Universidade de Aveiro
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Virgília Sofia Almeida Azevedo Silva, da Doutora Maria Benedicta Agostinho Donas-Bôtto Bordalo e Sá e do Doutor Marcelino Miguel Guedes de Jesus Oliveira, investigadores auxiliares do CESAM – Centro de Estudos do Ambiente e Mar e Departamento de Biologia da Universidade de Aveiro

Apoio financeiro da FCT (Fundação para a Ciência e a Tecnologia) no âmbito do acordo de parceria PT2020 e Compete 2020 cofinanciado pelo FEDER - Fundo Europeu de Desenvolvimento Regional. Trabalho inserido no projeto INSIDER (Impact of an invasive species under global climate change and anthropogenic contamination scenarios in coastal food webs). Programa Operacional da Competitividade e Internacionalização (02/SAICT/2017).

o júri

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agradecimentos

Gostaria de agradecer a todas as pessoas que ao longo destes dois anos se cruzaram comigo e me enriqueceram de alguma forma. Mas há pessoas que merecem um apreço especial pelo apoio e dedicação que me dispensaram.
Agradeço à Doutora Virgília Silva por ter aceitado orientar-me nesta caminhada, pela dedicação e paciência, por toda a confiança e incentivo em qualquer hora.
Agradeço ao Doutor Marcelino Miguel Oliveira por me ter recebido no seu laboratório com toda a disponibilidade e simpatia, pelo tempo despendido e contribuição para o meu crescimento científico. E à Mónica por ter estado sempre disponível para me ajudar e aconselhar.
Agradeço à Doutora Maria Bordalo e Sá por toda a disponibilidade, apoio e amabilidade.
E principalmente, aos três por me terem ajudado e orientado mesmo em tempos difíceis.
À Let, a minha melhor amiga e melhor pessoa que conheço, pela partilha diária ao longo destes dois anos, por me ouvir e aturar, por nunca me deixar cair ou pelo menos sozinha, e por estar sempre aqui. Como ela diz, fomos remando, batalhando, e mais marés iremos atravessar juntas.
À Tati por ter feito sempre o longe perto, por todas as mensagens e telefonemas só para me relembrar que está sempre ali para mim como sempre. Tal como ao computador dela, que em momentos de aflição salvou esta tese.
Às minhas meninas açorianas Cíntia e Mariana por tornarem os meus dias mais felizes e fazerem parte da minha família em Aveiro.
À Margarida por todas as horas de presença zoom, pela partilha de desespero e felicidade. Tal como ao Didi e Duarte, pela amizade, encorajamento e preocupação.
Aos meus pais e irmã por nunca duvidarem de mim, pelo apoio e amor incondicional, sem eles não estaria aqui. Sem as palavras que aconchegam à distância e os abraços na presença. E à Safira claro.
Agora que finalmente está concluída, não tenho como agradecer e retribuir a todas estas pessoas importantes por terem acreditado em mim mais do que eu própria. Obrigada

palavras-chave

Asparagopsis armata, linhas celulares de peixe, citotoxicidade, biomarcadores.

resumo

Os oceanos apresentam uma enorme importância no nosso planeta, representando quase 99% do espaço vital do planeta. A sua vastidão dá a ilusão de resistência à atividade antropogénica e recursos infinitos que tem levado à exploração imprópria ao longo dos anos. Consequência das pressões antropogénicas a introdução de espécies não-nativas representa uma ameaça devastadora à biodiversidade. A macroalga vermelha *Asparagopsis armata*, originária da Austrália Ocidental, está atualmente distribuída por todo o planeta sendo abundante na costa Portuguesa. Esta espécie representa uma ameaça para as espécies nativas dado que produz exsudados potencialmente tóxicos tornando-se altamente invasiva, sem predadores e com taxas de crescimento elevadas.

O presente trabalho teve como objetivo principal a avaliação da toxicidade do exsudado da *Asparagopsis armata*, recorrendo a ensaios *in vitro.* Assim, foi avaliada a sua citotoxicidade para uma linha celular de dourada (*Sparus aurata*), uma espécie de peixe de elevado valor comercial a nível nacional e Europeu. Foram realizados ensaios de 24 h, onde foi avaliada a viabilidade celular, recorrendo ao ensaio de redução de MTT e Resazurina, e avaliadas respostas bioquímicas associadas à atividade antioxidante e de biotransformação após exposição de 24 h. De uma forma geral, os dados revelaram que a viabilidade celular das células de dourada é significativamente reduzida quando expostas a mais de 25% de exsudato de *A. armata.* Para além de que induz um aumento na atividade de tióis não proteicos, indicativo de um aumento da capacidade antioxidante não enzimática em resposta aos compostos tóxicos presentes no exsudato.

keywords *Asparagopsis armata*, fish cell lines, cytotoxicity, biomarkers.

abstract

The oceans present an enormous importance on our planet, representing almost 99% of the planet's living space. Its vastness gives the illusion of resistance to anthropogenic activity and infinite resources that has led to inappropriate exploitation over the years. A consequence of anthropogenic pressures the introduction of non-native species poses a devastating threat to biodiversity. The red macroalgae *Asparagopsis armata*, originally from Western Australia, is now distributed all over the planet and is abundant along the Portuguese coast. This species represents a threat to native species since it produces potentially toxic exudates, becoming highly invasive, predatorless and with high growth rates.

The main objective of this study was to evaluate the toxicity of Asparagopsis armata exudates, using in vitro assays. Therefore, its cytotoxicity was evaluated for a cell line of gilthead sea bream (*Sparus aurata*), a fish species of high commercial value at national and European level. Assays were performed for 24 h, where cell viability was assessed, using the MTT and Resazurin reduction assay, and biochemical responses associated with antioxidant activity and biotransformation were evaluated after 24 h exposure. Overall, the data revealed that cell viability of gilthead seabream fish cells is significantly reduced when exposed to more than 25% *A. armata* exudate. It also induces an increase in non-protein thiol activity, indicative of an increased non-enzymatic antioxidant capacity in response to toxic compounds present in the exudate.

Content

List of figures and tables

Abbreviations and acronyms

3R	Reduction, Refinement, and Replacement
ASW	Artificial salt water
CAT	Catalase
CDNB	1-Chloro-2,4-dinitrobenzene
DMSO	Dimethyl sulfoxide
DTNB	5,5-Dithiobis-(2-nitrobenzoic acid)
FBS	Fetal bovine serum
GC-MS	Gas chromatography–mass spectrometry
GSH	Reduced Glutathione
GST	Glutathione *S*-transferases
IAS	Invasive alien species
L-15	Leibovitz's L-15 medium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NPSH	Non-protein thiols
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonyl fluoride
TCA	Trichloroacetic acid

Chapter I:

General Introduction

1. Introduction

1.1. Ocean

For many years there has been no doubt about the extreme importance of the ocean. After all, without it, humanity would not exist since it is an indispensable element for our life, our subsistence, and the environment that sustains us (IOC/UNESCO *et al.*, 2011). More than 70% of our planet's surface belongs to the ocean but it reveals an even bigger dimension when we consider that almost 99% of the planet's living space is in the ocean, given the immensity of life existing in the vast depths (Costanza, 1999).

Its vastness has created the illusion of infinite resources, and the difficulty of controlling and supervising these open resources has led to its improper exploitation (Costanza, 1999). According to a study of global impacts on the seas, just 13% of Earth's oceans can be considered wilderness, without the intervention of mankind and far from human populations. These wilderness areas contain high genetic diversity, unique functional traits, and endemic species (Jones *et al.*, 2018).

The oceans are part of us and reveal tremendous importance in sustaining life. However, their continuous and rapid decline has led to urgent calls for their protection, which is still not sufficiently known. Accordingly, it is crucial to proactively defend marine wilderness by incorporating these matters into global strategies aimed at conserving biodiversity (Jones *et al.*, 2018).

1.1.1. Invasive Alien Species

Concerning marine pollution, an increasingly discussed and taken into consideration topic in recent years is biological pollution, in particular the importance of the impact invasion species. Alien or non-native species are species introduced outside their original present or past distribution. They may or may not establish themselves and invade, outcompeting or predating on native species, becoming a pest without predators or other natural choices to control population growth (Boudouresque & Verlaque, 2002; Early *et al.*, 2016; Horan *et al.*, 2002). With this, species that are widespread and

established in ecosystems outside their natural range, and that their introduction or spread is a threat to biodiversity or ecosystem services (benefits to human well-being from ecosystems) are designated as invasive alien species (IAS) (Decreto-Lei n.º 92/2019 de 10 de Julho Da Presidência Do Conselho de Ministros, 2019; Regulamento (UE) n.º 1143/2014 de 22 de Outubro de 2014 Do Parlamento Europeu e Do Conselho Da União Europeia, 2014; Horan *et al.*, 2002).

The 2011 inter-agency report to the United Nations Conference on Sustainable Development, reported the impact of invasive species on aquatic ecosystems as the second most significantly occurring cause of biodiversity loss (IOC/UNESCO *et al.*, 2011). However, it is relevant to emphasize that for a species to be invasive, it must cause environmental or economic damage by spreading and establishing large populations (Namboothri *et al.*, 2012). The impact of invasive species on marine ecosystems is highly diverse, affecting not only global biodiversity but also other issues from human livelihoods to the economy (e.g., fisheries and industry). In contrast to other threats to ecosystems (e.g. overfishing), together with climate change, this major threat can be hard, if not impossible to reverse once the species has established itself (Early *et al.*, 2016; European Environment Agency, 2012; IOC/UNESCO *et al.*, 2011).

Ensuring the Regulation nº1143/2014, of the European Parliament and the Council of the European Union, of October 22nd, 2014, dedicated to the management and prevention of the introduction and spread of IAS, the decree-law nº92/2019 of July 10th, by the Presidency of the Council of Ministers present in the Official Journal (*Diário da República*), legally establishes the detention, control, and introduction of exotic species such as their repopulation, in order to protect the national biodiversity, the economy, and human health. This decree-law establishes the regime of interdiction of invasive species on the National List of Invasive Species, prohibiting their detention, trade, introduction, and cultivation (Decreto-Lei n.º 92/2019 de 10 de Julho Da Presidência Do Conselho de Ministros, 2019; Regulamento (UE) n.º 1143/2014 de 22 de Outubro de 2014 Do Parlamento Europeu e Do Conselho Da União Europeia, 2014).

1.1.1.1. *Asparagopsis armata*

In the National List of Invasive Species of the mentioned decree-law no. 92/2019, one of the species that is included is the red macroalgae *Asparagopsis armata* considered one of the 100 "Worst Invasive Marine Species" on the Mediterranean (Boudouresque & Verlaque, 2002; Decreto-Lei n.º 92/2019 de 10 de Julho Da Presidência Do Conselho de Ministros, 2019; Streftaris & Zenetos, 2006).

Although it is often impossible to determine the date of appearance of species in a region, it is documented that *Asparagopsis armata*, first observed by William H. Harvey in 1855 on the west coast of Australia and New Zealand, has spread along the European coast and the Mediterranean since 1925. Currently, it can be found on several coasts around the world as a consequence of its highly invasive behavior (Boudouresque & Verlaque, 2002; Harvey, 1855; Otero *et al.*, 2013; Pinteus *et al.*, 2021; Round, 1981). Its presence is registered from the Atlantic islands, Pacific islands, Subantarctic Islands, Caribbean islands, to the coasts of Africa, Central and North America, South Asia, the Middle East, and all over Europe (Guiry & Guiry, 2020; Pinteus *et al.*, 2021).

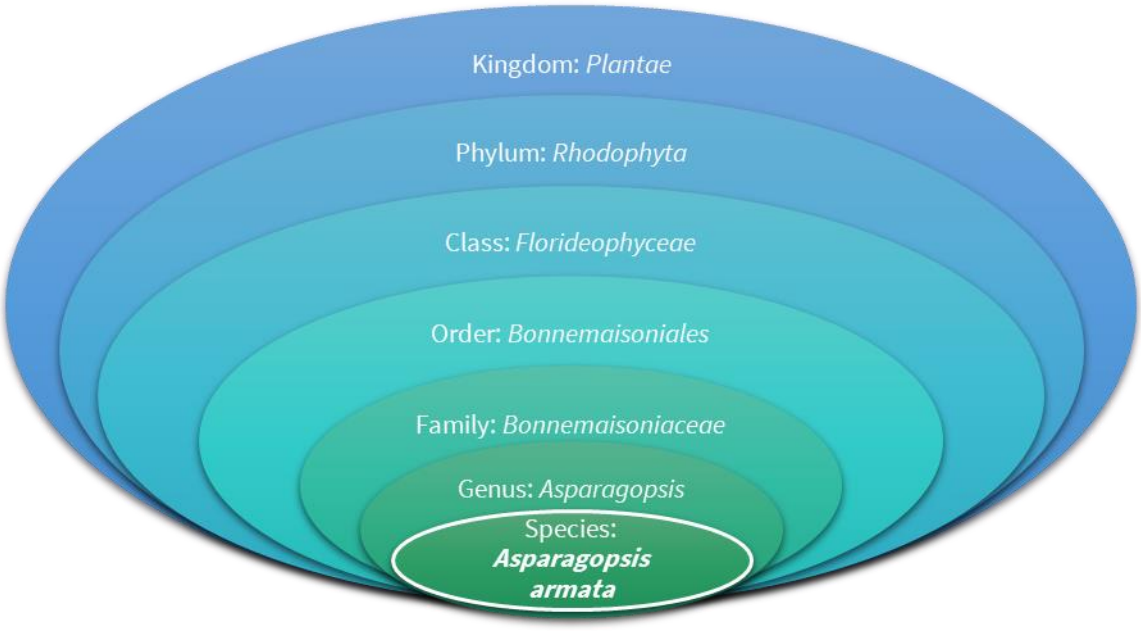

Figure 1: Taxonomic classification of *Asparagopsis armata*.

Asparagopsis armata (Class: *Florideophyceae,* Order: *Bonnemaisoniales* and Family: *Bonnemaisoniaceae*) belongs to one of the oldest groups of eukaryotic algae, the *Rhodophyta* Phylum (Figure 1), commonly known as red algae (Guiry & Guiry, 2020; Lee, 2008). This red seaweed has the peculiarity of having two morphologically different phases during its life cycle, so different that previously they were two distinct species (Chualáin *et al.*, 2004). The gametophyte phase (Figure 2A), has a stem diversely branched into small branches, especially in the upper part where it forms its characteristic aspect of "asparagus" (*Asparagus* in Latin), originating its name (Figure 2B). The harpoon-like branches gave its name of "*armata*" which means armed (Figure 2C). These distinct harpoon-like spines of *A. armata* also confer their common name of “harpoon weed” and allow the clear differentiation of *Asparagopsis taxiformis* of the same genus given their lack. This phase has a pale purplish-red color, that when removed from the water quickly degenerates to orange (Chualáin *et al.*, 2004; Guiry & Guiry, 2020; Pereira & Correia, 2015).

Figure 2: *Asparagopsis armata* seaweed in its gametophytic phase: A*) A. armata* in the Southwest of England, 2010; B) Branch with many tiny branches giving the appearance of "asparagus", 2016; C) Particular characteristic of the seaweed, close-up of a harpoon-like branch, 2016. Source: David Fenwick from www.aphotomarine.com

The tetrasporophyte, previously identified as *Falkenbergia rufolanosa*, is morphologically very distinct, brownish-red, very branched, with small filamentous tufts of cotton consistency (Figure 3) (Otero *et al.*, 2013; Pereira & Correia, 2015). Photophilic and epiphytic on other seaweeds, when compared to these hosts, this phase has a high potential for rapid nutrient uptake considering its high surface/volume ratio. As a result, it can densely cover other host species, reducing access to nutrients and light, a negative impact on ocean nourishment (Katsanevakis *et al.*, 2014; Pereira & Correia, 2015).

This seaweed is an annual species (each phase more predominant in certain months) usually found on infralittoral rocky bottoms from the surface to 40 meters depth (Otero *et al.*, 2013; Pereira & Correia, 2015).

Figure 3: Tetrasporophyte phase (*Falkenbergia rufolanosa*) of the seaweed *Asparagopsis armata*: A) *Falkenbergia*-phase of *A. armata* in Southwest England, 2011; B) Branch with dense cotton-wool-like tufts, 2018; C) Close-up of branched filaments, 2014. Source: David Fenwick from www.aphotomarine.com

With a very complex life cycle, able to reproduce vegetatively and sexually, this species presents a heteromorphic diplohaplontic life cycle. As it is represented in Figure 4, the gametophytic phase (*A. armata*) has male or female organs, followed by a microscopic carposporophyte stage, and then the tetrasporophyte phase (*F. rufolanosa*). Vegetatively, drifting gametophytes attach themselves to other algae by harpoon-like branches and produce new shoots. Tetrasporophyte also disperses by flotation and is essential for the invasive success of the seaweed (Otero *et al.*, 2013; Pinteus *et al.*, 2021).

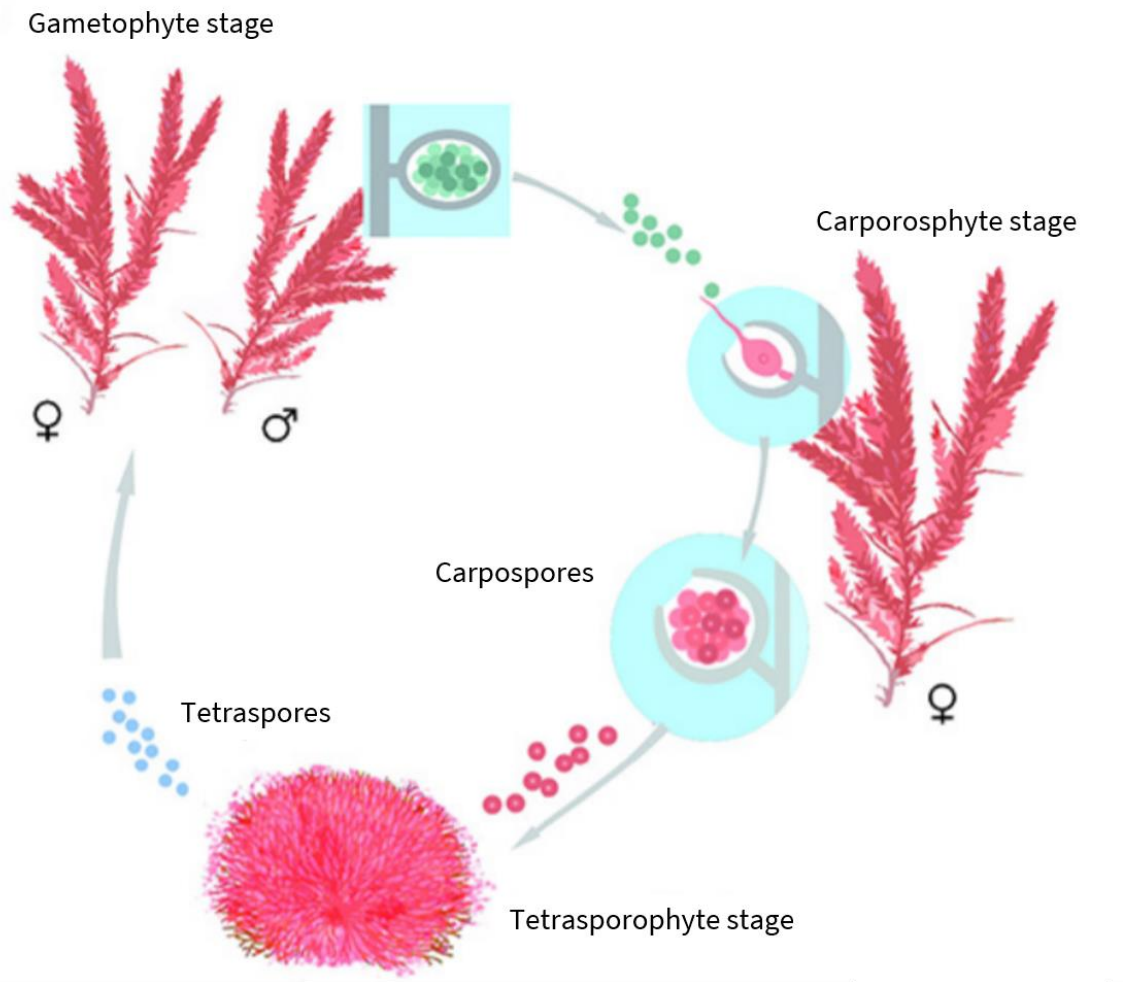

Figure 4: Schematic representation of the life cycle of *Asparagopsis armata*.
(Adapted from IUCN Centre for Mediterranean Cooperation, 2013)

The impact and/or potential of the *Asparagopsis armata* seaweed to negatively affect biological diversity and socioeconomics has been documented (Streftaris &

Zenetos, 2006), such as coastal fishing hindered by clogging in fishing nets and their growth in oysters (Katsanevakis *et al.*, 2014), or the ability to outcompete native species for light and space (Otero *et al.*, 2013). In addition, the producing brominated compounds, iodinated methanes, and acetone, may cause toxic effects on other species leading to their depletion. Being highly invasive in the ecosystem, *A. armata* leads to very important changes such as the replacement of "keystone species"(Pinteus *et al.*, 2018).

The great concern about the threats that IAS can bring with their introduction and establishment sometimes leads to bias, and the benefits and positive outcomes are underestimated (Katsanevakis *et al.*, 2014). However, during the last few years, the development of new products and technologies from marine organisms, such as seaweed, has considerably increased, especially with the growing awareness of environmental issues. This is possible to verify with the increasing number of scientific publications in which marine origin compounds have been used for various domains from the development of pharmaceuticals to cosmetics, bringing the marine environment to the center of attention in several industries (Genovese *et al.*, 2009; Pinteus *et al.*, 2018).

The red algae of the Bonnemaisoniaceae family are known for their halogenated compounds with strong antibiotic, antibacterial and antifungal activity (Genovese *et al.*, 2009; Soto, 2009). The red algae of the genus *Asparagopsis* have been reported as sources of many natural products, with emphasis on halogenated compounds such as haloforms, methanes, ketones, acetates, and acrylates. These algae contain an essential oil that gives it a strong aroma, composed mainly of bromoform with lower amounts of other metabolites (Genovese *et al.*, 2009).

It is important to consider the complexity of extracts since *A. armata* is known to generate over 100 compounds including halogenated compounds such as bromoform, bromines, chlorine, iodinated methanes, ethanes, ethanols, acetaldehydes, acetones, 2-acetoxypropanes, epoxypropanes, propenes, acroleins, butenones, and several halogenated acetic and acrylic acids (Genovese *et al.*, 2009; Pinteus *et al.*, 2021).

Producing so many compounds with a wide range of solubility and volatility makes it difficult to select an extraction and isolation method since a single one will not be satisfactory (Genovese *et al.*, 2009). In the evaluation of certain bioactivity of the extract, the concentration of bioactive substances can be very low. Furthermore, the demonstrated bioactivities can result from complex interactions between different molecules, thus an increase or loss of potency can occur in the purification process, depending on the synergies or antagonistic behaviors between molecules (Pinteus *et al.*, 2018). Through in-depth analysis by Gas chromatography–mass spectrometry (GC-MS) Pinteus *et al.* (2021) detected in the crude extract of *A. armata* fatty acids (hexadecanoic acid, dodecanoic acid, octadecanoic acid, and tetradecanoic acid) as the main compounds but also the presence of various brominated compounds such as tribromoethanol.

Asparagopsis armata is able to store these bioactive compounds in specialized structures avoiding their autotoxicity. This seaweed is known to contain specialized cells, typically known as vesicle or glands cells, with tubular connections allowing the transport of the secondary metabolites without cellular damage to the algae surface. The bioactive compounds are transported and secreted in the external region of the algae as a form of defense, being detected in the surrounding growth media (Amsler, 2008; Genovese *et al.*, 2009; Paul *et al.*, 2006).

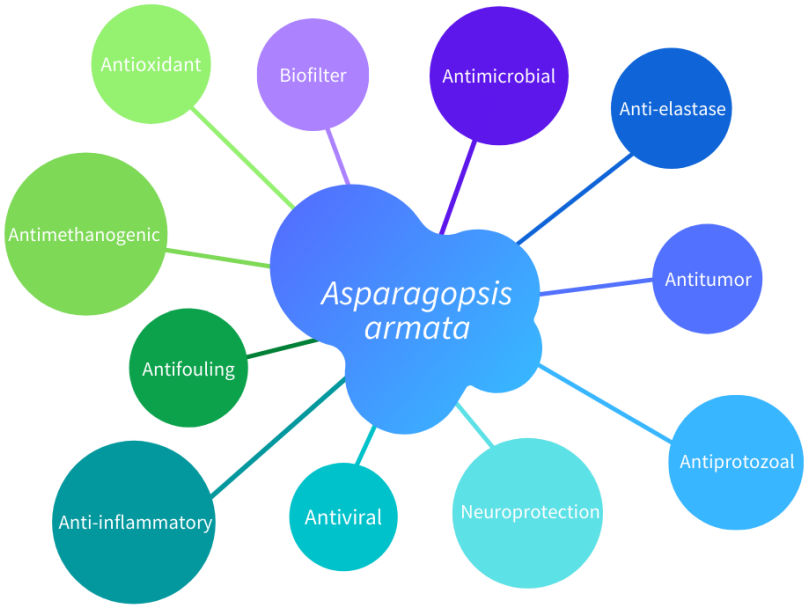

Figure 5: Main bioactivities that have been verified in *Asparagopsis armata*

The potential and/or bioactivity of the natural products of this seaweed has been diversely explored over the last years, and some of the main bioactivities described are represented in Figure 5. Accordingly, the following are some of the many examples described in the literature:

- Hornsey and Hide (1974) were pioneers in exploring the antimicrobial properties of *A. armata*, demonstrating inhibition of the growth of several bacteria with fresh portions of the algae. The antimicrobial potential has been widely exploited against immense pathogens of humans as well as fish and shrimp. For example:
  - Dichloromethane extracts from *A. armata* were used against fish pathogenic bacteria by Bansemir *et al.* (2006), being a potential alternative to conventional antibiotics (Katsanevakis *et al.*, 2014).
  - Rhimou (2010) performed tests with extract of *A. armata* which proved to be a potent antiviral against the herpes simplex virus.
- In the mid-90's, the cultivation of vegetatively propagated gametophytes in ropes to extract bioactive compounds (especially brominated and iodinated methanes and acetones) to be used in cosmetic and medical products, started in Brittany, north-west France (Katsanevakis *et al.*, 2014).
- Pinteus *et al.* (2018) reviewed the biotechnological potential presented by *A. armata* and *Sargassum muticum*, mentioning for example the use of antioxidant molecules of these algae in the pharmaceutical industry to increase the body's defenses against oxidative stress, or the possibility of the usefulness of the compounds of interest as a disinfection agent on ornamental fish species.
- Antifouling properties by the action of produced bromoform and dibromoacetic acid was demonstrated by Paul *et al.* (2006). Recently, a study confirmed that *A. armata* compounds have important anti-adherent

properties for the development of new greener antifouling formulations (Pinteus *et al.*, 2020).

- In 2011, researchers from the Polytechnic Institute of Leiria highlighted the antitumoral potential of the molecules produced by *Sphaerococcus coronopifolius* and *A. armata*, in which methanolic extract from each seaweed induced toxicity and inhibited the proliferation of cells of the human cancer cell line HepG2 (Alves *et al.*, 2011, 2016).
- Genovese *et al.* (2009) revealed a high potential of *A. armata* and *Asparagopsis taxiformis* as a source of natural products with antiprotozoal activity against *Leishmania*, which causes severe human leishmaniasis disease.
- For cosmetic purposes, it is already possible to acquire *A. armata* extract (Ysaline100®) with the purpose of being used in skin products and shampoo (Pinteus *et al.*, 2018).
- In a commercial fish farm in the south of Portugal, the tetrasporophyte of this species was successfully cultivated as a continuous biofilter for its effluents (Schuenhoff *et al.*, 2006). Mata (2010) has shown that *A. armata* cultivated in tanks is a biofilter more efficient for aquaculture when than the green seaweed *Ulva rigida* that is conventionally used.
- Roque *et al.* (2019) results showed that the inclusion of *A. armata* as an additive in dairy cows' feed caused enteric methane emissions to decrease.

Despite all the beneficial potential that *A. armata* has revealed, the cytotoxic potential and the threat it may represent cannot be ignored. By releasing halogenated compounds into its environment, it can be toxic to the surrounding biota, potentially inducing changes that may represent an ecological imbalance of the invaded habitat. Guerra-Garcia *et al.* (2012) revealed that although *A. armata* hosts several crustacean assemblages, its invasive presence involves an impoverishment of peracarid species.

Paul *et al*. (2006) observed a feeding deterrence by two marine mesograzers (the amphipod *Hyale nigra* and the abalone *Haliotis rubra*) under exposure to halogenated compounds from *A. armata*. Silva *et al*. (2020) identified a physiological impairment of the common shrimp *Palaemon elegans* and the marine snail *Gibbula umbilicalis*, as well as a behavioral impairment of the marine snail, which presented reduced food consumption, when under the effect of *A. armata* exudate. Recently, Coelho *et al*. (2021) demonstrated a decline in the clearance rate and attachment strength of the mussel *Mytilus galloprovincialis* together with an increased non-enzymatic antioxidant capacity and consequent energy consumption after exposure to low concentrations of *A. armata* exudate.

Accordingly, it becomes important to assess the potential impact of *A. armata* exudate on the surrounding biota. Thus, the present research work consists in the evaluation of the cytotoxicity of exudate in fish cells derived from gilthead seabream (*Sparus aurata*), a species of high economic importance in the Atlantic and Mediterranean.

1.2. *Sparus aurata* as a biological model

The use of fish as biological models in scientific research has increased significantly in recent decades worldwide. This is mainly due to the rapid expansion of the fish farming industry and the increasing use as substitutes for mammalian model organisms, for example, in chemical testing (Schaeck *et al*., 2013). For this research, the selected species was gilthead seabream (*Sparus aurata*) that represents a key element of the coastal ecosystem widely used by the scientific community as good biological model, particularly for marine ecotoxicology. This species is among the most important in aquaculture in Europe, presenting high commercial value (Almeida *et al*., 2019; Franch *et al*., 2006; Rodrigues *et al*., 2018). Gilthead seabream is common throughout the Mediterranean and along the East Atlantic coast, usually found living on rocky or sandy bottoms or in grasslands of weeds. In Europe, this fish species has high economic

importance considering its production, being one of the major products of European aquaculture. Most of the commercialized gilthead seabreams come from aquaculture, with the largest producer being the European Union (Comissão Europeia, 2012). According to the 2019 Fishery Statistics, 228.576 tons of gilthead seabream were produced globally in 2018, of which 898 tons, just in Portugal (INE & DGRM, 2019)

Asparagopsis armata and *Sparus aurata* have already been associated in scientific research before but in different contexts. For example, in the fish farm in southern Portugal mentioned earlier, they used *A. armata* was used a biofilter of effluents generated by gilthead seabream (Schuenhoff *et al.*, 2006). Castanho (2017) tested the *A. armata* extract (Ysaline100®) in the feed for larval aquaculture of *Sparus aurata* to reduce the bacterial load and understand the effects on the development of the larvae. With the use of the extract, the number of bacteria was significantly reduced in the water and the larval guts, and the growth of the larvae increased. However, increased mortality especially up to 10 days after hatching was observed, which indicated a higher susceptibility at this stage of larval development and the need to reduce the extract concentrations at this stage.

1.2.1. Replacement of the animal model

The animal model is the most widely adopted for toxicity evaluation, although its use gives rise to ethical and moral discussions, given the number of animals required and the suffering caused (Cazarin *et al.*, 2004). More than 60 years ago, William Russell and Rex Burch published "The Principles of Humane Experimental Technique", in which they proposed for the first time the concept of the 3Rs (Reduction, Refinement, and Replacement), intending to reevaluate the use of animals in research processes. They proposed that if animals had to be used in experiments, they should be Replaced by less sentient forms or species, the number of animals used Reduced to a minimum, and the experiments Refined so that the pain and distress caused would be as little as possible (Flecknell, 2002). The 3Rs are now globally established and have become rooted in

legislation as the ethical approach to animal experimentation in many countries (Sneddon *et al.*, 2017). Several successful alternatives to the use of animals have been developed, such as *in vitro* models, computer model approaches, cell cultures, and new imaging or analytical techniques (Doke & Dhawale, 2015; Sneddon *et al.*, 2017). With this in mind, following the law and moral ethics, a gilthead seabream cell line was used in all experimental work.

The gilthead seabream fish was not used in this experimental process following the concept of 3Rs and with the Decree-Law No. 113/2013, that transposes the Directive 2010/63/EU which establishes measures for the protection of animals used for scientific purposes, in order to protect animals but at the same time allow the evolution of science (Decreto-Lei n.º 113/2013 de 7 de Agosto Do Ministério Da Agricultura, Do Mar, Do Ambiente e Do Ordenamento Do Território, 2013; Directiva 2010/63/UE Do Parlamento Europeu e Do Conselho 22 de Setembro de 2010 Relativa à Protecção Dos Animais Utilizados Para Fins Científicos, 2010).

1.2.1.1. *Fish cell culture*

Cell culture is routinely applied as a preliminary screening in toxicological tests, since the process of integrating toxicological knowledge often starts from the simplest biological organization, namely, at the cellular and molecular level (Bols *et al.*, 2005; Doke & Dhawale, 2015). Research using cell culture offers many advantages such as a controlled and defined environment, and conditions that are easier to reproduce (pH, temperature, osmotic pressure, and O_2 and CO_2 pressure) (Bols *et al.*, 2005; Freshney, 2010). In contrast to whole animal studies in toxicology, it is an inexpensive technology with faster results, easier dosing, and no interference from the complexities and variations of non-target processes. In the field of ecotoxicology, cell culture enables comparison at the cellular level between multiple species on exposure to toxic substances under identical conditions. In addition, it is possible to identify and study biomarkers, to evaluate the potential environmental impact of individual chemicals, or

for identification of eco-toxicants in samples taken from the environment (Bols *et al.*, 2005; Freshney, 2010).

In cell culture it is possible to use cell lines and/or primary cultures, the two types are interrelated since cell lines are developed from primary cultures. Primary cultures are freshly isolated directly from cells, organs, or tissues of the animal and when they reach the maximum capacity of substrate or space (confluence is reached) the subculture is performed, becoming a cell line (Bols *et al.*, 2005; Segner, 1998). They are considered finite if incubated for a limited time variable, days or weeks until proliferation stops, or they reach senescence, and this is one of the great differences in regard to the continuous cell lines (Bols *et al.*, 2005; Thermo Fisher Scientific, 2019; Uysal *et al.*, 2018). These become immortal through a process known as transformation, which can occur spontaneously or in a chemically or virally induced process. As a result, they acquire the ability to propagate indefinitely in culture due to becoming immortalized. Continuous cell lines often lose their original structural, metabolic, and functional properties. In contrast, they provide an unlimited number of cells while primary cultures/finite lines may present difficulties in providing sufficient cells of homogeneous quality, as very precise cell separation techniques are required to avoid heterogeneity. Cell culture, although often used, cannot replace the complex interactions of the animal model, but provides an initial insight into biological processes involved at the cellular level (Bols *et al.*, 2005; Freshney, 2010; Segner, 1998; Uysal *et al.*, 2018). The major limitation of cell culture is the expenditure of effort and materials to produce relatively few cells (Freshney, 2010).

Cell cultures from fish are important model systems in a wide variety of research areas, e.g. ecotoxicology, virology, biotechnology, biomedical, immunology, carcinogenesis, transgenics, and disease control (Schaeck *et al.*, 2013). In 1962, the first fish cell line was first established from the gonad of rainbow trout *Salmo gairdneri* (RTG-2) and is still used for many applications, such as virology and toxicology (Wolf & Quimby, 1962). Since then, the development of fish cell lines has progressed enormously, and their number has increased significantly. Rachlin and Perlmutter (1968) pioneered the

use of fish cell lines for *in vitro* cytotoxicity assays and performed their research in the study of aquatic toxins.

1.3. Cell viability assays

Cell viability assays are frequently applied to determine if test compounds affect cell proliferation or cause cytotoxic effects. Most of these methods are based on the same principle, the number of viable cells present is estimated by measuring the activity of a biochemical marker associated with viable cell (Präbst *et al.*, 2017; Riss *et al.*, 2016). In most cell viability assays, cells are incubated with a substrate that will be converted into a fluorescent or colored product by the viable cells. This product can be measured by basic spectroscopic and will be proportional to the number of viable cells present since dead cells are unable to convert the substrate (Präbst *et al.*, 2017; Riss *et al.*, 2016). Two of the most widely used methods are MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay and resazurin reduction assay (Adan *et al.*, 2016; Stockert *et al.*, 2018).

1.3.1. MTT tetrazolium reduction assay

The MTT tetrazolium reduction assay was developed by Mossman in 1983 as a rapid colorimetric assay for the quantification of living cells on a scanning multiwell spectrophotometer. It has been and remains widely applied by the scientific community as can be verified by the vast amount of published articles containing it (Mosmann, 1983; Präbst *et al.*, 2017; Riss *et al.*, 2016; Stockert *et al.*, 2012, 2018). This assay is based on the ability of the yellow tetrazolium salt MTT to penetrate easily into the viable cells, given its positive charge, and be converted into insoluble purple formazan crystals by mitochondrial enzymes such as dehydrogenases using NADH as an electron donor (Präbst *et al.*, 2017; Riss *et al.*, 2016). As the formed crystals accumulate inside the cells, their solubilization is necessary for their spectroscopic measurement. Solubilization can be performed with different solutions such as acidified isopropanol originally used by

Mossman (1983) or DMSO, which several authors defend as a better alternative (Adan *et al.*, 2016; Präbst *et al.*, 2017; Riss *et al.*, 2016; Stockert *et al.*, 2018). To perform it, the assay causes total death of the cells under study, being a disadvantage especially for follow-up studies. Although it involves an optimization of cell density and incubation time performed, it is a simple, inexpensive, and fast method for measuring mitochondrial activity using a substrate metabolized by most cell types (Adan *et al.*, 2016; Präbst *et al.*, 2017; Stoddart, 2011).

1.3.2. Resazurin reduction assay

Discovered by Weselsky, resazurin has been an indicator of cell metabolism to estimate cell viability and proliferation widely applied over the years. Since the 1920s it has been used in the analysis of bacterial and yeast contamination of milk. It was also adopted to assess semen quality and antifungal susceptibility testing (Czekanska, 2011; Nixon & Lamb, 1945; O'Brien *et al.*, 2000; To *et al.*, 1995; Twigg, 1945; Weselsky, 1871). This redox dye (blue and nonfluorescent) is a cell-permeable indicator that can be reduced in the resorufin product (pink and fluorescent) by cells with an active metabolism (through mitochondrial, microsomal, or cytosolic enzymes) (O'Brien *et al.*, 2000; Präbst *et al.*, 2017; Riss *et al.*, 2016). This method, also known as Alamar Blue, is very popular for its simplicity, versatility, homogenous nature, low cost, and high sensitivity (more than tetrazolium reduction assays). Besides that, it can be measured either absorbance or fluorescence and allows multiplex assays with a sequential protocol (O'Brien *et al.*, 2000; Präbst *et al.*, 2017; Riss *et al.*, 2016; Stoddart, 2011). However, in fluorescence measurement, there may be fluorescence interference produced by the compounds under study (Riss *et al.*, 2016). The resazurin reduction assay involves the optimization of cell density per well and especially incubation time since, even though resazurin is not toxic to cells in short exposures, cell viability may be affected when contact is excessive (Czekanska, 2011; Riss *et al.*, 2016).

1.4. Biochemical biomarkers

The concept of a biomarker is quite diffuse in the literature and several definitions have been given. However, it can be considered as a biological response to exposure or the toxic effects of environmental chemicals (van der Oost *et al.*, 2016). Biochemical biomarkers can play an important role as early warning signals of adverse biological responses as they are measured at the molecular and cellular level, anticipating changes at higher levels of biological organization (organelle, cell, tissue, organ, individual, population) (Rodrigues *et al.*, 2018; van der Oost *et al.*, 2016). In the environment, these signals can be essential for understanding ecotoxicological effects induced by exposure to environmental contaminants or climate change (Barbosa *et al.*, 2019).

Glutathione *S*-Transferases (GST) as one of the widely used biomarkers is associated with antioxidant defense and biotransformation. It is involved in the reactions that occur in phase II of metabolism in the biotransformation of xenobiotics in organisms (Rodrigues *et al.*, 2018). In addition, it is implicated in the antioxidant defense system of organisms, protecting cells and their components against reactive oxygen species (ROS) and other free radicals. When ROS are in excess, they can lead to oxidative stress or oxidative damage of cellular components, such as proteins, lipids, and DNA (Jemec *et al.*, 2010; Rodrigues *et al.*, 2018). Also, indicators of antioxidant activity are non-protein thiols (NPSH) and the widely applied enzyme catalase (CAT), which acts against ROS by converting hydrogen peroxide in water and oxygen (Almeida *et al.*, 2019; Oliveira *et al.*, 2010). In the group of non-protein thiols, the most abundant is glutathione (GSH), which is important in detoxification and excretion of xenobiotics, and important modulator of cellular homeostasis (Oliveira *et al.*, 2004, 2010)

Aims of the study

This work aimed to evaluate the toxicity of *Asparagopsis armata* exudate in a fibroblast-like cell line, derived from the fin tissue of an adult gilthead seabream (*Sparus aurata*). For this purpose, cell viability and proliferation as well as the enzymatic activity

of catalase, glutathione *S*-transferases, and non-protein thiol groups were evaluated after 24 hours exposure. Considering that the exudate was not isolated from artificial salt water, the effect of artificial salt water was also evaluated

Chapter II:

Materials and Methods

2. Materials and Methods

2.1. *Asparagopsis armata* sampling and exudate production

Asparagopsis armata (gametophyte phase) was collected by hand through free diving in the subtidal zone at the Terceira island Azores, Portugal (38°38'59.2"N 27°13'16.4"W). Seaweed were kept in aerated seawater tanks until next day and packed in sealed containers to be transported to the University of Aveiro (Portugal). Immediately upon arrival, *A. armata* individuals were cleared from debris and associated epiphytes. They were then allocated to a tank with artificial salt water (ASW) (marine RedSea® Salt premium grade) in a 1:10 proportion (salinity 35 ± 1, pH 8.0 ± 0.1, temperature 20.0 ± 0.5°C) in the dark with no aeration for 24h to produce the exudate. Afterwards, algae were removed from the tank and the resulting media (stock solution – 100 %) was preserved at – 20°C, until further use (Coelho *et al.*, 2021).

For this research, the exudate was carefully defrosted on over ice protected from light. After defrosting and already inside the flow chamber with the light off, the exudate was filtered with a syringe and 0.2 µm filter (cellulose acetate membrane) and stored in 1 mL aliquots. As the Eppendorf tubes were being filled, they were wrapped in aluminum foil and placed on ice to avoid their degradation. All aliquots were preserved at -20°C for future use.

For the control, the same filtration and storage procedure was performed for the artificial salt water (marine RedSea® Salt premium grade) with salinity 35.

2.2. Cell culture

For the cytotoxicity assays and biomarkers, the SAF-1 (ECACC 00122301) continuous cell line was used, which was established and characterized by Julia Bejar, Juan Borrego, and M. Carmen Alvarez in Spain in 1997. This immortalized fibroblast-like cell line is derived from the fin tissue of an adult gilthead seabream (*Sparus aurata*). When compared to other cellular fish lines, SAF-1 presents some advantages in maintenance,

such as optimal growth capacity in a standard environment, with a low concentration of fetal bovine serum (FBS), and without requiring the addition of NaCl (Bejar *et al.*, 1997).

The cells were cultured in Leibovitz-15 medium (L-15) (Gibco, Thermo Fisher Scientific), supplemented with 10% fetal bovine serum (FBS) (SIGMA-Aldrich) and 1% antibiotics (100 µg/mL streptomycin and 100 U/mL penicillin G) (SIGMA-Aldrich). The cells were maintained in 10 cm culture dishes or 25 cm^2 flasks in a cell incubator at 25ºC and with 85% relative humidity (Almeida *et al.*, 2019).

When the cell confluence was around 80%, the cells were routinely subcultivated following the standard trypsinization method, using a dissociation enzyme, TrypLE Express (Gibco), to detach the cells from the petri dish/ flask, which was neutralized with medium with serum.

2.3. Cytotoxicity assays

Trypan blue dye exclusion test was used for the quantitative evaluation of viability and cell proliferation. All the cells that exclude the dye are considered viable, as it selectively stains dead cells or cells with damaged cell membranes (Stoddart, 2011). Cell counting was performed using a hemocytometer (Neubauer chamber).

Viable cells were seeded at a density of 2.0×10^4 cells/well in 96-well plates and kept in the incubator until the following day to allow their adherence. This cell density seeded was set after previous assays where the cell growth rate and optimal absorption readings were evaluated.

Dilutions of exudate in the culture medium were freshly performed for each assay in the following percentages: 5, 10, 15, 20, 25, 30, 35, 40, 45, 50%. In parallel, dilution of artificial sea water, in the same concentrations, was prepared as controls.

After 24 hours of seeding, the culture medium was discarded from the plate, and 150 µL of each condition (dilution of algae exudate and respective artificial salt water control) was added to the cell monolayer. The cells were exposed for 24 hours to control

(culture medium) and to dilutions of exudate and artificial salt water (ASW), and during the exposure, the 96-well plate was maintained in the incubator at 25ºC protected from light. For each experimental condition (concentration or control), four technical replicates were performed in each assay.

The entire exposure procedure was analogous for the resazurin and MTT assays, only differing after the exposure.

2.3.1. Resazurin assay

The resazurin reduction assay was used to evaluate the cytotoxicity of the *Asparagopsis armata* exudate on SAF-1 cell line. After exposure of the cells to the exudate and ASW, the resazurin assay protocol described by Riss *et al.* (2016) was applied to access cell viability. Resazurin solution, 30 µL of 0.15 mg/mL (Sigma-Aldrich) prepared previously, was added to each well (with the light off) to achieve a final concentration of 0.025 mg/mL. The plate was placed in incubator for 4 hours at 25ºC, protected from light (Riss *et al.*, 2016). The absorbance was read at 570 nm (resorufin) and 600 nm (resazurin) using a spectrophotometer plate reader.

2.3.2. MTT assay

For the determination of cytotoxicity following the MTT method, the protocol MTT-based cell growth determination kit (Sigma-Aldrich) and described by Riss *et al.* (2016) were adapted. The MTT substrate (Sigma-Aldrich) was previously prepared in a physiologically balanced solution (5 mg/mL in PBS), sterilized by filtration (syringe with 0.2 µm filter), and stored protected from light at -20ºC.

After 24 hours exposure, the medium was discarded and 100 µl/well of phosphate-buffered saline (PBS) was added to wash the cells. Then, 100 µl of MTT diluted in PBS at the final concentration of 0.5 mg/mL was added to each well, following the incubation of 3 hours at 25ºC with the plate protected from light. When completed, the MTT solution

was removed and for cell lysis, 100 µl/well of 100% DMSO was added to dissolve the crystals formed inside the cells. After 20 minutes, the absorbance was measured spectrophotometrically at 570 nm (formazan) and 690 nm.

2.3.3. Preparation and biochemical biomarkers assays

2.3.3.1. *Sample preparation*

Based on the cell viability results of the assays described earlier, the SAF-1 cell line was exposed to equal dilutions of artificial salt water and exudate of the algae *A. armata* in a culture medium (25, 30, and 35%). For each condition, 3 biological replicates were used in 25 cm^2 flasks with $7x10^5$ cells each for 24 hours, including control cells that were incubated with culture medium only. After 24 hours of exposure, cells were extracted from the flasks with the standard trypsinization method.

Viable cell count was performed with an automated cell counter (Countess™ II FL Automated Cell Counter) to confirm the existence of cells required to perform the following assays. A preliminary assay with only control samples was performed to ensure that the number of cells used was satisfactory for protein quantification and biomarker activities determination. Each replicate was frozen in homogenization buffer (20 mM Tris-HCl pH 7.5, 1 mM phenylmethylsulfonyl fluoride (PMSF)) at -80°C until further measurements of total protein quantification, and analyses of catalase (CAT), glutathione *S*-transferases (GST), and non-protein thiols (NPSH) activities.

Samples were individually homogenized using a sonicator (Branson Ultrasonics™, Sonifier 250), with caution, and on ice to prevent enzyme degradation. All assays for biomarker activity determination and protein quantification were performed in micro-assays set up in 96 flat-bottom plates and read spectrophotometrically. For all assays measuring biomarker activity, blanks were the homogenization buffer used for the samples. All samples were measured in quadruplicates or triplicates according to sample availability.

2.3.3.2. *Protein quantification*

Protein quantification was determined for all samples based on the Bradford method (1976), adapted for micro-assay set up in a 96-well flat bottom plate, using bovine γ-globulin as the standard. In each well of the microplate 10 µL of each sample/control/standard and 250 µL of freshly prepared and diluted Bio-Rad reagent (1:4 ultrapure H_2O) was added. The 96-well plate was then deprived of light for 15 min and the absorbance was read at 595 nm.

2.3.3.3. *Catalase activity*

Catalase (CAT) activity was determined following the procedure of Aebi (1984) with some modifications analyzing the decomposition of the substrate of hydrogen peroxide (H_2O_2) at 240 nm. CAT activity was calculated as µmol H_2O_2 consumed per min per mg of protein (ε = 43.5 M^{-1} cm^{-1}). A volume of 25 µL of sample was and 225 µL of reaction mixture (K-phosphate buffer 0.05 M, pH 7.0 and H_2O_2 30 mM) was added to each well. Absorbance was read for 3 min at 240 nm.

2.3.3.4. *Glutathione S-Transferases*

An adaptation of the procedure of Habig *et al.* (1974) was followed for the determination of Glutathione *S*-Transferases (GST) activity with the conjugation of glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB). In each well, 50 µL of sample/blank was added along with 200 µL of reaction mixture (165 µL K-phosphate buffer (0.1 M, pH 6.5), 30 µL GSH (10 mM), and 5 µL CDNB (60 mM). Absorbance was read at 340 nm for 3 min at 25°C and expressed as millimoles CDNB conjugate formed per minute per milligram protein (ε = 9.6 mM^{-1} cm^{-1}).

2.3.3.5. *Non-protein thiols levels*

Non-protein thiols were determined by the method of Sedlak and Lindsay (1968) with adaptations, based on the reaction of the -SH groups with the color reagent 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB). For protein precipitation, 150 µL of sample were incubated with 150 µL of 10% trichloroacetic acid (TCA) on ice for 1 hour. Then, the samples were centrifuged at 12000 rpm for 3 min at 20°C. In the microplate 50 µL supernatants, 230 µL sodium potassium buffer (0.1M, pH 7.4) and 20 µL DTNB were added. Absorbance at 412 nm was determined and activity expressed as nmol per mg protein.

3. Data analysis

All results are presented as the mean ± standard deviation of the mean of replicates performed. Statistical differences between two groups were analyzed by the unpaired two-tailed Student's *t* test. Differences were considered significant when *p*-values were lower than 0.05.

CHAPTER III:

RESULTS AND DISCUSSION

1. Results and Discussion

1.1. Cytotoxicity assessment

The cell viability data acquired by spectrophotometry of the MTT and resazurin reduction assay were presented in percentage in relation to the control, considered as 100% viability. Figure 6 represents the cell viability for each experimental condition after 24 hours exposure to *A. armata* exudate and artificial salt water (ASW) evaluated by MTT assay. This cell viability parameter shows a reduction with increasing concentration of exudate or ASW added to the cell culture, noticeable for >30% of both, where a significant difference ($p<0.05$) compared to the control is revealed.

At lower concentrations, significant differences from the control are noted, indicating higher proliferation when exposed to 5% and 10% ASW, and 5% exudate. Given the results we can also note a statistically significant difference at 10% of both treatments, where ASW shows a higher percentage of cell viability.

A significant difference between the effects of ASW and exudate was observed from 40%, more pronounced for >45%, indicating a significant reduction of cell viability by the exposure of cells to the exudate *per si*, probably due to the increased secondary metabolites of the exudate in the cells. The difference between exudate and ASW effects may be considered the actual effect of the exudate on the cells, as possible interferences from the artificial salt water in which the exudate was produced were excluded. In fact, it can be observed a reduction of cell viability of ~14%, ~23% and ~38% induced by, respectively, 40%, 45% and 50% of exudate *per si*.

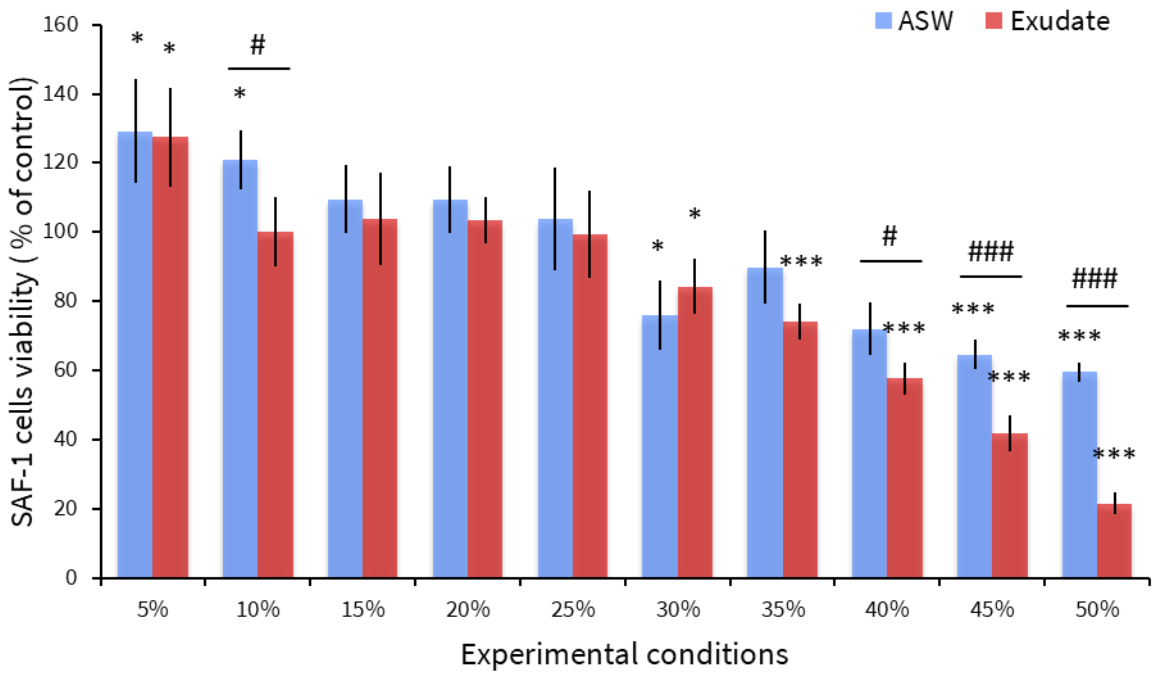

Figure 6: Cell viability assessed by MTT assay of SAF-1 cells after 24 hours exposure to different concentrations of artificial salt water (ASW, blue) and *Asparagopsis armata* exudate (Exudate, red). Values in percentage correspond to mean ± standard deviation (n=4 replicates). * represents statistically significant differences to control (*p<0.05). # represents statistically significant differences between exposure groups (#p<0.05).

Figure 7 represent the resazurin assay for evaluation of cell viability under the same experimental conditions of exposure time and concentrations as the MTT assay. The exposure of SAF-1 cells to ASW show statistically significant differences from the control for concentrations higher than 40%. However, the exudate shows cytotoxic potential starting at 25%, notably reducing cell viability from this concentration onwards, almost disabling all cells at 50% concentration in the culture (with 3.5% viability), suggesting a significantly toxic effect. In fact, it is observed a significant reduction of cell viability of ~25%, ~32%, ~39%, ~40%, ~50% and ~44% induced by 25%, 30%, 35%, 40%, 45%, 50% of the algae exudate *per si*, leaving aside possible interferences from the artificial salt water. A significant increase in cell viability at 5% ASW is also noticeable, indicating increased proliferation when in the presence of low percentages of ASW.

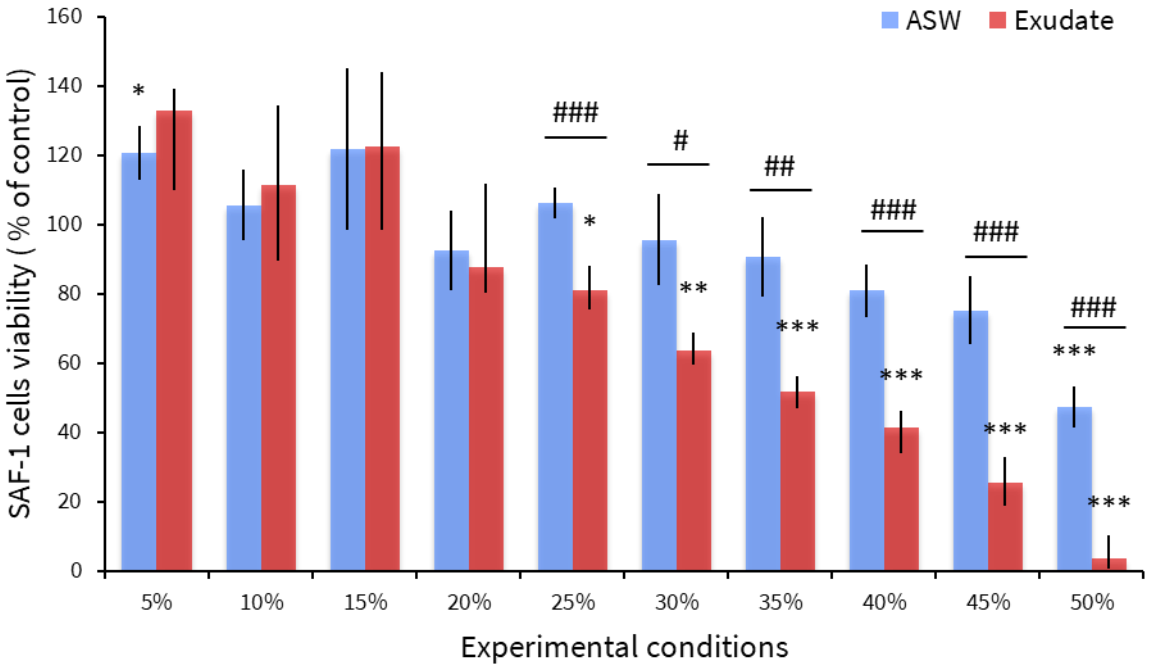

Figure 7: Cell viability of SAF-1 cells after 24 hours exposure to different concentrations of artificial salt water (ASW, blue) and *Asparagopsis armata* exudate (Exudate, red) assessed by resazurin assay. Values in percentage correspond to mean ± standard deviation (n=4 replicates) to control. * represents statistically significant differences to control (*p<0.05). # represents statistically significant differences between exposure groups (#p<0.05).

When comparing the two cytotoxicity experiments, the resazurin assay shows significantly lower values of cell viability on exposure to *A. armata* exudate starting at 30%, while exposure to ASW only reveals a significant difference at 50% concentration.

Paul *et al.* (2006) reported the presence of compounds in the media surrounding the algae that are typically present in extracts of the algae. So, the effect of *A. armata* extract that is present in the literature will be considered for comparison with the data obtained, where applicable. However, there is a noticeable gap in knowledge for studies performed in cell culture concerning the potential cytotoxic impact of either extracts or exudates of *Asparagopsis armata*. Zubia *et al.* (2009) developed their study on 3 cancer cell lines exposed to 24 different red algae extracts, including *A. armata*. During 24 hours at 100 µg/mL, the extract of *A. armata* in two of the three lines, the Daudi and Jurkat cell

lines, stood out with high cytotoxicity together with *Brongniartella byssoides* and *Heterosiphonia plumosa*. This was the first report of cytotoxic activity of *A. armata*. After that, Alves *et al*. (2016) also evaluated the cytotoxic activity of extracts of 12 seaweeds on a cancer cell line, namely Hep-G2, for 24 h at the concentration of 1000 µg/mL. The authors reported *A. armata* as one of the analyzed algae with more cytotoxic activity and an interesting antitumor potential, together with *Plocamium cartilagineum* and *Sphaerococcus coronopifolius*. In accordance with the observed in these studies, the present results also demonstrate the cytotoxic effect of *A. armata*. However, it is not possible to compare in terms of concentration with any of the mentioned studies since they were carried out with extract concentrations incomparable to the practiced conditions.

1.2. Trypan blue and biochemical biomarkers assays

1.2.1. Trypan Blue

Before performing the biomarker assays, viable SAF-1 cells after exposure for 24 hours were counted with trypan blue in an automated cell counter. Figure 8 shows the number of viable cells recorded, revealing significant differences from control in almost all conditions except ASW at 30% and 35%, the latter showing an unusual standard deviation relative to the others. Being a direct method for measuring viability, requires more manipulation of the cells, implies trypsinization and removal of the cells from the vial where the exposure occurred, which can lead to the loss or unavailability of cells in its handling.

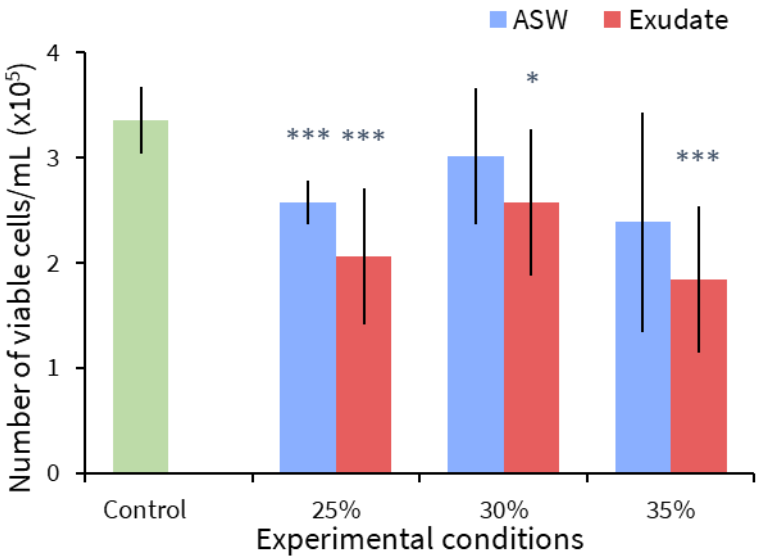

Figure 8: Representation of the number of viable SAF-1 cells present per milliliter using an automated cell counter (Countess™ II FL Automated Cell Counter) with trypan blue after 24 hours of exposure to 25, 30, and 35% exudate of *A. armata* (red) and artificial salt water (ASW, blue) equally. Values correspond to mean ± standard deviation (n=3) to control. * represents statistically significant differences compared to control (* $p<0.05$ and *** $p<0.005$)

1.2.2. Biochemical biomarkers

Figure 9 represents the enzymatic activity of Catalase (A), Glutathione *S*-Transferases (B), and Non-Protein Thiol groups (C) under the different experimental conditions. Catalase (CAT) activity did not reveal any significant differences between the experimental conditions. In the case of glutathione *S*-transferases (GST) activity, no condition showed significant alterations compared to the control, although a significant difference existed between the exudate and ASW, both 30%. Nevertheless, it was in the non-protein thiol groups where a significant increase in activity was recorded at the 30% exudate exposure compared to the control and ASW, proposing an increased non-enzymatic antioxidant capacity, and increased detoxification activity in response to exposure to the metabolites present in the exudate.

The results presented for the enzymatic activity of the biomarkers coincide with those presented by Coelho *et al.* (2021), despite the fact that different types of tests were performed on different organisms and different exposure periods, there was a similar

response in the enzymatic activity of the biomarkers reported. Mussel *M. galloprovincialis* exposed to the same exudate of *A. armata*, found no changes in the enzymatic activity of CAT and GST in different tissues (gills and digestive gland).

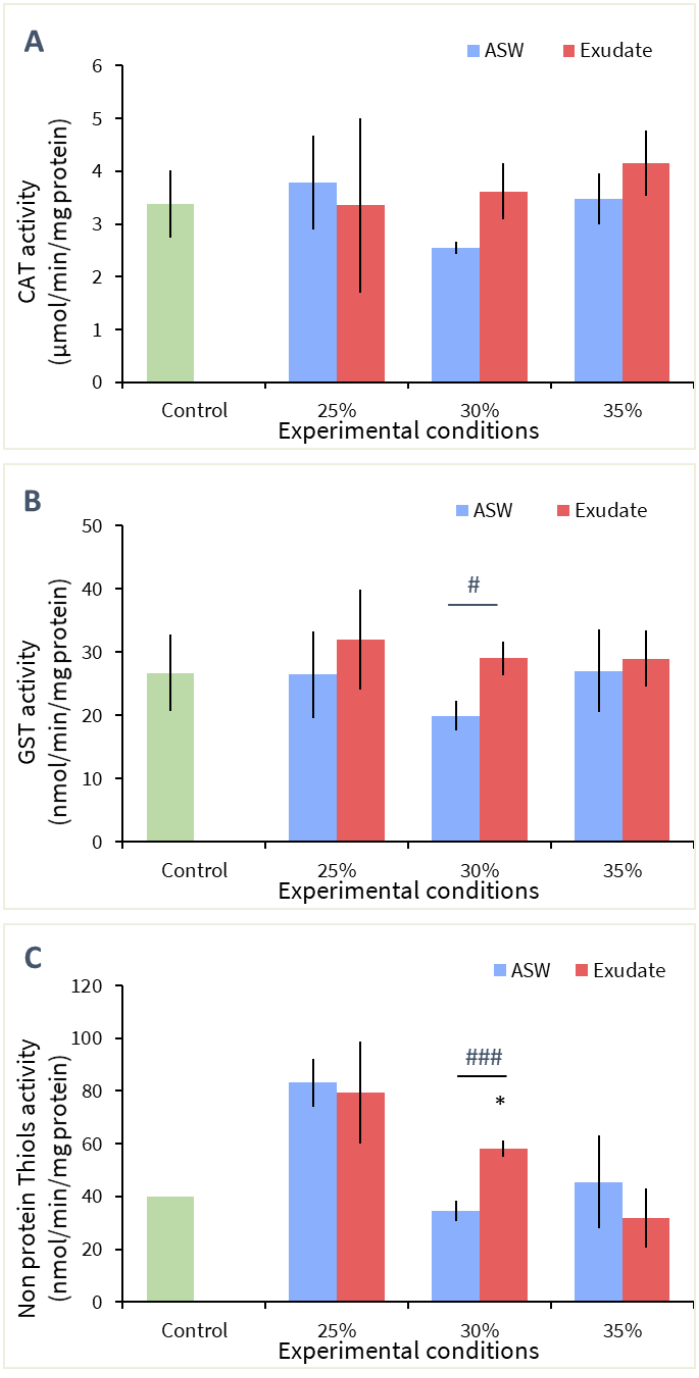

Figure 9: Enzymatic activity of catalase (A), glutathione *S*-transferases (B), and non-protein thiol levels (C) of SAF-1 cells exposed to different percentages of *Asparagopsis armata* exudate (red) and artificial salt water (ASW, blue) for 24h. Results are expressed as mean ± standard deviation (n=3 replicates). * represents statistically significant differences to control (* $p<0.05$). # represents statistically significant differences between exposure groups (# $p<0.05$).

CHAPTER IV:

CONCLUDING REMARKS

To understand the potential impact of the exudate of the invasive alga *Asparagopsis armata*, cytotoxicity tests were performed to assess the viability and cell proliferation of SAF-1 cells when exposed to it. Antioxidant enzymatic and biotransformation activities through catalase and glutathione *s*-transferases, and non-enzymatic antioxidant activity through non-protein thiols were also evaluated (Almeida *et al.*, 2019; Oliveira *et al.*, 2010).

Although there is a significant amount of research on the potential of *Asparagopsis armata*, there is limited knowledge on the impact of the released exudates on the surrounding environment, especially on cell lines. Nevertheless*, A. armata* revealed cytotoxicity in the tested gilthead seabream fish cells that support the toxicity reported in other models (Coelho *et al.*, 2021; Guerra-garcía *et al.*, 2012; Paul *et al.*, 2006; Silva *et al.*, 2021). Therefore, this research reveals a significant contribution, since for the first time the effect of exudate produced by invasive algae on a fish cell line was tested.

The increased non-protein thiol content in SAF-1 cells after exposure to exudate, suggests a response to altered oxidative status leading to increased antioxidant defenses. Also suggesting the increase in glutathione, which may be due to the high presence of halogenated metabolites in the exudate of *A. armata*. Given that the toxicity of these compounds seems to be related to their efficiency as alkylating agents, and the resistance of cells to these agents has been associated with high concentrations of glutathione in the cells (Colvin *et al.*, 1993; McConnell & Fenical, 1977). It may also indicate alterations in important biochemical processes since the glutathione is involved directly or indirectly in several processes such as maintenance of enzyme activity, amino acid transport, or protection against oxidative stress (Coelho *et al.*, 2021; Oza *et al.*, 2002). These biochemical responses could be more thoroughly explored in the future and used as warning signals in determining the effects provoked by the compounds released from *A. armata*.

There is still no information on the concentration of exudates released by this seaweed in the wild. Thus, the impact may be more severe, since the toxic effect will

presumably depend on the density of the algae present in the invaded areas, such as the volume of water present for example in tide pools where competition with other species for resources and nutrients is higher. Furthermore, the stress to which the algae are subjected may also cause variations in the toxicity, since the amount of bioactive compound produced may differ depending on the physical stresses that they are subjected to (such as temperature or sun exposure).

Since the algal exudate is a mixture of several compounds, it is crucial to determine the effective concentrations of each compound and ascertain which contribute the most to the observed effects, the types of interactions between the active substances, such as the influence of abiotic conditions. Validating the equivalence of the exudate components with extracts reported in the literature is also essential.

CHAPTER V:

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