x		x			x		
	Nassarius incrassatus	x	x	x	x		x		
	Limapontia sp.							x	
	Runcina sp.			x	x			x	x
	Gibbula cineraria								x
	Opisthobranchia								x
	Aplysia sp.				x				x
	Tritia incrassata								x
	Nudibranchia								x
Polyplacophora									
	Acanthochitona carinatus					x			
	Lepidochitona cinerea	x	x	x	x	x	x		x
	Acanthochitona fascicularis	x	x	x	x		x		x
	Lepidochitona sp.	x	x	x	x	x	x		x

Ophiuiroidea									
	Amphipholis squamata	x	x			x	x		
Anthozoa									
	Actinaria		x				x		x
Picnogonida									
	Pycnogonia		x	x	x		x	x	x

Chapter VII

General discussion and concluding remarks

Chapter VII - General discussion and concluding remarks

The aim of this thesis was to understand the overall effects, and gain insight on the mechanisms of toxicity of the invasive macroalgae *Asparagopsis armata*, studying different levels of biological organization. This thesis aimed to fill this gap though an integrative approach, unraveling potential mechanisms for the macroalgae toxicity and impacts. To achieve this goal, several experiments were conducted with the objective of determining effects on various endpoints.

Assessing lethal and sublethal effects of contaminants at the organismal level, provides sensitive information that can be easily used to predict possible outcomes at the population level, and there is a need to develop new and sensitive early warning tools for fast detection of ecological adverse effects. In this sense, in Chapter II, fatty acid profile is proposed to be used as a biomarker of ecological risk assessment. In fact, in this Chapter, the metal exposures, used as reference substance, showed to influence FA profile, with mechanisms that may act as potent inducers of fatty acid modulation in *G. umbilicalis.*

Despite the responses at the FA profile level were more evident for Hg, the metals Ni and Cd induced similar response trends in the snails, suggesting a common effect of metal contamination. Five fatty acids (palmitic, eicosatrienoic, arachidonic, eicosapentaenoic, and docosahexaenoic acids), with a high functional link to membrane organization (homeoviscous adaptation) and immune responses, demonstrated to be especially good indicators of *G. umbilicalis* responses to the array of metals used. The commonly used lipid related endpoints in contamination effect assessment, like LPO and total lipid content, did not show clear responses to the array of metals in the conditions tested. However, under same conditions, FA profile suggested an alteration in the fatty acid metabolism and showed to be suitable multibiomarker integrative tool in coastal environments risk assessment using a sea snail as model species.

The approach used in this Chapter offers the possibility of applying this tool for this and other organisms exposed to different pollutants, and many other FAs involved in other functions that were not affected with metals might be affected in exposures with other pollutants. Having this new tool in place, it was used to evaluate FAP responses also to

Asparagopsis armata exudate [Chapter V] contamination. Moreover, with this tool, most interesting and novel information on the mechanisms of chemical toxicity of this invasive alga was obtained.

The activity of ChEs has also been widely used as a biomarker of neuronal and neuromuscular changes in an organism (Howcroft *et al*., 2011). However, ChEs are extremely complex; they display differential forms, expression, biologic functions, location, and catalytic activity (Nunes, 2011). This high variability and complexity of ChE requires their characterization before their use as biomarkers. In this thesis, this characterization was made in Chapter III for *Gibbula umbilicalis* since this information was already available for *Palaemon elegans (*Frasco *et al*., 2006) The results indicated that the main cholinesterase form present in *G. umbilicalis* is acetylcholinesterase (AChE), by inhibition by excess of substrate and inhibition with BW284C51.

The *in vivo* exposure to a chlorpyrifos-based formulation (Dursban) showed that this pesticide was capable of exerting significant effects both on AChE activity and flipping behavior. In this Chapter the link between AChE activity inhibition and adverse effects on behavioural changes was established: AChE inhibition was positively correlated with the flipping test, indicating a mechanistic relationship between the two endpoints determined in *in vivo* exposures. This study highlights the importance of linking biochemical endpoints such as AChE activity with higher level endpoints like behavioural alterations, increasing the ecological relevance of the effects observed. These endpoints are transversal throughout this thesis and used in Chapters IV and V. Also, knowing that the main ChE form present in the marine snail *G. umbilicalis* is AChE, is important for the successful application of this biomarker in the next Chapters, using this gastropod. Regarding the shrimp, the cholinesterase (ChE) enzyme present in the eyes of the shrimp *Palaemon* showed typical properties of acetylcholinesterase, as stated in the work of Frasco *et al*. 2006.

To understand the mechanisms of toxicity of the invasive *A. armata* in marine invertebrates, lethal and non-lethal concentrations of the exudate of this alga were tested in *G. umbilicalis* and *P. elegans* and biochemical biomarkers and behavioural responses were analyzed. The Chapter IV shows that organisms have different energy requirements to deal with the stress caused by the macroalgae exudate. In fact, the acute tests revealed

that *P. elegans* is more tolerant (96 h LC_{50} [95% CI] of 5.04% [4.84-5.25]) than *G. umbilicalis* (96 h LC_{50} [95% CI] of 2.79% [1.66-4.69]) with significantly higher LC_{50}. Feeding activity revealed that only *G. umbilicalis* was affected after *A. armata* exposure. Also, Paul *et al*. 2006 detected feeding deterrence in *A. armata* dichloromethane extracts. Regarding effects on *G. umbilicalis*, *A. armata* induced a significant increase in total lipids, along with decreased activities of LDH and ETS. This lipid increase may be related to the maintenance of energy reserves as they alter their behaviour, decreasing their activity. In *P. elegans*, there was an increase in lipids and proteins, that may reflect an indution in protein synthesis for detoxification and other defence mechanisms (Smolders *et al*., 2003). Thencrease of ETS and LDH activities indicates that the organisms are spending energy to fight stress caused *by A. armata*, which could compromise other biological functions (Sokolova *et al*., 2012).

These results indicate that organisms have different energy requirements to deal with the stress caused by the complexity of this exudate and provide important insights into the heterogeneous effects of the *A. armata* exudate, driven by species-specific metabolic susceptibility patterns. Also, this Chapter highlighted the importance of researching the natural toxic exudates released into the environment and the mechanisms on how they can affect the surrounding organisms and their mode of action in the invaded ecosystems.

Following the results obtained in Chapter IV we further addressed potential mechanisms of action involved in the previously observed effects. Antioxidant defences like SOD and GST, oxidative stress parameters like LPO and DNA damage, and neuronal parameters as AChE, were assessed in *G. umbilicalis* and *P. elegans*. The results from this study showed that *A. armata* exudate induced shifts in the studied endpoints, yet with different responses between species. The inhibition of GST in mid exudate concentrations in G. *umbilicalis* could be due to the direct action of the exudate compounds on the enzyme since some allelochemicals of plants are also GST inhibitors (Lee, 1991). Regarding the neuronal parameter, a significant induction of AChE was observed in *G. umbilicalis* and this is also stated for the crab *Barytelphusa guerini* (Reddy *et al*., 1990) after 4d of exposure to fluoride, a halogenated compound. Regarding the shrimp, and similar to *G. umbilicalis*, an

over-production of ROS might have resulted in the seen inhibition of SOD activity and the trend for a decreased level of GST. This inhibition might have led to the accumulation of ROS which in turn led to an increase of LPO. Further, *P. elegans* neuronal enzyme activity, was inhibited. In fact, in literature, some macroalgae extracts including *A. armata* reveled potent inhibitory capacity on ChEs (Custódio *et al*., 2016). Differentiated fatty acids profiles shifts were detected due to exudate exposure. Differences were more pronounced for *P. elegans* with a general increase in PUFA which commonly means a defence mechanism protecting from membrane disruption (Fokina *et al*., 2013). At the same time, the increasing of ARA in both invertebrates means that this FA was possibly required for activation of eicosanoid synthesis for the regulation of inflammation and immunity responses (Delaporte *et al*., 2006), which is in accordance with the Chapter II, where levels of ARA in *G. umbilicalis* increased after metal exposure and might indicate a general response to chemical stress. DGLA and EDA, clustered in a different group, also present an increase, being involved in eicosanoid synthesis. For *P. elegans*, in the last concentration, there was a decrease of n3 PUFA due to the loss of ability of *the* shrimp to cope with the stressor at higher concentrations, leading to the increase of LPO levels. In the same concentration, n6 PUFA and short chain increased, activating the post-inflammatory response (Mirmonsef *et al*., 2012). These dissimilarities between biomarkers from both species, suggest different metabolic processes being affected, the same way as previous Chapter (IV). For both invertebrates there was a similar response not only in ARA but also with DPA, indicating a common mechanism regulation of inflammation and immunity responses, triggered by the exudate. In general, the non-monotonic responses detected from both works may derive from the complexity in the media and differentiated mechanisms of action of different individual compounds and their different concentrations in the mixture at a given exudate dilution, which constitutes an extra challenge to the interpretation of results of such nature.

Despite these results obtained in the laboratory, it is of great importance to evaluate the effects of this invasive alga in a more realistic scenario. The field investigations (Chapter VII), carried out in 2018, showed that *A. armata* induces significant changes into the

invaded community. *A. armata* had a significant impact on the native intertidal algal assemblage but not so evident over macroinvertebrate assemblage. *A. armata* exhibits seasonal development patterns having therefore a temporal variation in biomass (Klein & Verlaque, 2009). In this study, *A. armata* reached the peak biomass score in spring and similar seasonal patterns in biomass of this species have been recorded in the Azores (NE Atlantic archipelago) (Neto, 2000). In summer, with lower *A. armata* biomass, a higher algal richness is noted. This result agrees with the concept known as "biotic resistance" proposed by Elton (1958) that predicts that diverse native communities are more resistant to invasion. In spring, where *A. armata* registered higher biomass, invaded pools registered a higher biomass of native macroalgae. In fact, increased light intensity, daylight duration and temperature during the spring and summer stimulates the growth of many native sub-canopy algal (Vye *et al*., 2018). In general, high densities of *A. armata* inhabiting the tide pools could be responsible for a decrease in the macroalgae abundance through competitive interactions. *Ellisolandia elongata* contributed substantially to the total dissimilarity between different pools. This species decreased its biomass when *A. armata* was present. Guerra-García *et al*. (2012) also that reports *E. elongata* is the main algal species affected by the presence of this invasive species. Rock pools type did not differ in the abundance of macrofauna species, although there is a tendency towards increased variability of assemblage structure and towards decreased species richness in the presence of invasive species. However, there was a tendency of Gastropoda, Bivalvia, and Crustacea to preferer invaded pools and Polyplacophora, Polychaeta, and Ophiuroidea to prefer the native macroalgal assemblage pools. In fact, crustaceans are the dominant epifaunal group on *A. armata*, representing more than 50% of the total fauna in Pacios *et al*., 2011. Also, due to the great differences in morphology and size of the two phases of *A. armata*, their ability to harbour mollusc assemblages can differ in macrofauna composition.

Results suggest that there was limited effect of *Asparagopsis armata* on community assemblages, due to short time follow-up, but the spreading of this species ought to drive to a homogenisation of communities (lower differences between sampling stations). Nevertheless, the constant increase in the algal biomass and its presence along the intertidal suggests that the effect will be greater in the future.

The present thesis novelty relied on the ecotoxicological and ecological information to provide insight in how *A. armata* affects specific invertebrates and in general coastal communities, embracing methods at different biological organization levels which prepare the way for various hypotheses testing in assessing *Asparagopsis armata* exudates toxicity. Moreover, some interesting and novel information on the mechanisms of chemical toxicity of the *A. armata* exudate, was obtained. This work was done taking into account an integrative approach. Effects seen at higher levels of biological organization (populations and communities) are the consequence of the sum of effects on individuals, which resulted from impacts at the cellular and molecular levels.

Despite the fact that the chemistry of *Asparagopsis armata* was well characterized, with over 100 halogenated compounds, by McConnell and Fenical (1977), a significant step forward after this thesis would be to understand which are the most specific compounds released through the exudate and under which conditions, in order to measure more correctly the inherent risks of their release through the environment. The chemicals identified could be used in future experiments using single and combined exposures assays. Also, the application of other behavior endpoints such as avoidance, swimming velocity, growth and reproduction could be can be useful to complement *A. armata* toxicity information. Regarding molecular endpoints, the application of "omics" methodologies, such as transcriptomics, genomics and proteomics would also be interesting to consider and of utmost value to amplifying the knowledge of the mechanisms of toxic action of these algae exudates.

This thesis contributed to the growing knowledge as an important step in the research of natural toxic exudates released to the environment and prospective effects of this seaweed in invaded communities under increasing global change scenarios.

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