Universidade de Aveiro

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SUSETE FILIPA GONÇALVES PINTEUS

ACRESCENTAR VALOR AOS INVASORES MARINHOS *ASPARAGOPSIS ARMATA* E *SARGASSUM MUTICUM*

ADDING VALUE TO THE MARINE INVADERS *ASPARAGOPSIS ARMATA* AND *SARGASSUM MUTICUM*

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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 (Do*Mar), realizada sob a orientação científica do Doutor Rui Filipe Pinto Pedrosa (Investigador Principal do Centro de Investigação MARE-IPLeiria e Professor Coordenador do Politécnico de Leiria), do Professor Doutor Luis Miguel Botana López (Professor Catedrático da Universidade de Santiago de Compostela) e do Doutor Marco Filipe Loureiro Lemos (Professor Adjunto do Politécnico de Leiria).

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"Para ser grande, sê inteiro: Nada teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és no mínimo que fazes.
Assim em cada lago, a lua toda brilha,
porque alta vive."

Ricardo Reis

o júri:

presidente

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Professor Coordenador, Instituto Politécnico de Leiria

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Palavras-chave Algas, antioxidantes, antimicrobianos, anti-incrustantes, biotecnologia marinha, espécies invasoras, extratos bioativos, produtos naturais.

Resumo

As espécies invasoras marinhas são uma das principais ameaças à integridade dos ecossistemas marinhos, promovendo um desequilíbrio nas comunidades nativas, resultando em sérios impactos ecológicos e económicos. Entre as algas marinhas, a *Sargassum muticum* e a *Asparagopsis armata* são reconhecidas como invasoras de sucesso na Europa e América. Apesar de já terem sido efetuadas várias tentativas para controlar o avanço de invasores marinhos, nenhuma provou ser efetivamente eficaz, sendo, portanto, necessário desenvolver estratégias alternativas.

Nas últimas décadas as algas marinhas têm sido estudadas devido às suas múltiplas propriedades biológicas. No entanto, um dos principais problemas associados à exploração destes componentes bioativos prende-se com a abundância de matéria prima que, na maioria dos casos, é escassa e sazonal, resultando a sua exploração em sérios danos ecológicos. Neste contexto, a exploração de organismos invasores marinhos oferece-nos uma dupla oportunidade – elevada disponibilidade do material biológico para extração de compostos bioativos com características únicas com vista ao desenvolvimento de novos produtos, e, por outro lado, através da sua recolha, a mitigação dos efeitos negativos provocados pela sua invasão, contribuindo deste modo para a integridade e sustentabilidade do ecossistema marinho.

Neste contexto, o presente trabalho pretendeu avaliar o potencial antioxidante, antimicrobiano e anti-incrustante de extratos destas duas algas invasoras, bem como a quantificação e caracterização química dos compostos bioativos através de técnicas cromatográficas e espetroscópicas. Em acréscimo, foram também estudados os mecanismos de ação envolvidos nas bioatividades mais potentes.

As propriedades antioxidantes foram avaliadas através da determinação do conteúdo total em polifenóis (TPC), da capacidade de destoxificação do radical 1,1-difenil-2-picril-hidrazil (DPPH) e da avaliação da capacidade de absorção do radical oxigénio (ORAC).

A atividade antimicrobiana foi avaliada em *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, *Escherichia coli* e *Salmonella enteritidis*, através da análise da inibição do seu crescimento. Neste âmbito, foi também avaliado o potencial de inibição de produção de biofilmes em *Pseudomonas aeruginosa*.

As propriedades anti-incrustantes foram avaliadas através da análise da capacidade de inibição do crescimento de nove microalgas, nomeadamente *Tetraselmis suecica*, *Tetraselmis chui*, *Thalassiosira weissflogii, Chaetoceros calcitrans*, *Cylindrotheca closterium, Nannochloropsis oculata, Phaeodactylum tricornutum, Isocrysis galbana*, *Nannochloropsis gaditana* e em cinco microrganismos marinhos, nomeadamente *Vibrio anguillarum*, *Photobacterium damselae* ssp. *piscicida*, *Aeromonas hydrophila*, *Aeromonas aquariorum* e *Edwardsiella tarda*. Foi também testado o potencial dos extratos e das frações de ambas as algas na inibição da produção de biofilmes nos microorganismos *Vibrio parahaemolyticus* e *Bacillus subtilis.*
Também no âmbito do potencial anti-incrustante foi proposto um novo modelo para avaliar as propriedades de anti-aderência dos extratos em pólipos de medusas, nomeadamente de *Aurelia aurita e Phyllorhiza punctata.*

De uma forma geral, *Sargassum muticum* revelou ser a alga com maior potencial antioxidante, revelando propriedades citoprotetoras em condições de stress oxidativo que poderão estar relacionadas com a redução da produção de peróxido de hidrogénio e com bloqueio do processo apoptótico.

Por outro lado, a alga *Asparagopsis armata* revelou o maior potencial antimicrobiano com um largo-espectro de atividade e com capacidade para inibir o desenvolvimento de biofilmes bacterianos. A ação antimicrobiana parece estar relacionada com danos na membrana citoplasmática e danos no ADN.

Ambas as algas apresentam potencial anti-incrustante. Esta bioatividade foi mais evidente nos extratos brutos de ambas as algas, tendo-se verificado propriedades anti-algais, anti-bacterianas e de anti-aderência.

Demonstra-se nesta investigação que ambas as algas apresentam propriedades bioativas relevantes com elevado potencial para serem usadas no desenvolvimento sustentável de novos produtos, resultando em benefícios económicos importantes. Por outro lado, na pesquisa de produtos naturais marinhos, um dos principais obstáculos para o desenvolvimento industrial de novos produtos prende-se com a escassez da matéria prima e os impactos ambientais negativos inerentes à recolha do material biológico. Neste contexto, as algas *Sargassum muticum* e *Asparagopsis armata* são invasores que ocorrem em abundância, não só na costa Portuguesa, mas em praticamente todo o mundo e, portanto, a sua recolha do meio ambiente para a exploração industrial dos seus compostos bioativos contribuirá para a restauração e equilíbrio do ecossistema marinho, transformando uma ameaça ambiental numa grande oportunidade sócio-económica.

Keywords Seaweed, antioxidant, antimicrobial, antifouling, marine biotechnology, invasive species, bioactive extracts, natural products

Abstract

Marine invasive species are widely recognized as one of the worst threats to marine ecosystems integrity, unbalancing native communities, which may lead to paramount ecological and economic impacts. Within seaweeds, *Sargassum muticum* and *Asparagopsis armata* are recognized as successful invaders in Europe and America. Despite several attempts to control the spread of marine invaders, until now, all have proven to be elusive, and therefore, alternative strategies should be embraced.

Worldwide, seaweeds have been increasingly explored due to their ability to produce bioactive compounds. However, one of the main problems associated with the production/extraction of these bioactive compounds for new products development, is the scarce availability of target species and the potential negative environmental consequences of their exploitation. Accordingly, the use of invasive species to obtain natural bioactive compounds presents us with a two-folded opportunity - high availability of the biological material for the extraction of unique bioactive compounds for new products development, and through specimen collection, the mitigation of the negative effects caused by alien species, contributing to ecosystem integrity and sustainability.

Within this framework, the present work aimed to evaluate the antioxidant, antimicrobial, and antifouling properties of these seaweed extracts, as well as the concentration of the bioactive compounds by chromatographic and spectroscopic means. In addition, the mechanisms of action possibly involved in the most potent antioxidant and antimicrobial activities were also studied.

The antioxidant capacity was estimated through the quantification of the total phenolic content (TPC) and by evaluating the scavenging properties through the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) assays.
The antimicrobial potential was evaluated on *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, *Escherichia coli,* and *Salmonella enteritidis* through growth inhibition analysis.
The antifouling properties of both seaweed was evaluated on the growth inhibition of nine microalgae growth, namely *Tetraselmis suecica, Tetraselmis chui, Thalassiosira weissflogii, Chaetoceros calcitrans*, *Cylindrotheca closterium, Nannochloropsis oculata, Phaeodactylum tricornutum, Isocrysis galbana*, *Nannochloropsis gaditana,* and 5 marine microorganisms, namely *Vibrio anguillarum*, *Photobacterium damselae* ssp. *piscicida*, *Aeromonas hydrophila*, *Aeromonas aquariorum,* and *Edwardsiella tarda*. The biofilm inhibitory capacity was tested on *Vibrio parahaemolyticus* and *Bacillus subtilis*.

Within this framework a new model to evaluate the anti-adherence properties of seaweed extracts on the marine invertebrates *Aurelia aurita* and *Phyllorhiza punctata* polyps is also proposed.

Sargassum muticum revealed to contain compounds with high antioxidant potential exhibiting cytoprotective properties in oxidative stress conditions, which can be related with the reduction of hydrogen peroxide production and apoptosis blocking.

A. armata revealed to produce compounds with high antimicrobial potential with a broad-spectrum activity and with potential to interfere in bacterial biofilms development. Results suggest that the antimicrobial activity may be linked to microorganisms cytoplasmatic membrane damage and DNA damage.

Both, *Sargassum muticum* and *Asparagopis armata* presented relevant antifouling potential. This property was the most expressive on both seaweed crude extracts, which revealed high anti-algae effects, anti-bacterial and anti-adherence properties.

The present work shows that the potential of both seaweeds to provide valuable compounds is enormous, pointing therefore to an opportunity for sustainable development with high economic benefits.

In marine natural products research and development, one of the main bottlenecks for further industrial applications is the scarcity of the biological target and the negative impact that is associated with its collection from the ecosystem. Since *Asparagopsis armata* and *Sargassum muticum* are successful invaders and occur in great amount in Portugal and many other coasts of the world, their collection could contribute for marine ecosystem restoration and equilibrium by means of profitable harvesting for a high revenue industry, turning a threat into a major socio-economic opportunity.

Thesis publications

The present work is based on the following manuscripts:

Pinteus, S., Lemos, M.F.L., Alves, C., Neugebauer, A., Silva, J., Thomas, O. P., Botana, L. M., Gaspar, H., Pedrosa, R. 2018. Marine Invasive Macroalgae: turning a real threat into a major opportunity - the biotechnological potential of *Sargassum muticum* and *Asparagopsis armata*. Algal Research, 34, 217-234.

Pinteus, S., Lemos, M. F., Silva, J., Alves, C., Neugebauer, A., Freitas, R., Duarte, A., Pedrosa, R. 2017. An Insight into *Sargassum muticum* cytoprotective mechanisms against oxidative stress on a human cell *in vitro* model. Marine Drugs, 15(11), 353.

Pinteus, S., Lemos, M. F., Simões, M., Alves, C., Silva, J., Gaspar, H., Martins, A., Rodrigues, A., Pedrosa, R. 2020. Marine invasive species for high-value products exploration – Unveiling the antimicrobial potential of *Asparagopsis armata* against human pathogens. Algal Research, 52, 102091.

Pinteus, S., Lemos, M. F., Alves, C., Silva, J., Pedrosa, R. 2021. The marine invasive seaweeds *Asparagopsis armata* and *Sargassum muticum* as targets for greener antifouling solutions. Science of the Total Environment, 750, 141372.

Pinteus, S., Lemos, M. F., Freitas, R., Duarte, I. M., Alves, C., Silva, J., Marques, S.C., Pedrosa, R. 2020. Medusa polyps adherence inhibition: A novel experimental model for antifouling assays. Science of the Total Environment, 136796.

Index

List of figures and tables

Figures

Tables

Abbreviations

AA – *Asparagopsis armata*

AAPH – 2,2'-Azobis(2-methylpropionamidine) dihydrochloride

AFS – Antifouling systems on ships

AS – Auto sampler

ATCC – American type culture collection

ATCI – Acetylthiocholine iodide

ATP – Adenosine triphosphate

AUC – Area under curve

BHA – Butylated hydroxyanisole

BHT – Butylated hydroxytoluene

CC - Column chromatography

CH – Cyclohexane

CMD – Collateral membrane damage

DCM – Dichloromethane

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DPPH - 1,1-Diphenyl-2-picryl-hydrazyl

DSMZ – *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (German collection of microorganisms and cell cultures)

DTNB – 5,5-Dithio-bis(2-nitrobenzoic acid)

EtAC – Ethyl acetate

FAO – Food and agriculture organization

FBS – Fetal bovine serum

FID – Flame ionization detector

GAE – Gallic acid equivalents

GC – Gas chromatography

GC-FID – Gas chromatography – flame ionization detector

GC-MS – Gas chromatography - mass spectrometry

HO• - Hydroxyl radical

HPLC – High performance liquid chromatography

IMO – International maritime organization

JC-1 – 5,5’,6,6’-Tetrachloro-1,1’,3,3’-tetraethyl-imidacarbocyanine iodide

LB – Luria broth

MCF-7 – Human breast adenocarcinoma cell line

MD – Methanol:dichloromethane

MDE – Methanol:dichloromethane extract

MDFx – Fraction x of the vacuum liquid chromatography from methanol:dichloromethane extract

MD-VLC - Vacuum liquid chromatography of the methanol:dichloromethane extract

MeOH – Methanol

MFx – Fraction x of the the vacuum liquid chromatography from methanolic extract

MS – Mass spectrometry

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

M-VLC – Vacuum liquid chromatography of the methanolic extract

NMR – Nuclear magnetic resonance

$O2^{\bullet-}$ - Superoxide radical

ORAC – Oxygen radical absorbance capacity

PE – Phloroglucinol equivalents

RNA – Ribonucleic acid

ROS – Reactive oxygen species

SD – Standard deviation

SEM – Standard error of the mean

SM – *Sargassum muticum*

TBHQ – Tertiary butylhydroquinone

TBT – Tributyltin

TE – Trolox equivalents

TLC – Thin layer chromatography

TMD – Total membrane damage

TPC – Total phenolics content

TSB – Trypticase soy broth

UV – Ultra violet

VLC – Vacuum liquid chromatography

WHO – World health organization

YPD - Yeast extract–peptone–dextrose

Chapter 1

General Introduction

This chapter is based and includes the following manuscript:

Pinteus, S., Lemos, M.F.L., Alves, C., Neugebauer, A., Silva, J., Thomas, O. P., Botana, L. M., Gaspar, H., Pedrosa, R. 2018. Marine Invasive Macroalgae: turning a real threat into a major opportunity - the biotechnological potential of *Sargassum muticum* and *Asparagopsis armata*. Algal Research 34, 217-234.

1.1. Marine invaders – the success case of *Asparagopsis armata* and *Sargassum muticum*

Marine environments are among the richest and most complex ecosystems, encompassing a wide and yet not fully known diversity of species, genetic diversity, and also a vast array of habitats molding organism's presence and responses (Hay and Fenical, 1996; Rocha et al., 2011). Normally, these ecosystems are balanced, however with marine resources over-exploration, pollution, and climate changes, marine ecosystems are continuously changing, promoting drivers for exotic species emergence and marine environment invasion success.

An invasive species was defined as "one that has been introduced by human activity, either deliberately or accidentally, to a new geographic area or ecosystem outside of its natural distribution range, and which has then established and spread" (Council of Europe/UNEP, Pan-European biological and landscape diversity strategy, Invasive Alien Species 2002; Carlton, 2002; Shine, 2007). The emergence of invasive species is a serious environmental problem that affects entire biological communities. They compete with native ones (leading to their displacement) and alter habitat's structures promoting biodiversity loss and cascading effects or trophic web shifts that can result in major negative impacts on the ecosystem (Goodenough, 2010; Hussner, 2012; Molnar et al., 2008; Streftaris and Zenetos, 2006). Ultimately, invasive species can promote the eradication of native ones impoverishing local genetic variability, in a process known as "biotic homogenization" (Marchetti et al., 2001). As a result, invasive seaweeds play a key role in marine ecosystem's equilibrium, structure and function by modifying, creating, maintaining, or destroying habitats (Thomsen et al., 2016). Within marine seaweeds, *Sargassum muticum* and *Asparagopsis armata* exhibit a highly invasive behavior being included in the "100 Worst Invasive Species" list for the Mediterranean (Boudouresque and Verlaque, 2002; Streftaris and Zenetos, 2006).

The brown macroalgae *Sargassum muticum* (Yendo) Fensholt (Class: Phaeophyceae, Subclass: Cyclosporeae, Order: Fucales, Family: Sargassasseae) (Guiry and Guiry, 2017) is a brown canopy forming seaweed, native from Asia, that was introduced in North America in the 1940s and in Europe in the early 1970s (Critchley et al., 1990; Farnham et al., 1973) probably attached to imported Japanese oysters (Scagel, 1956). It is known to have a direct impact on the indigenous flora assemblages by affecting the water movement, light penetration, sediment accumulation, and anoxia (Stæhr et al., 2000). This species is a fast grower, with great photosynthetic activity, a high reproductive capacity with an effective sexual reproduction, and the possibility of vegetative propagation. Moreover, it has an efficient dispersion by fertile branches or floats, and a great capacity to attach to any single mobile solid object facilitating its dispersal (Davison, 2009; Hanif

and Idrissi, 2014).These different characteristics provide a clear understanding of its evolutionary and establishment success.

The red seaweed *Asparagopsis armata* (Harvey) (Class: Florideophyceae, Subclass: Rhodymeniophycidae, Order: Bonnemaisoniales, Family: Bonnemaisoniaceae) (Guiry and Guiry, 2017) was first described in 1855 by Irish botanist William H. Harvey on Western Australia and New Zealand coastline (Harvey, 1855). It is encountered in European coasts since 1925 and can also be found in the Northeast Atlantic (from the Shetland Islands to Morocco), Mediterranean, South Africa, Middle East, and Indo-Pacific region (Boudouresque and Verlaque, 2002; Chualáin et al., 2004; Guiry and Guiry, 2017).

In *A. armata*'s life-cycle, two phases are distinguished, an haploid (sexual) and a diploid (asexual), with the haploid phase occurring from June or July - August or September (in Western Europe). The plant is up to 20 cm tall, with barbed branches and is rosy pink, yellowish-pink color, and after removed from water the color becomes reddish. The diploid phase (tetrasporophyte) is occurring all year, but most evident from October until March. In this phase, the plant is more brownish-red, greatly branched, filamentous, with a dense cotton-wool-like tufts organization up to 15 mm in diameter (Guiry and Guiry, 2017). The physical and biological characteristics of *A. armata* are crucial for its invasive success due to its free-floating tetrasporophyte (*Falkenbergia*), and an active propagation *via* fragmentation and attachment to other floating structures on the haploid phase. *A. armata* also shows a strong epiphytic relationship with other seaweed and marine debris. Due to the tetrasporophytes high surface-volume ratio, this seaweed has a greater potential for rapid uptake of nutrients comparing to other seaweeds inputting a negative impact on ocean nourishment. On the other side, it also has the ability to densely cover other species limiting light and nutrients access (Katsanevakis et al., 2014). Additionally, studies revealed that this invasive species has the ability to produce brominated compounds, iodinated methanes and acetones (Marshall et al., 2003; Marshall et al., 1999), which may produce toxic effects on other species leading to their depletion. This phenomenon is quite evident during low-tide in rocky pools were organisms remain several hours without water exchanges (Engström-Öst and Isaksson, 2006; Katsanevakis et al., 2014).

Due to their highly invasive behavior*, A. armata* and *S. muticum* induce important changes in the ecosystem replacing "keystone species" (Boudouresque and Verlaque, 2002; Chualáin et al., 2004). Although it is widely recognized that invasive seaweeds are serious threats to ecosystems, few attempts have been made to control their spread, and less have proven to be efficient. The most obvious method to control invasive seaweed spreading is their collection from the ocean. However,

their removal by hand is extremely time-consuming and will be dependent on low tides or boats and diving, resulting in unaffordable costs if there is no commercial value for these species (Davison, 2009; Kraan, 2008). Some approaches have also included the use of toxic substances that affect not only the target species but also all surrounding environments (Anderson Lars, 2007; Andreakis and Schaffelke, 2012; Kraan, 2008). Therefore, alternative and cost-effective measures should be undertaken to control or mitigate the damaging effects of invasive seaweeds.

The harvesting of the seaweeds for commercial purposes can be an efficient alternative. In the 1980´s, the collection of seaweeds was a common practice in several countries, mainly for fertilization purposes and phycocolloids extraction (McHugh, 2003; Pereira, 2010). However, due to new commercial alternatives and to keep up with industrial development, these activities were placed aside. Nowadays, with societies increasingly aware for environmental issues and demanding natural products, the marine environment is again on the focus of several industries, including highly valued pharmaceutical and biotechnological industries. In fact, it is currently known that the marine environment is a rich source of original bioactive structures, which remains scarcely explored. The quest for new natural products opens, therefore, an opportunity for the exploitation of marine resources being imperative to find sustainable strategies.

Under this framework, the invasive species *S. muticum* and *A. armata* may turn out to be excellent and abundant sources of bioactive compounds, providing high socio-economic revenue, while their collection from the ocean may be included in effective and sustainable management practices contributing to the marine ecosystem equilibrium and site restoration.

1.2. Seaweeds potential for added-value compounds production

The ocean coast is full of diverse macro and microorganism that developed unique metabolic strategies to assure survival in one of the harsher and constantly changing environments. The production of secondary metabolites is therefore crucial for survival and, alike primary metabolites, have a more restricted taxonomic distribution that is often limited to a specific species or genus (Wink, 2003). Seaweeds are extremely plastic, physiologically and morphologically, with the capacity to rapidly adapt to new conditions (Fogg, 2001; Ramus et al., 1976). They are so adaptable that, depending on environmental factors, their metabolites production may be different, even within the same species (Sacristán-Soriano et al., 2012). This unique ability makes them ideal candidates for the screening of both new and known compounds with bioactive potential for the development of sustainable, economic and environment-friendly drugs, cosmetics and chemicals for human benefits. In the last decades, seaweed derived compounds (e.g. polyunsaturated fatty

acids (PUFAs), polyphenols, polysaccharides, proteins and pigments) have gained an increasing biotechnological interest (Brown et al., 2014; Chojnacka et al., 2012) mainly due to their high bioactive potential such as antioxidant, antiviral, antitumor, antimicrobial, antifouling, among others (Holdt and Kraan, 2011; Prabhakaran et al., 2012; Wijesinghe and Jeon, 2011).

Nevertheless, the extraction of bioactive compounds is always a challenging task, and several bottlenecks have to be overcome. The most common challenge is associated with the yield of extraction, which is normally very low. Thus, the amount of biological material required to obtain a reasonable amount of the isolated molecule for chemical characterization and biological screenings, is in the kg order. This is a serious problem for some marine species which are very small, seasonal, or species that occur on specific places in reduced amounts. In these situations, the collection of the biological material may induce negative pressures in the marine ecosystems and is not environmentally or economically sustainable. In this point of view, if dully explored, marine invasive species are an opportunity for drugs discovery and development.

1.3. The importance of seaweeds-derived antioxidant compounds

Antioxidants are molecules that have a wide range of applications as reducing agents, preventing undesirable oxidation processes by interacting and neutralizing free radicals. Therefore, antioxidants are powerful defenses against lipid and protein oxidation, cell damage and mutagenesis. These characteristics confer antioxidant molecules a variety of uses in different industries, such as in the pharmaceutical, cosmetic, and food industries (Devi et al., 2008; Murray et al., 2013).

Antioxidant molecules can be used to increase the body defenses against oxidative stress. Oxidative stress may arise as a result of the imbalance between reactive oxygen species (ROS) originated from regular or enhanced metabolic processes and antioxidant defenses. The most active ROS are the free radicals due to unpaired electrons in their structure. These are highly reactive and can alter the biological functions of several macromolecules, such as carbohydrates, nucleic acids, lipids, and proteins, leading to cell death (Birben et al., 2012). In fact, several studies point out that the accumulation of ROS are implicated in the genesis and development of a wide range of diseases, including cancer, neurological disorders, cardiovascular diseases, diabetes and are also presumed to be involved in the aging process (Bickers and Athar, 2006; Choi and Choi, 2015; Finkel and Holbrook, 2000). Knowing the potential of antioxidants to scavenge ROS, one line of approach to delay or prevent pathogenesis is based on the administration of antioxidants as an effort to restore homeostasis, additionally to the organisms' own defenses.

On another point of view, with increasing life expectancy, the size of the elderly population has substantially increased and, consequently, modern society is presented with new medical, social, and financial challenges allied to an increasing pressure on aesthetical appearance.

During a lifetime, skin is exposed to chemical challenges generated by its own metabolism as well as by the environment, mostly pollution, chemicals, and ultra-violet radiation. To counteract these aggressions skin comprises its own defense strategies, which include an array of antioxidative mechanisms involving enzymatic and non-enzymatic molecules. Although highly effective, this defense system is often overwhelmed resulting in an accumulation of ROS which ultimately results in dermatological disorders and aging. Antioxidants can help to protect skin from the damaging effects of the sun and other physical aggressions combating the free-radical damage that is responsible for the visible (and hidden) signs of aging. They also play a crucial role in cellular repair and healing by stimulating cell and tissues' growth, helping the body to repair itself (Bickers and Athar, 2006).

The finding of new and more effective antioxidant substances will have, therefore, a great economic impact in the pharmaceutical and cosmetic industries.

On the other hand, in the food industry, it is critical to find alternative solutions for the development of pre-prepared food to which, in many cases, synthetic antioxidants are added. The convenience and enhanced quality of "fast food" and semi-processed refrigerated foods makes them an attractive choice with high profits for food processors. However, there are many safety and quality challenges that food industry must consider when marketing refrigerated foods, especially those with extended shelf lives. Synthetic antioxidants such as BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), TBHQ (tertiary butylhydroquinone) and propyl gallate are very effective and easy to add into a variety of food products, with reduced costs. However, some properties of these synthetic antioxidants, such as their high volatility and instability at high temperatures, strict legislation for synthetic food additives use, and consumer preferences and concerns, are pressuring the manufacturers to create a new paradigm into shifting from synthetic to natural antioxidants (Shahidi, 2000). Moreover, several studies have revealed alarming results about the use of synthetic antioxidants such as carcinogenic effects and liver damage in laboratory animals (Saad et al., 2007). Consequently, the use of natural antioxidants in food is a promising alternative for synthetic antioxidants, especially due to their high compatibility with dietary intake and no harmful effects to the human organism (Heo et al., 2005; Lobo et al., 2010; Patra et al., 2008).

1.4. The importance of seaweeds-derived antimicrobial compounds

Despite the great advances in clinical microbiology, the concerns about resistant pathogens continue to be a mainstream subject. Like all living beings, microorganisms constantly adapt to new environments and changes on surroundings, developing new metabolic strategies in order to survive. This capacity is quite evident when considering the continuous signalization of resistant strains.

The development of microorganism resistances to antimicrobial agents is closely associated with clinical practices. The extensively prescription of antibiotics, together with its neglecting use by patients, allows bacterial populations to adapt and survive by acquiring resistance and passing it to their descendants (Giedraitienė et al., 2011).

The development of drug resistant strains is a public health concern. It is estimated that on 2050 about 13 million people all over the world will die from infection diseases pointing to the urgent need for a new generation of antimicrobial agents (Dye, 2014). Moreover, scientists suggest that emergent resistances are "largely a question of when and not if" (Council, 2006). Until now, the strategies to counteract resistant strains have mainly encompassed the redesigning of known structures (both natural and synthetic). It is clearly evident that more and different approaches to overcome this global concern are needed.

The pharmaceutical industry has been focused on terrestrial resources for more than 50 years, however the marine environment continues to be mainly unexplored for its ability to yield antimicrobial compounds. In the last decade, several studies on marine organisms have proven their ability on the production of new structures, with unique characteristics, many of them not found in terrestrial sources. These can be further used for molecular and chemical modeling for therapeutic approaches (Abad et al., 2011). In line with this, seaweeds have shown to have a successful defense mechanism against microfouling and epiphytic organisms that involves the production of secondary metabolites with antimicrobial properties.

Among seaweeds, *S. muticum* and *A. armata* were already targets on different studies concerning antimicrobial activity (Pinteus et al., 2018; Pinteus et al., 2015; Madhiyazhagan et al., 2015, Tanniou et al., 2014; Oumaskour et al., 2013; Villareal-Gómez et al., 2010; Salvador et al., 2007; Bansemir et al., 2006), nevertheless the screenings were mainly conducted on crude extracts and the mechanisms of action involved in the antimicrobial activity is still to be understood.

1.5. The importance of seaweeds-derived antifouling compounds

Biofouling can be defined as the "settlement and subsequent growth of marine organisms on submerged surfaces" (Wahl, 1989). Because these surfaces are often artificial (e.g. boat hulls, oil and gas platforms, seawater pipelines, nets and enclosures) the unwanted fouling organisms present significant economic costs and problems to human activities in the marine environment (Fitridge et al., 2012; Iyapparaj et al., 2014; Yebra et al., 2004). In aquaculture systems, fouling induces severe negative economic and structural impacts due to structural loading and corrosion; on the other hand, in industrial fishing, the biofouling on ships increases the surface roughness of the hull causing an increased frictional resistance and fuel consumption up to 50%, decreasing top speed and range (Schultz et al., 2011). The increased fuel usage, dry-docking, structural inspection and maintenance resulting from biofouling have been estimated to cost the world's marine industries and governments over $6.5 billion dollars per year (Manilal et al., 2010). Further ecological implications of biofouling include increased carbon emissions and potential dispersion of invasive species (Bellas, 2006; Iyapparaj et al., 2014; Silkina et al., 2012), which are still intangible bust estimated to account for billions when considering ecosystem services impairment.

For long time, there has been an attempt to produce compounds that reduce or inhibit fouling. These anti-fouling systems were defined in the International Convention on the Control of Harmful Anti-fouling Systems on Ships, 2001 (AFS Convention) as "a coating, paint, surface treatment, or device that is used on a ship to control or prevent attachment of unwanted organisms" (Yebra et al., 2004). By other words, antifouling is, therefore, the process of controlling, regulating or preventing the fouling of a surface.

The control of biofouling can be divided into three categories: biological, chemical, and physical control. The traditional chemical methods include treatment with biocides, toxic antifouling coatings (polymeric systems), or coatings described as foul-release or easy cleaning coating. Generally, antifouling coatings are based on heavy metals, particularly copper or tin based compounds for their active ingredients, therefore presenting high toxicity for marine organisms and remaining in the ecosystems for long periods of time.

Although many antifouling compounds were deemed toxic and banned due to their environmental risks (e.g. tributyltin, commonly addressed as TBT), copper-based coatings, often containing booster biocides, have continued to be used - these compounds are normally very toxic affecting the marine ecosystems by killing target and non-target organisms through its leaching to the media (Dalley, 1989; Yebra et al., 2004). Hence, alternative and environmentally friendly, safe, and

efficient antifouling substances are necessary to find and these can be targeted in marine organisms.

In the marine environment, marine organisms are prone to biofouling. This is particularly evident for sessile organisms. As a result, seaweeds produce a wide variety of chemically active metabolites, with recognized antifouling properties (Fusetani, 2011; Satheesh et al., 2016), revealing therefore, to be interesting sources to explore the production of bioactive natural compounds with antifouling potential. Moreover, since these compounds occur naturally in the marine environment, they will be certainly less damaging for the environment than the current heavy-metal based paints (Burgess et al., 2003).

1.6. Aim and outline of the thesis

The introduction of invasive alien species is a major threat to ecosystem biodiversity, structure and function. The marine red algae *Asparagopsis armata* and the brown algae *Sargassum muticum* exhibit strong invasive behavior being included in the list of the 100 "Worst Invasives in the Mediterranean Sea" (Streftaris and Zenetos 2006).

In this work, the economic value of these algae will be enhanced by revealing their potential as sources of antioxidant, antimicrobial, and antifouling substances. These will have further sustainable use as natural environment-friendly compounds in several industries, resulting in high valuable products.

Within the marine environment a number of biologically active molecules have been identified that have commercial as well as societal and environmental benefits, which can have important applications in pharmaceutical, food, cosmetic, and paint industries, mainly as antimicrobial, antioxidant, and antifouling molecules (Murray et al., 2013).

This would agree with the view that new natural antimicrobial (A), antioxidant (B), and antifouling (C) compounds are needed:

A) Bacterial infections especially those associated with emerging multidrug resistance organisms are a primary health care problem. An excessive use of antimicrobials has led to a rapid increase in the prevalence of drug-resistant microorganisms. In fact, many antibiotics have become either ineffective of far less reliable than they used to be (Paphitou, 2013).

B) One major action of antioxidants in cells is to prevent damage caused by the action of reactive oxygen species (ROS). Excessive amounts of ROS, decrease of the antioxidant defenses or both generate oxidative stress, that is linked to numerous diseases such as cancer, diabetes, neurological disorders and skin aging (Bickers and Athar, 2006; Choi and Choi, 2015; Finkel and

Holbrook, 2000). On the other hand, in food industry, lipid oxidation by ROS causes a decrease in nutritional value of lipids, in their safety and appearance. Oxidation is the predominant cause of qualitative decay of foods, which leads to rancidity and toxicity. Several synthetic antioxidants, are currently used, however, these antioxidants are linked to diverse health problems and diseases such as cancer (Saad et al., 2007).

C) The need for antifoulants to prevent the settlement and growth of marine organisms on submerged structures, such as oil rig supports, buoys, fish cages and ship's hulls is recognized universally. However, most of the commercially available compounds cause severe negative impacts on the aquatic ecosystem by their toxicity. Natural marine antifouling molecules may provide less toxic and more specific antifouling promise inhibiting the settlement fouling organisms. The development and research on marine derived antifouling products was promoted by the International Maritime Organization convention as alternatives to current antifouling paints (Konstantinou and Albanis, 2004).

Accordingly, this thesis was divided into five chapters (Figure 1.1), including the current general introduction (Chapter 1); Chapter 2 concerning the antioxidant potential of both seaweeds; Chapters 3 related to the antimicrobial potential of both seaweeds; Chapter 4 describing the antifouling potential of both seaweeds comprising the development of a new model for antifouling tests (Chapter 4B); and a final chapter with final considerations, summarizing the general conclusions and future perspectives of this work (Chapter 5).

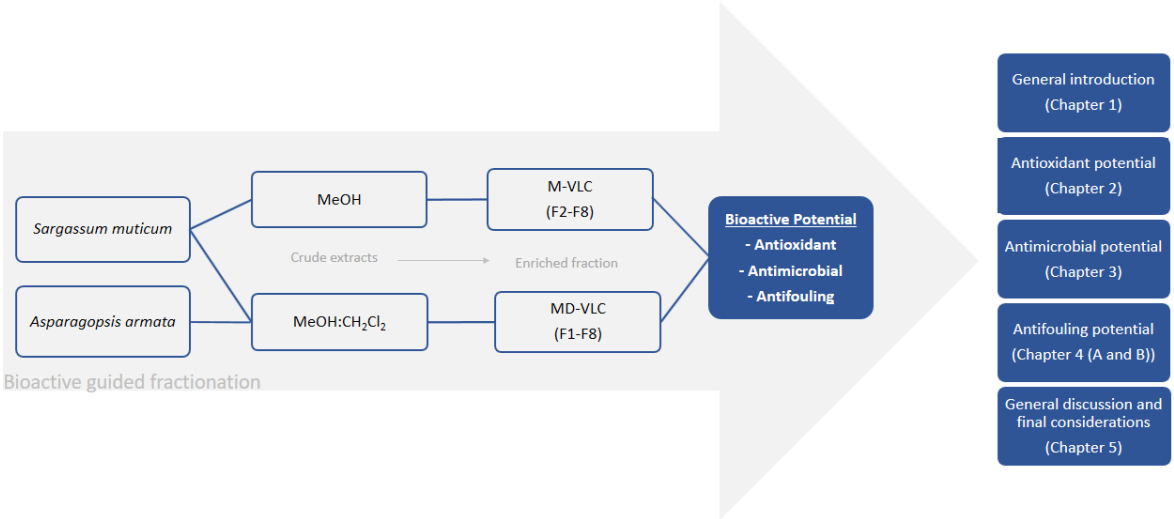

Figure 1.1. Overview of thesis outline. MeOH – methanol; CH_2Cl_2 – dichloromethane; M-VLC – vacuum liquid chromatography of the methanolic extract; MD-VLC – vacuum liquid chromatography of the methanol:dichloromethane extract.

In detail:

Chapter 2 is entitled "The antioxidant potential of the marine invaders *Asparagopsis armata* and *Sargassum muticum*" and assesses the quantification of the total phenolic content, the capacity to scavenge the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical, and the oxygen radical absorbance capacity (ORAC). The most potent samples are also evaluated for their potential to protect human cells in an induced oxidative stress condition, as well as the mechanisms associated to the observed effects unraveled by addressing the real-time H_2O_2 production, mitochondrial membrane potential, and Caspase-9 activity.

Chapter 3 is entitled "The antimicrobial potential of the marine invaders *Asparagopsis armata* and *Sargassum muticum*" and assesses the screening of the antimicrobial potential of both seaweeds on human pathogens *Escherichia coli, Salmonella enteritidis*, *Pseudomonas aeruginosa*, *Candida albicans,* and *Staphylococcus aureus*. The most potent samples were further studied in order to understand the possible mechanisms of action involved in the antimicrobial effects.

Chapter 4 is entitled "The antifouling potential of the marine invaders *Asparagopsis armata* and *Sargassum muticum*" and assesses the potential of both seaweeds to inhibit the growth of microalgae and marine microorganisms, as well as the capacity to inhibit biofilm formation (4A). To test the anti-settlement potential, a novel *in vivo* model was developed with the species *Aurelia aurita* and *Phyllorhiza punctata* (medusa polyps) (4B).

Chapter 5 is entitled "General discussion and final considerations" and resumes the main results and findings of chapters 2, 3, and 4, while future research perspectives are addressed.

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Chapter 2

The antioxidant potential of the marine invaders *Asparagopsis armata* and *Sargassum muticum*

This chapter is based and includes the following manuscript:

Pinteus, S., Lemos, M. F., Silva, J., Alves, C., Neugebauer, A., Freitas, R., Duarte, A., Pedrosa, R. 2017. An insight into *Sargassum muticum* cytoprotective mechanisms against oxidative stress on a human cell *in vitro* model. Marine Drugs 15(11), 353.

2.1. Introduction

There has been a paradigm change in consumers behavior associated with the increasing knowledge that health is directly related with food quality. In fact, it is now evident that healthy food, rich in vitamins and minerals, can prevent or delay the development of several oxidative stress associated diseases (Anand et al., 2015; Rahal et al., 2014). Biological systems produce different oxidative molecules that in normal conditions are balanced by enzymatic and non-enzymatic defenses. However, due to several factors, such as stress, smoking, alcoholism, pollution, and poor food intake quality, the production of oxidative molecules increases promoting an imbalance between these molecules and the antioxidant defense mechanisms. Reactive oxygen species (ROS) include unstable metabolites of molecular oxygen (O_2) that have higher reactivity than O_2 - such as the superoxide radical ($O2^{\bullet -}$) and the hydroxyl radical ($HO^{\bullet}$), and non-radical molecules such as hydrogen peroxide (H_2O_2). These highly reactive molecules often oxidize important biological molecules such as lipids, DNA, and proteins, producing toxic substances that accumulate in cells leading to pathologies such as diabetes, neurodegenerative diseases, cardiovascular diseases, and cancer, among others (Rahal et al. 2014). Since many of these diseases do not have an effective cure, it is of utmost importance to search for new compounds with capacity to decrease oxidative stress conditions. For thousands of years, seaweeds have been valued and widely consumed as a direct human food in many Asian countries (McHugh, 2003). Indeed, many studies address the lower incidence of diseases like cancer and diabetes in these countries, which can be directly related to dietary habits (Sharifuddin et al., 2015; Teas et al., 2013). Due to this evidence, and an increasing need for new natural products for drug development, in the last decades, seaweeds have been targeted for bioactive compounds discovery, and many important bioactivities have been recognized, such as antitumor, anti-inflammatory, antimicrobial and antioxidant activities (Dore et al., 2013; Mayer et al., 2011; Pinteus et al., 2017a; Pinteus et al., 2017b; Rodrigues et al., 2015; Yende et al., 2014).

Being exposed to extremely variable conditions such as a wide range of temperatures, high solar exposition, nutrients privation, desiccation and osmotic stress, intertidal species developed strategies to protect themselves and prosper. These include a well-orchestrated complex of enzymatic and nonenzymatic antioxidant responses, which comprises, among others, the production of secondary metabolites (Flores-Molina et al., 2014).

In the specific case of brown seaweeds, it is known that these organisms produce a unique class of phenolic compounds known as phlorotannins. These are mainly composed by oligomers or polymers of phloroglucinol (1,3,5-trihydroxybenzene), connected by aryl–aryl bonds (fucols), ether bonds (phlorethols, hydroxyphlorethols, fuhalols), or both (fucophlorethols), or with a dibenzodioxin linkage (eckols and carmalols) (Balboa et al., 2013), and are also involved in chemical defenses against herbivory, structural functions in cell walls, growth and reproduction (Kubanek et al., 2004).

Within this context, the aim of this study was to evaluate the antioxidant potential of *Sargassum muticum* and *Asparagopsis armata* compounds. To accomplish this task, both seaweeds were subjected to several chromatographic steps, and all the resulting fractions were screened through the DPPH radical scavenging assay, ORAC assay, and the TPC estimated. Finally, the most potent fractions were evaluated for their potential to protect human cells in an oxidative-stress induced condition using human breast adenocarcinoma cells (MCF-7 cells - ACC 115) as model (Figure 2.1). The mechanisms associated to the observed effects were also unraveled by addressing the real-time H_2O_2 production, mitochondrial membrane potential and Caspase-9 activity.

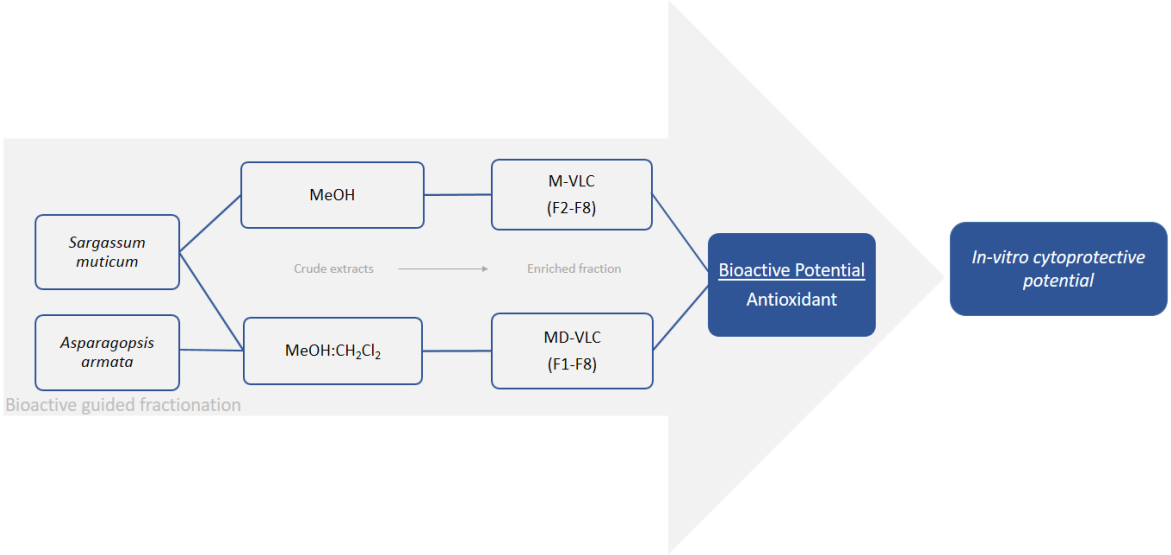

Figure 2.1 – Overview of chapter 2 design experiments. MeOH – methanol; CH_2Cl_2 – dichloromethane; M-VLC – vacuum liquid chromatography of the methanolic extract; MD-VLC – vacuum liquid chromatography of the methanol:dichloromethane extract.

2.2. Material and methods

2.2.1. Chemicals and reagents

Methanol (M) and dichloromethane (D), cyclohexane (CH), ethyl acetate (EtAc) of analytical grade were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). All the other chemicals and reagents, molecular biology and analytical grades, were purchased from Merck (Merck KGaA, Darmstadt, Germany).

2.2.2. *Sargassum muticum* collection and extraction

Sargassum muticum specimens were collected at Praia Norte beach, Viana do Castelo, Portugal (41°41'44.2'' N 8°51'08.1'' W) and transported to laboratory in cooled boxes, in August 2014. The samples were washed firstly with seawater to remove epibionts, sand and debris, and then with distilled water. Finally, samples were frozen at -80 °C and freeze-dried.

Two independent extractions were carried out, one with 100% methanol (M), and another with 1:1 methanol:dichloromethane (MD) as follows: freeze-dried samples were extracted overnight with constant stirring in a 1:40 biomass:solvent ratio, in the dark. The solutions were then filtered and evaporated in a rotary evaporator at 40 °C, 150 rpm, and the resulting biomass (crude extracts) stored at -20 °C until further use.

2.2.2.1. *Sargassum muticum* fractionation by vacuum liquid chromatography

The fractionation procedure was conducted as described by Rodrigues and coworkers (2015) with some modifications as follows: a normal phase vacuum liquid chromatography (VLC) was prepared in a Büchner filter funnel with 120 g of silica beads 40-60 µm, 60A, and equilibrated with cyclohexane. The solvent system (400 mL), of the methanolic crude extract, consisted in cyclohexane with increasing amounts (25%) of ethyl acetate, resulting in 5 fractions (MF1-MF5). The last 3 fractions were prepared with dichloromethane, methanol:dichloromethane (1:1), and methanol (MF6, MF7, and MF8, respectively). For the MD crude extract, the same column was used and the solvent system was the same for the first 6 fractions (MDF1-MDF6); however, fraction MDF7 was eluted with 100% M, and fraction MDF8 was eluted with M:D (1:1). Finally, all the solvents were evaporated at 40 °C, under low pressure, in a rotary evaporator (Heidolph, Laborota 4000, Germany) and the dried fractions solubilized in DMSO (100 mg/mL) and stored at −20 °C until

further use. The first elution (MF1) of the methanolic extraction did not resulted in enough biomass for the bioassays, and therefore the tests were assessed on MF2–MF8 fractions.

2.2.3. *Asparagopsis armata* collection and extraction

Asparagopsis armata was collected in the Berlengas Natural Reserve (39° 24'43'' N 9° 29'56'' W) by scuba diving and transported to laboratory in cooled boxes, in August 2014. The samples were washed firstly with seawater to remove epibionts, sand and debris, and then with distilled water. Finally, samples were frozen at -80 °C and freeze-dried.

The freeze-dried seaweed powder was extracted overnight in a 1:40 biomass:solvent ratio with M:D (1:1) mixture with constant stirring in the dark. The solvent was evaporated under low pressure, at 40 °C and 150 rpm, in a rotary evaporator (Heidolph, Laborota 4000, Germany), and the resulting biomass (crude extract) stored at -20 °C until further use.

2.2.3.1. *Asparagopsis armata* fractionation by vacuum liquid chromatography

The fractionation procedure was conducted as previously described by Rodrigues and coworkers (2015) as follows: a VLC was prepared in a Büchner filter funnel with 120 g of silica beads 40-60 µm, 60A, and equilibrated with cyclohexane. The crude M:D extract was then eluted with 400 mL of cyclohexane with increasing amounts (25%) of ethyl acetate, resulting in 5 fractions (F1-F5), the last three fractions were obtained with dichloromethane, methanol and methanol:dichloromethane (1:1), resulting in the fractions F6, F7 and F8, respectively. Finally, all the solvents were evaporated, and the dried fractions solubilized in DMSO (100 mg/mL) and stored at −20 °C until further use.

2.2.4. Analysis of the total phenolic content

Total phenolic content (TPC) of *A. armata* and *S. muticum* fractions was determined using the Folin–Ciocalteu method (Singleton and Rossi, 1965) adapted for microplate. Briefly, 2 µL of extract were added to 158 µL of distilled water and 10 µL of Folin–Ciocalteu reagent, vortexed and then, 30 µL of 20% Na_2CO_3 (w/v) was added. After one hour of reaction in the dark, the absorbance was measured at 755 nm (Synergy H1 Multi-Mode Microplate Reader, BioTek® Instruments, Winooski, VT, USA) against a blank solution. The TPC was expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g extract) for *Asparagopsis armata*, while for *Sargassum muticum* results are expressed as phloroglucinol equivalents per gram of dry extract (mg PE/ g extract).

2.2.5. Analysis of the DPPH radical scavenging activity

The DPPH radical scavenging activity was performed according to Brand-Williams and co-workers (1995) adapted for microplate. The reaction occurred in the dark with 2 µL of each test sample (1000 µg/mL) and 198 µL of the DPPH solution (0.1 mM in ethanol). After 30 min incubation, the absorbance was measured at 517 nm. Butylated hydroxytoluene (BHT) was used as standard. The scavenge potential was calculated in percentage of control. For the extracts that scavenged the DPPH radical in more than 50%, IC_{50} values (µg/mL) were determined.

2.2.6. Analysis of the oxygen radical absorbance capacity

Oxygen radical absorbance capacity (ORAC-fluorescein assay) was evaluated as described by Dávalos and co-workers (2004). Briefly, the reaction was carried out in 75 mM phosphate buffer (pH 7.4), for a final reaction mixture of 200 µL. Sample (20 µL) and fluorescein (120 µL; 70 nM, final concentration) were placed in the wells of 96-well microplates and pre-incubated for 15 min at 37 °C. AAPH solution (60 µL; 12 mM, final concentration) was added, and the fluorescence ($\lambda_{excitation}$: 458 nm, $\lambda_{emission}$: 520 nm) recorded every minute for 240 min. The microplate was automatically shaken prior to each reading. A blank, using phosphate buffer instead of fluorescein, and eight calibration solutions using Trolox (1–8 µM, final concentration) were also carried out in each assay. All the reaction mixtures were prepared in duplicate, and at least three independent assays were performed for each sample. Antioxidant curves (fluorescence *versus* time) were first normalized to the curve of the blank, corresponding to the same assay by multiplying original data by the factor $fluorescence_{blank,t=0}/fluorescence_{sample,t=0}$. From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as:

$$AUC = 1 + \sum_{i=1}^{i=80} \frac{fi}{f0}$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i. Results were expressed in µmol of trolox equivalents/g of dry extract (µmol TE/g).

2.2.7. Analysis of the oxidative-stress protective potential in an *in vitro* human cell model

2.2.7.1. Cells maintenance culture conditions

Human breast adenocarcinoma model (MCF-7 cells—ACC 115) were acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ). All cells were maintained according to DSMZ procedure.

2.2.7.2. Cytotoxicity evaluation

The cytotoxicity of seaweed fractions in MCF-7 cells was determined after cells reached the total confluence in 96-well plates. Samples (1 mg/mL) were dissolved in RPMI 1640 medium without fetal bovine serum (FBS), sterile filtered (0.2 µm), and incubated with cells during 24h. Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method (Yuan and Walsh, 2006).

2.2.7.3. Protective effects of *S. muticum* fractions on MCF-7 cells exposed to H_2O_2

All the fractions resulting from *A. armata* revealed high cytotoxicity and therefore were excluded for cytoprotective tests.

Concerning *S. muticum* fractions with no cytotoxicity, they were evaluated for their cytoprotective potential, namely fractions MF4, MF6, MF7, and MF8 from the M-VLC, and MDF1, MDF2, MDF3, MDF4, and MDF7 obtained from the MD-VLC.

Hydrogen peroxide (H_2O_2) is a cytotoxic agent capable of disrupting normal cells functioning, leading to cells death. In this assay, cells were co-exposed to H_2O_2 (0.2 mM) and seaweed fractions during 24h. After this period, cells were washed and their viability verified by evaluating the mitochondrial dehydrogenase activity through the MTT method. The results were expressed as percentage of control.

2.2.7.4. Real-time qantification of H_2O_2 production

Quantification of H_2O_2 was assessed with the "AmplexTM Red hydrogen peroxide Assay" Kit (Life Technologies, Carlsbad, CA, USA). The Amplex Red is a fluorophore that evidences a low basal fluorescence, which reacts with H_2O_2 in a 1:1 ratio. This reaction is initiated with horseradish peroxidase and successive reactions occur, leading to the appearance of resofurin, a highly fluorescent product (Mohanty et al., 1997). H_2O_2 production was quantified on MCF-7 cells after

24h of treatment with H_2O_2 (0.2 mM) and seaweed fractions (1 mg/mL). The fluorescence was measured at excitation/emission 590/530 nm, along 60 min, immediately after the addition of the fluorophore. The levels of H_2O_2 were calculated through the slope of the linear phase of fluorescence curve and the results expressed as percentage of control.

2.2.7.5. Mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨm) was determined using the fluorescent probe, JC-1 (Molecular Probes, Eugene, OR, USA). MCF-7 cells were treated with H_2O_2 (0.2 mM) in the absence or presence of seaweed fractions (1 mg/mL) during 24h. Cells were then washed with Hank's buffer and incubated with JC-1 (3 µM) during 15 min at 37 °C. The formation of JC-1 aggregates (excitation/emission 490/590 nm) and the monomeric form of JC-1 (excitation/emission 490/530 nm) was accompanied simultaneously during 30 min. Results were expressed as the ratio of the monomers/aggregates of JC-1 as percentage of control.

2.2.7.6. Caspase-9 activity

Caspase-9 activity was assessed using the "Caspase-9 Assay kit" K118 (Biovision, Milpitas, CA, USA). Cells were cultured in six-well plates and treated with H_2O_2 (0.2 mM) and *S. muticum* fractions (1 mg/mL—24h), washed twice with Hank's buffer, collected by centrifugation (2300 *g*, 10 min, 4 °C), resuspended in 50 µL of lysis buffer, incubated 20 min on ice, and centrifuged (15,870 *g*, 20 min, 4 °C). After, 50 µL of supernatant was transferred to a 96-well plate and 50 µL of reaction buffer containing dithiothreitol (10 mM) and 5 µL of substrate added. This reaction was followed at excitation/emission: 400/505 nm along 90 min. Caspase-9 activity was calculated through the slope of the fluorescence resulting from 7-amino-4-(trifluoromethyl) coumarin accumulation and expressed as % of control (Δ fluorescence (u.a)/mg of protein/min).

2.2.8. Statistical analysis

The IC_{50} concentration was calculated from nonlinear regression analysis using GraphPad Prism software with the equation: $Y = 100/(1 + 10^{(X-LogIC50)})$. All data were checked for normality and homoscedasticity using the Shapiro–Wilk and Levene's test, respectively. Comparisons concerning variables, which did not meet variance or distributional assumptions, were carried out with the Kruskal–Wallis nonparametric tests (Zar, 2010). One-way analysis of variance (ANOVA) was carried out when evaluating the effects of seaweed fractions (1 mg/mL) in an oxidative stress condition

promoted by H_2O_2 addition (0.2 mM) on MCF-7 cells after 24h of incubation. The statistical comparisons among the groups were performed with the Newman–Keuls multiple comparison test (Zar 2010). For all statistical tests, the significance level was set at *p*-value < 0.05, and results were expressed as mean ± standard error of the mean (SEM). All calculations were performed on GraphPad InStat v. 3.5 (GraphPad Software, La Jolla, CA, USA).

2.3. Results

2.3.1. *Sargassum muticum* and *Asparagopsis armata* antioxidant potential

To understand the antioxidant potential of each fraction, the total phenolic content (TPC), the oxygen radical absorbance capacity (ORAC), and the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging capacity were evaluated. The results are shown in Tables 2.1 and 2.2 for *Sargassum muticum* and *Asparagopsis armata*, respectively.

Table 2.1. Total phenolic content and antioxidant activity of *Sargassum muticum* crude extracts and VLC fractions. Values correspond to the mean ± standard error of the mean of three independent experiments. The values in parenthesis represent the confidence intervals for 95%.

Fraction	TPC [a]	DPPH [b]	ORAC [c]
Crude MDE	75.75 ± 0.03	28.4 (26.9–30.0)	12634.2 ± 312.5
MDF1	25.79 ± 0.02	>1000	1.5 ± 0.5
MDF2	55.87 ± 0.03	>1000	16.1 ± 0.3
MDF3	38.85 ± 0.02	>1000	29.1 ± 5.8
MDF4	34.31 ± 0.01	>1000	11.4 ± 3.4
MDF5	60.06 ± 0.02	57.3 (51.6–63.7)	281.0 ± 13.2
MDF6	36.78 ± 0.01	81.1 (68.0–96.9)	136.8 ± 14.4
MDF7	21.67 ± 0.01	98.8 (84.6–115.5)	770.0 ± 26.3
MDF8	84.08 ± 0.03	32.3 (29.0–36.0)	3040.1 ± 266.2
Crude ME	85.26 ± 1.16	53.1 (46.7–67.8)	2672.8 ± 54.2
MF2	20.45 ± 0.96	106.4 (92.9–121.9)	14.7 ± 0.1
MF3	16.86 ± 0.56	291.0 (261.9–323.3)	17.5 ± 0.2
MF4	25.62 ± 1.05	>1000	42.5 ± 0.1
MF5	26.65 ± 0.83	>1000	77.9 ± 22.6
MF6	11.66 ± 0.41	>1000	42.9 ± 0.2
MF7	36.72 ± 1.31	>1000	44.4 ± 15.1
MF8	61.67 ± 1.50	36.4 (32.4–41.0)	52.0 ± 4.3
BHT	-	50.3 (36.0–54.3)	330.7 ± 37.5

[a] mg Phloroglucinol equivalents/g extract (PE/g); [b] DPPH radical scavenging activity (IC_{50} µg/mL); [c] µmol Trolox equivalents/g extract (TE/g); MD—methanol:dichloromethane; M—Methanol; E—extract.

In this study, *S. muticum* and *A. armata* were investigated through different assays to give an overview of the antioxidant potential found in the crude extracts and VLC fractions.

Concerning *S. muticum* (Table 2.1), in both VLCs, the last fraction, F8, presented the highest phenolic content, namely 84.08±0.03 and 61.67±1.50 mg PE/g extract, for MDF8 and MF8, respectively. Regarding the DPPH radical scavenging ability, within the MD-VLC fractions, MDF5–MDF8 presented the highest potential, while within the M-VLC, fractions MF2, MF3, and MF8 presented the best radical scavenging capacity. In both VLCs, fractions F8 were the most potent with an IC_{50} of 32.3 (29.0–36.0) µg/mL and 36.4 (32.4–41.0) µg/mL for MDF8 and MF8, respectively. Concerning oxygen radical absorbance capacity, the results suggest that, within the MD-VLC, the antioxidant activity increases along polarity, where the MDF8 fraction was found to be the most potent with 3040.1±266.2 µmol TE/g extract. Concerning the M-VLC fractions, the results revealed a weak oxygen radical absorbance capacity transversely to all fractions, with the highest potential exhibited by MF5, with 77.9±22.6 µmol TE/g extract.

Table 2.2. Total phenolic content and antioxidant activity of *Asparagopsis armata* crude extract and VLC fractions. Values correspond to the mean ± standard error of the mean of three independent experiments. The values in parenthesis represent the confidence intervals for 95%.

Fraction	TPC [a]	DPPH [b]	ORAC [c]
Crude	0.68 ± 0.04	804.6 (727.7-889.7)	1502.3 ± 43.7
F1	0.58 ± 0.02	1001.0 (903.0-1110.0)	1658.1 ± 34.5
F2	0.99 ± 0.33	894.7 (837.9-955.4)	1728.7 ± 23.7
F3	2.81 ± 0.2	790.5 (617.3-1012.0)	2334.8 ± 21.8
F4	2.78 ± 0.19	523.3 (418.7-654.0)	2156.5 ± 20.4
F5	3.99 ± 0.09	691.7 (586.0-816.4)	2248.7 ± 94.0
F6	2.16 ± 0.05	402.9 (319.7-507.7)	1624.4 ± 53.7
F7	0.35 ± 0.02	>1000	1143.4 ± 56.8
F8	0.81 ± 0.02	>1000	970.0 ± 83.8
BHT	-	40.6 (27.4-60.1)	-

[a] mg gallic acid equivalents/g extract (GAE/g); [b] DPPH radical scavenging activity (IC_{50} µg/mL); [c] µmol Trolox equivalents/g extract (TE/g).

Regarding *A. armata* (Table 2.2.), the antioxidant potential was much lower in all assays than for *S. muticum*. The TPC was found to be the highest in fraction F5 (3.99 mg GAE/g extract), followed by

fractions F3, F4, and F6 (2.81-2.16 mg GAE/g extract). The remaining fractions varied the TPC between 0.35 and 0.99 mg GAE/g extract.

As regards to the DPPH radical scavenging ability, all VLC fractions revealed a low scavenging potential, with the fraction F6 revealing the highest potency with an IC_{50} of 402.9 (319.7-507.7 µg/mL).

Further evaluation of the antioxidant activities was conducted by ORAC assay. The peroxyl radical scavenging activities of crude extract and VLC fractions of *A. armata* varied from 970.0 µM to 2248.7 µM of TE/ g extract. The highest potential was exhibited by fraction F5 and the lowest by F8 and F7 (Table 2.2).

Since *S. muticum* revealed an unequivocal superior antioxidant potential, it was selected for further analysis, namely for its potential to protect human cells on an oxidative stress condition.

2.3.2. Evaluation of cytoprotective potential of *Sargassum muticum* on an *in vitro* cellular model, the MCF-7 cells.

2.3.2.1. Evaluation *of Sargassum muticum* fractions cytotoxicity.

To assess the cytoprotective potential of *S. muticum* fractions, firstly, their toxicity was evaluated on MCF-7 cells through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method (Figure 2.2). Fractions with no cytotoxicity were then selected for further analysis.

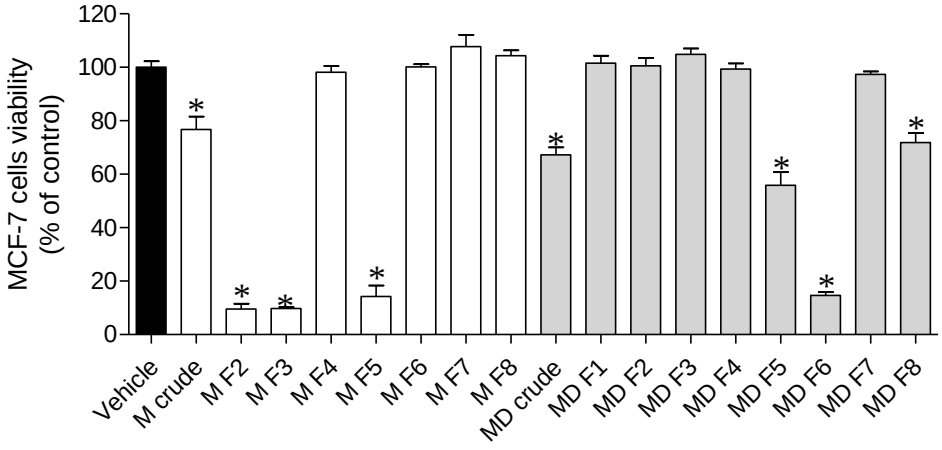

Figure 2.2. Evaluation of *Sargassum muticum* fractions cytotoxicity on MCF-7 cells (1 mg/mL; 24h). Results were obtained by the MTT method. Columns represent the mean of 3 independent

experiments ± standard error of the mean (SEM). Symbols (*) represent statistically significant differences (ANOVA, Dunett's test, $p < 0.05$) when compared to vehicle.

According to the results observed in Figure 2.2, fractions MF4, MF6, MF7, MF8 and MDF1, MDF2, MDF3, MDF4, and MDF7 did not exhibit toxicity, and therefore were selected for further assays to understand their potential to protect human cells from an induced oxidative stress condition.

2.3.3. Protective effects of *Sargassum muticum* fractions on MCF-7 cells exposed to H_2O_2.

Hydrogen peroxide (H_2O_2) is a cytotoxic agent capable of disrupting normal cells functioning, leading to cells death. In this assay, cells are co-exposed to H_2O_2 (0.2 mM) in the presence and absence of the seaweed fractions during 24h. After this period, cells were washed and their viability verified by evaluating the mitochondrial dehydrogenase activity through the MTT method. The results are shown in Figure 2.3.

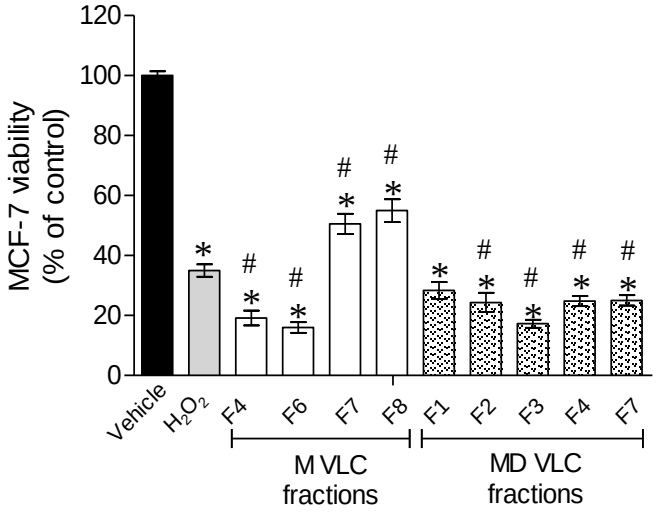

Figure 2.3. Effect of *Sargassum muticum* fractions (1 mg/mL) in an oxidative stress condition promoted by H_2O_2 (0.2 mM), after 24h of treatment, on MCF-7 cells. Results were obtained by the MTT method. Columns represent the mean ± standard error of the mean of three independent experiments. Symbols represent statistically significant differences (Kruskal Wallis, ANOVA, Dunett's test, $p < 0.05$,) when compared to: * vehicle and $^{\#}$ H_2O_2.

The results presented in Figure 2.3 reveal that when exposing cells to H_2O_2, cells viability decrease in more than 50%. In the presence of *Sargassum muticum* VLC fractions, two fractions were able to

partially revert the H_2O_2 effects, namely fractions F7 and F8, both obtained with a methanolic extraction. To understand the relevance of the observed results, the cytotoxicity of MF7 and MF8 fractions were also assessed on the non-tumor cells, the primary mouse embryonic fibroblast cells 3T3, revealing no toxicity (MF7—118.7 ± 4.8% viable cells; MF8—121.7 ± 5.7% viable cells).

2.3.4. Cellular mechanisms involved in the protective effects evidenced by *Sargassum muticum* fractions

2.3.4.1. Real-time quantification of H_2O_2 production

Cells produce H_2O_2 as a result of metabolic processes. This production may increase under stress conditions. In this assay, the basal H_2O_2 production as well as the H_2O_2 produced after exposure to this cytotoxic agent, in the presence or absence of *S. muticum* fractions, was evaluated. Therefore, MCF-7 cells were incubated with H_2O_2 (0.2 mM) in the presence and absence of *S. muticum* fractions (24h). The medium was then removed and the Amplex Red, together with horseradish peroxidase was added, which in the presence of H_2O_2 is converted to resoforin - a highly fluorescent compound. This conversion was accompanied along 60 min allowing the real time quantification of the production of H_2O_2 (Figure 2.4).

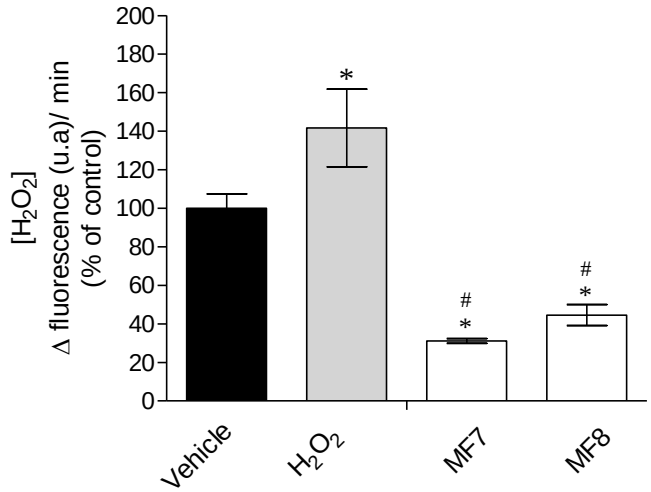

Figure 2.4. Real time production of H_2O_2 by MCF-7 cells in the absence or presence of the MF7 and MF8 (1 mg/mL; 24h) fractions of *Sargassum muticum*. Columns represent the mean ± standard error of the mean (SEM) of three independent experiments. Symbols represent statistically significant differences (ANOVA, Dunett's test, $p < 0.05$) when compared to: * vehicle and $^{\#}$ H_2O_2.

Results reveal that fractions MF7 and MF8 induced a significant decrease in the real time H_2O_2 production (Figure 2.4) in about 70% and 56%, respectively, regarding the vehicle situation (basal H_2O_2 production), and about 78% and 68.5%, respectively, comparing to the H_2O_2 situation.

2.3.4.2. Mitochondrial membrane potential

Mitochondria are the engines of cellular respiration being responsible for adenosine triphosphate (ATP) production, which is mainly regulated by the mitochondrial membrane potential (ΔΨm) generated by an electrochemical gradient across their inner membrane. Therefore, changes in the ΔΨm have been originally proposed as early and obligate events in the apoptotic signaling pathway (Cook et al., 1999; Satoh et al., 1997). Figure 2.5 shows the effects of MF7 and MF8 fractions on MCF-7 cells mitochondrial membrane potential.

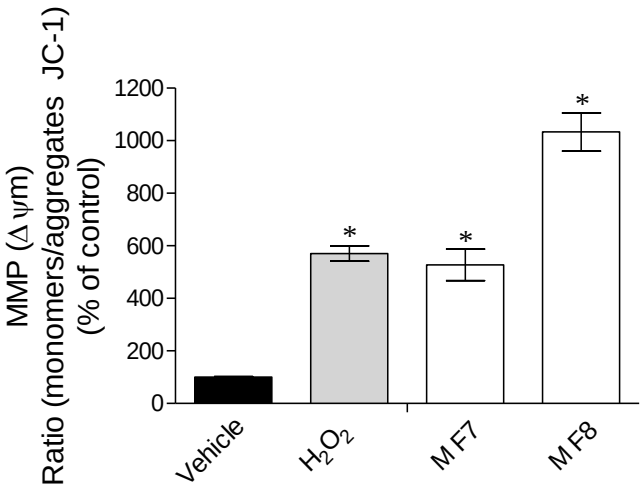

Figure 2.5. Effects of H_2O_2 (0.2 mM) in the presence or absence of *Sargassum muticum* fractions (1 mg/mL) in mitochondrial membrane potential of MCF-7 cells after 24h of incubation. Results were obtained by the ratio between the monomers/aggregates of JC-1. Columns represent the mean ± standard error of the mean (SEM) of three independent experiments. Symbols represent statistically significant differences (ANOVA, Dunett's test, $p < 0.05$) when compared to: * vehicle and $^{\#}$ H_2O_2.

The H_2O_2 treatment promoted a mitochondrial membrane depolarization (Figure 2.5). The presence of the seaweed fractions did not reduce the depolarization induced by H_2O_2. Additionally, fraction MF8 seemed to potentiate the H_2O_2 effects.

2.3.4.3. Caspase-9 activity

Caspase-9 is a member of the caspase family of cysteine proteases, a key element in apoptotic events. Once activated, Caspase-9 triggers a succession of reactions known as caspase cascade, leading to cell death by apoptosis. In response to the oxidative stress insult, Caspase-9 is activated, therefore it is appropriate to understand if the cellular protection evidenced by *S. muticum* fractions is due to the inhibition of this enzyme. The results are shown in Figure 2.6.

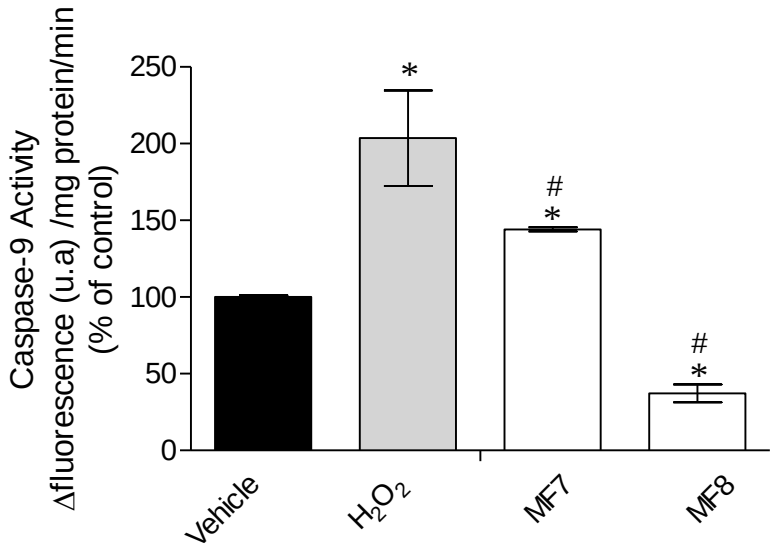

Figure 2.6. Effects of H_2O_2 (0.2 mM) in the presence or absence of *Sargassum muticum* fractions (1 mg/mL) on Caspase-9 activity of MCF-7 cells after 24h of treatment. Results are presented in arbitrary units of fluorescence per mg protein per minute (% control). Columns represent the mean ± standard error of the mean (SEM) from three independent experiments. Symbols represent statistically significant differences (ANOVA, Dunett's test, $p < 0.05$) when compared to: * vehicle and $^{\#}$ H_2O_2.

It is possible to see that Caspase-9 activity is significantly increased in the presence of H_2O_2 and, in the presence of *S. muticum* fraction MF8, Caspase-9 activity was significantly decreased (Figure 2.6).

2.4. Discussion and conclusion

2.4.1. *Sargassum muticum* and *Asparagopsis armata* antioxidant potential

Seaweeds have been recognized as a healthy food ingredient, while there are also increasing evidence that their secondary metabolites can be also used as new structures for drug development. *Sargassum muticum* has been widely used in traditional oriental medicine, but there is scarce science-based data available on its bioactive properties, specially data sustained on cellular models. On the other hand, since oxidative stress is associated with the development of numerous diseases, it is fairly pertinent to evaluate its potential to protect cells from oxidative stress conditions. *S. muticum* was already evaluated for its potential antioxidant compounds production (Namvar et al., 2013; Pinteus et al., 2017a), but few studies have been conducted to concentrate and purify its bioactive molecules. Moreover, the lack of studies contributing to the understanding of the mechanisms of action is also worthy of note. Here, two extraction methods and subsequent sub-fractions were tested for their antioxidant potential and also for their effective protection on an *in vitro* cellular model—the MCF-7 cells. The total phenolic content increased in the MDF8 fraction comparing with the crude MD extract (MDE), this fraction also revealed a high scavenging capacity, yet losing potential in peroxyl radicals scavenging. Since the crude extract is a complex mixture of compounds, along the chromatography process many compounds may be lost and others concentrated, which may justify the increase in the concentration of antioxidant molecules capable of reducing DPPH radical. Concerning the methanolic extraction, the fractioning also promoted the reduction of the total phenolic content and a considerable loss of compounds with the capacity to scavenge peroxyl radicals, while the capacity of scavenging the DPPH radical was slightly increased. Not surprisingly, the last fractions of the VLC are the ones revealing the highest antioxidant activity, since many antioxidant compounds, including phlorotannins, have polar characteristics and therefore could be retained longer in the column (Koivikko et al., 2007).

Concerning *Asparagopsis armata*, the antioxidant potential is much less expressive than the verified for *Sargassum muticum.* Nevertheless, when comparing with other works, the antioxidant results reveal to be more promising. For instance, in the work of Zubia and co-workers (2009), 24 red algae from the Brittany coast, including *Asparagopsis armata*, were screened for their ability to

scavenge the DPPH radical. In this work *Asparagopsis armata* scored an IC_{50} of 6250 µg/mL, and the remaining 23 seaweeds, excepting *Brongniartella byssoides,* which presented an IC_{50} of 140 µg/mL, presented IC_{50} between 1390 and 29720 µg/mL. On the other hand, as regards to the TPC, in the same work (Zubia et al. 2009) *A. armata* scored 1.13 ± 0.05 mg phloroglucinol equivalents / g extract, which is also much lower than the obtained in all fractions and crude extract in the present work. The differences verified between different works within the same species can be related to chemical and ecological diversity as well as different extraction approaches (Stengel et al., 2011).

2.4.2. Protective effects of *Sargassum muticum* fractions on an oxidative stress condition induced by H_2O_2

Since a previous study identified cytotoxic characteristics on *S. muticum* (Namvar et al., 2013), it was important to understand the toxic effects on the human cellular model used in this work, the MCF-7 cells, since along the purification process it could be possible to separate non-toxic substances, conferring importance to the purification process. As a result, fractions MF2, MF3, MF5, MDF5, MDF6, and MDF8 presented toxicity and thus, their cytoprotection was not tested. All other fractions were tested for their potential for protecting cells on an oxidative stress condition induced by H_2O_2.

H_2O_2 is one of the most widely used agents to induce oxidative stress on *in vitro* models (Gille and Joenje 1992). This molecule is involved in many cellular mechanisms and presents high membrane permeability, allowing it to enter cells readily, inducing toxicity. Moreover, its relative stability enables a reproducible oxidative stress condition in the cells (Rhee et al., 2010; Ryter et al., 2007).

Fractions MF7 and MF8 presented capacity to reduce the H_2O_2 induced effects, which resulted in the increase of cell's viability.

The following step involved the quantification of the cells H_2O_2 production, where it is possible to verify that the production of H_2O_2 was strongly inhibited by the presence of these enriched fractions. H_2O_2 is one of the main intercessors of oxidative stress-induced cytotoxicity (Halliwell, 1992). Thus, the results strongly suggest that the increase of cell's viability is related to the capacity of MF7 and MF8 to block the damage effects of H_2O_2, as well as the ability to decrease its production. Accordingly, other studies assessing the protective effects of seaweed have already revealed that these marine organisms contain compounds with capacity to protect cells from oxidative stress through H_2O_2-mediated disruption (Heo and Jeon, 2009; Kang et al., 2012; Pinteus et al., 2017a; Pinteus et al., 2017b). O'Sullivan and co-workers (2011) also evaluated the capacity of seaweed to protect cellular damage induced by H_2O_2 and verified that *Fucus serratus* and *Fucus*

vesiculosus (Fucales) reduced H_2O_2-mediated DNA damage. Kang and collaborators (2012) also showed that phlorotannins from the brown seaweed *Ecklonia cava* produced neuroprotective effects in murine hippocampal HT22 cells against H_2O_2-induced oxidative stress. Moreover, the effects exhibited by MF7 and MF8 fractions become more relevant since they did not demonstrated cytotoxicity when tested in 3T3 cells.

2.4.2.1. Mechanisms of action insight: mitochondrial membrane potential and Caspase-9 activity evaluation

Concerning the effects of H_2O_2 in the mitochondrial membrane potential (ΔΨm), it was observed that this induced a noticeable membrane depolarization. The mitochondrial membrane potential is essential to maintain the physiological function of the respiratory chain, and therefore changes on ΔΨm can promote cell death since it is involved in early apoptotic signaling pathways (Cook et al., 1999; Satoh et al., 1997). In the presence of MF7 fraction, the depolarization induced by H_2O_2 was maintained, but the MF8 fraction seemed to increase the H_2O_2 effects by increasing membrane polarization. These results suggest that the protective effects of these fractions are not directly associated to the mitochondrial membrane's potential changes. Other possibility for the observed results was the involvement of the compounds in apoptotic mechanisms, and therefore, to deepen the protective effects of these fractions, the Caspase-9 activity was also assessed. Caspase-9 is one of the downstream regulators of apoptosis and has being associated to oxidative stress pathways (Blanc et al., 2000). Although the increase of ΔΨm is associated with the activation of caspases cascade, MF8 induced a marked reduction of more than 80% of Caspase-9 activity. Similarly, Chia and co-workers (2015) evaluated the ability of seaweed to reduce cytotoxicity on MCF-7 cells and verified that *Turbinaria ornate* (also belonging to the Fucales order) also reduced Caspase-9 activity. Lee and co-workers (2012) also showed that seaweed phlorotannins promoted cellular protection of HepG-2 cells by reducing Caspase-3 activity.

Together, our results suggest that *Sargassum muticum* contains compounds with the capacity to decrease cell's oxidative stress levels by blocking the production of H_2O_2 and acting as downstream blockers of apoptosis. These characteristics now depicted, reveal the potential of *Sargassum muticum* as a source of functional ingredients against oxidative stress conditions, including those related with neurodegenerative diseases.

2.5. References

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Chapter 3

The antimicrobial potential of the marine invaders *Asparagopsis armata* and *Sargassum muticum*

This chapter is based and includes the following manuscript:

Pinteus, S., Lemos, M. F., Simões, M., Alves, C., Silva, J., Gaspar, H., Martins, A., Rodrigues, A., Pedrosa, R. 2020. Marine invasive species for high-value products exploration – Unveiling the antimicrobial potential of *Asparagopsis armata* against human pathogens. Algal Research, 52, 102091.

3.1. Introduction

Since the discovery of penicillin in 1928 (Fleming, 1929), more than 350 antimicrobials have been developed, derived from natural sources (mainly terrestrial), or chemically synthetized (semi-synthetics and synthetics) (Silber et al., 2016). These antimicrobials were life changing as they significantly increase life expectancy by counteracting infectious diseases. Soon, it would be discovered that these drugs could also function for preventing animal death and as growth promoters in agriculture practices (WHO, 2017, 2014; Cantas et al., 2013).

The wide use of antimicrobials allied with the lack of knowledge on the microorganisms' adaptation abilities led to the emergence of resistant strains. As a result, nowadays, infection diseases represent a major threat against human health and one of the major causes of mortality. On the other hand, with more profitable therapeutics to invest in (*e.g.* cancer and chronic diseases therapeutics), many pharmaceutical companies have disregarded the screenings for new antimicrobial drugs development. Only in the last decade, due to the dramatic increase of deaths resulting from infectious diseases, the discovery and development pipeline for new antimicrobials has become a priority (WHO, 2014).

Antibiotics can be categorized based on their targets and mechanisms of action, in addition to whether they induce cell death (bactericidal drugs) or inhibit cell growth (bacteriostatic drugs). Accordingly, several groups can be highlighted: quinolones (DNA synthesis inhibition), β-lactams and glycopeptides (cell wall synthesis inhibition), macrolides, tetracyclines and aminoglycosides (protein synthesis inhibition), polymyxins (cell membrane permeability changes), and rifamycins (inhibition of RNA synthesis) (Kohanski et al., 2010; Neu, 1973). With an incredible adaptation capacity, microorganisms developed strategies to defend themselves against antimicrobial drugs, resulting in resistant strains, also called "superbugs". Those strategies include the prevention of the antimicrobial drug accumulation by either decreasing their uptake or increasing efflux from the cell (changes in membrane), changes in the target site, inhibition of drug binding through bacterial gene mutations, and inactivation of the antimicrobial drug through enzymatic mechanisms (Kapoor et al., 2017; Giedraitienė et al., 2011). Also, once resistances are acquired, microbial cells have the ability to transfer the acquired resistance to descendants and even to other strains and species by several mechanisms including gene transference through plasmids (conjugation or transformation), transposons (conjugation), integrons or bacteriophages (transduction). These mechanisms explain the extraordinary evolutionary success of microbial species. In addition to these mechanisms,

several microorganisms have also developed another strategy to survive in hostile environments, the production of biofilms.

Microbial biofilms are complex organized structures of static microbial communities which have the capacity to adhere to different matrices. The biofilm allows the microbial community to survive in hostile environments by maintaining a slow growth or a starved state with a low nutrients uptake rate. Additionally, the diffusion rate of antimicrobials is retarded by the polymeric substances present in the biofilm matrices, making their total penetration more difficult (Kumar et al., 2017; Costerton et al., 1999). As a result, bacteria that are located deeper in the biofilm may develop resistance to antimicrobials and pass the resistance mechanism to future generations or by horizontal gene transfer. In fact, one of the triggers to the biofilm formation is the presence of non-effective antimicrobial drugs, and therefore the biofilm formation is intimately related with antimicrobial drugs resistance development (Ciofu and Tolker-Nielson 2019; Kumar et al., 2017).

From a medicinal point of view, these mechanisms result in serious challenges, being of utmost importance to develop effective strategies to counteract all forms of resistant microbes.

Since the great majority of the available antimicrobials derived from terrestrial sources (plants or microorganisms) (Hayashi et al., 2013), it is important to explore other sources of new chemical structures upon which new classes of antimicrobial drugs can be based on. In line with this view, recent trends in drug research emphasize that the marine environment has a high potential for the discovery of new bioactive molecules (Murray et al., 2013).

In the last decades, marine organisms have been studied for new bioactive compounds, being already known that these organisms produce bioactive compounds with unique chemical structures, mainly through their secondary metabolism. These compounds are synthetized as part of a defense mechanism against predators being also involved in the regulation of internal mechanisms associated with physical parameters, such as pH, U.V. radiation exposure, CO_2, and temperature (Murray et al., 2013; Hay, 1997).

Within marine organisms, seaweeds have already revealed to be an excellent source of bioactive compounds (Blunt et al., 2018; Stengel and Connan, 2015; Kiuru et al., 2014). Nevertheless, the low yields of extraction, together with biomass losses resulting from the extraction and purification processes, demand the collection of significant quantities of biological material (Murray et al., 2013). For some seaweed specimens, which occur in small quantities and in specific places, their collection is not environmentally sustainable. In line with this view, the highly invasive seaweeds *Asparagopsis armata* and *Sargassum muticum*, due to the large biomass availability and vast

impacts in the recipient ecosystem represent an excellent opportunity for bioactive compounds discovery and new products development.

In this chapter the antimicrobial potential of these species will be evaluated and discussed, and the most promising fractions will be further analyzed to unveil the mechanisms of action eventually involved in the observed effects (Figure 3.1).

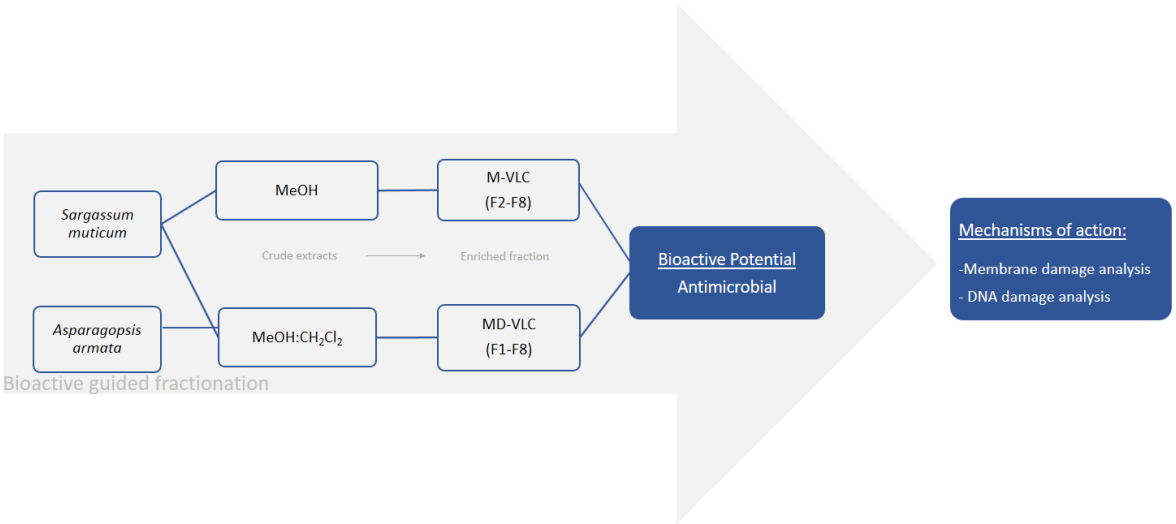

Figure 3.1 – Overview of chapter 3 design experiments. MeOH – methanol; CH_2Cl_2 – dichloromethane; M-VLC – vacuum liquid chromatography of the methanolic extract; MD-VLC – vacuum liquid chromatography of the methanol:dichloromethane extract.

3.2. Material and methods

3.2.1. Chemicals and reagents

Chemicals and molecular biology reagents of analytical grade were purchased from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany). Methanol, dichloromethane, ethyl acetate, cyclohexane and *n*-hexane (95-99 % purity grade) used for extractions and for chromatographic purposes (TLC, VLC, and preparative CC), were obtained from VWR BDH Chemicals (Fonteney-sous-Bois, France), while HPLC grade solvents dichloromethane, acetonitrile, methanol and water (Fischer Chemical, Leicestershire, UK) were used for HPLC and GC- MS analysis. Gene Ruler 1 Kb DNA Ladder was obtained from ThermoFisher (ThermoFisher Scientific, Waltham, USA).

3.2.2. Microorganisms and culture media

Bacterial strains *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 10231), *Salmonella enteritidis* (ATCC 13076), and *Escherichia coli* (ATCC

25922) were obtained from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures. Luria-Bertani (LB) broth and yeast extract peptone dextrose (YPD) were obtained from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany).

3.2.3. *Sargassum muticum* and *Asparagopsis armata* collection, extraction and fractionation

Sargassum muticum and *Asparagopsis armata* collection and extraction was conducted as previously described in Chapter 2, section 2.2.2. and 2.2.3., respectively.

The fractionation procedure was conducted as detailed in Chapter 2, sections 2.2.2.1 and 2.2.3.1 for *Sargassum muticum* and *Asparagopsis armata*, respectively.

3.2.4. Antimicrobial activity of *Sargassum muticum* and *Asparagopsis armata*

All microorganisms were grown in LB broth, except *C. albicans,* which was grown in YPD broth, in the presence/absence of seaweeds extracts (1 mg/mL) at 37 °C. Negative controls were conducted with DMSO. Optical density (600 nm) was read in a microplate reader (Synergy H1 Multi-Mode Microplate Reader, BioTek® Instruments, Winooski, USA). The growth inhibitory capacity was determined as percentage of control. For seaweed extracts presenting an inhibitory capacity >50% at 1 mg/mL, a dose response analysis (1000-10 µg/mL) was conducted to determine the IC_{50} (concentration which inhibits 50% microorganisms' growth).

3.2.5. Membrane permeability analysis

Membrane damage analysis was performed according to Ojeda-Sana and co-workers (2013) with slight modifications, as follows. A freshly overnight grown culture was gently centrifuged to remove culture medium and gently washed twice with 0.85% sterile saline solution. A working solution was then prepared by adjusting the microorganism's culture absorbance to Abs_{600}= 0.5 in 0.85% sterile saline solution. To the working solution was added seaweed samples or DMSO (negative control) and incubated 4 hours at 37 °C. Blanks were prepared with saline solution and fractions (without microorganism). A positive control was prepared by subjecting the working solution to a thermic treatment (90 °C, 10 min) to induce total membrane permeability. All the suspensions were transferred to a black microplate and incubated with 5 µM Sytox Green for 10 min, in the dark. The resultant fluorescence of the DNA-bound dye was quantified on a fluorescence microplate reader

(Synergy H1 Multi-Mode Microplate Reader, BioTek® Instruments, Winooski, USA) with excitation/emission wavelengths of 535/595 nm, respectively.

It is known that healthy cell membranes are impermeable to the Sytox Green; however, when the integrity of the cell membrane is affected, this fluorophore enters the cells freely resulting in intense green fluorescence. The membrane damage was determined using the following equations:

$$(1)\ TMD\ (\%) = \left[\left(\frac{(Fsample - Fblank)}{Fctr(+)}\right)\right] x\ 100$$

$$(2)\ BMD\ (\%) = \left[\left(\frac{Fctr(-)}{Fctr(+)}\right)\right]\ x\ 100$$

$$(3)\ Membrane\ Damage\ (\%) = \%TMD - \%BMD$$

Where: TMD is the total membrane damage promoted by seaweeds extracts; F_{sample} is the fluorescence of the working solution with extracts; F_{blank} is the fluorescence of the blank samples without microorganism); $F_{ctr(+)}$ is the fluorescence of the positive control; BMD is the basal membrane damage (damages resulting from the process and DMSO); $F_{crt(-)}$ is the fluorescence of the working solution with DMSO.

3.2.6. DNA damaging potential

DNA damaging potential was conducted following the methodology described by Hu and collaborators (2017), in brief: plasmid (pGADT7 – 7987bp) DNA (10 µL; 100 ng) was mixed with seaweeds samples (10 µL; 500 and 250 µg/mL). The reaction mixture was incubated at 37 °C for 2 h before being loaded on a 0.7% agarose gel with 1% red-safe. Five µL of Gene Ruler 1 Kb DNA Ladder was also loaded in the gel. Electrophoresis was then performed for 50 min under 80 V. DMSO was used in the same conditions as negative control, and 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) was used as positive control).

3.2.7. Biofilm formation inhibitory activity

Biofilm formation was performed according to O'Toole and co-workers (2011), as follows: *Pseudomonas aeruginosa* was grown overnight at 37 °C in LB medium. Five µL of the overnight grown bacteria was added to a 96-well microplate with 93 µL of fresh medium and 2 µL of seaweeds samples or DMSO (control) followed by incubation at 37 °C for 48h. Blanks were performed with culture medium and test sample without bacteria. The medium was then removed, and wells

washed twice with water and left to dry at 37 °C for 30 min. Following, 125 µL of a 0.1% solution of crystal violet in water was added, and the plate incubated at room temperature for 15 min. After this period, the crystal violet was discarded, the wells washed three times with water and left to dry at 37 °C. The crystal violet was then solubilized by adding 150 µL of 30% acetic acid in water, incubated for 15 min and transferred to a new microplate. The absorbance was then read at 550 nm and the results presented as percentage of control.

3.2.8. Bioactive compounds concentration process

Fractions with the highest antimicrobial potential, and with enough biomass, were selected for further isolation processes. In a first approach, samples with enough biomass were fractionated on a Kieselgel 60–200 µm (VWR, Ref. 84893.290, Leuven, Belgium) preparative chromatography column (CC). Further purification of lesser complex fractions was achieved on a Kieselgel 40–63 µm (VWR, Ref. 84894.290, Leuven, Belgium) CC and/or by preparative thin layer chromatography on Kieselgel 60 F_{254} glass plates (Merck, Ref. 5744, Darmstadt, Germany). Elution was performed by gradient polarity with mixtures of *n*-hexane/ethyl acetate and dichloromethane/methanol. Fractions´ composition was followed by analytical thin layer chromatography (TLC) on Kieselgel 60 F_{254} aluminium sheets (Merck, Ref. 5554, Darmstadt, Germany) and spots visualized under UV light (254 and 364 nm). TLC plates were revealed by spraying with H_2SO_4 in MeOH (10%, v/v), followed by heating at 120 °C. Fractions having an identical profile were combined. More refined separations were accomplished in a HPLC equipment (Jasco, LC – 4000 series, Easton, EUA) through analysis with H_2O/CH_3CN: isocratic step from 0 to 5 min (50:50), followed by a gradient elution from 5 to 15 min (50:50 to 100) (flow rate 2.0 mL/min, injection volume 200 µL, 25 °C) with a semi-preparative column (Synergi Fusion-RP 80ª, Phenomenex, 10 x 250 mm, 4 µm). The mobile phase was filtered through a membrane filter (0.45 µm) and degassed for 15 min in an ultrasonic bath before use.

3.2.9. Bioactive compounds identification

Crude extracts and bioactive fractions were analyzed by gas chromatography coupled to mass spectrometry (GC-MS) and to flame ionization detectors (GC-FID).

3.2.9.1. GC-FID analysis

Test samples were dissolved in methanol (3 mg/mL), added to 1.5 mL acetyl chloride:methanol (1:19 v/v) and heated in a water bath at 80 °C for 1 h. After cooling, 1 mL of ultrapure water and 1 mL *n*-hexane were added and the solution vortex-stirred for 1 min followed by centrifugation at

1500 *g* for 5 min. 500 µL of the organic upper phase was recovered and analyzed by gas chromatography. A Finnigan Ultra Trace gas chromatograph equipped with a Thermo TR-FAME capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness), an auto sampler AS 3000 from Thermo Electron Corp. (Boston, Mass., U.S.A.), and a flame ionization detector (FID) were used to detect the fatty acid methyl esters. The injector (operating in splitless mode) and the detector temperatures were set at 250 and 260 °C, respectively. The column temperature was initially set at 60 °C for 1 min, then raised 15 °C /min to 150 °C and held for 1 min, followed by an increase of 5 °C /min to 220 °C, and maintained for 10 min. Helium was used as carrier gas at a flow rate of 1.5 mL/min. Air and hydrogen were supplied to the detector at flow rates of 350 and 35 mL/min, respectively.

3.2.9.2. GC-MS analysis

GC–MS qualitative analysis were performed in a Shimadzu QP2010-Plus GC/MS system equipped with a TRB5MS (30 m × 0.25 mm i.d. × 0.25 µm film thickness) capillary column (Teknokroma, Barcelona, Spain) operating in the linear velocity mode. The carrier gas was helium 5.0 (Linde, Portugal), at a constant flow of 1 mL/min. Samples were dissolved in dichloromethane and automatically injected. Injections were performed in split mode, with a ratio of 1/9. The injector port was heated to 280 °C. The initial column temperature of 60 °C was held for 2 min, followed by a temperature ramp of 30 °C/min to 300 °C held for 15 min. All mass spectra were acquired in electron impact (EI) mode at 70 eV. The operate temperatures were 200 °C for MS ion source, and 250 °C for the liner interface. The analyses were performed in full scan mode with mass ranging from 10 to 800 *m/z.* Compounds were identified by matching the mass fragmentation patterns with those stored in the GC-MS mass spectral databases (Wiley 229 and NIST-National Institute of Standards and Technology libraries).

3.2.10. Data analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison of group means to determine significant differences relatively to control. All data were checked for normality and homoscedasticity using the Shapiro–Wilk and Levene's test, respectively. Comparisons concerning variables, that did not meet variance or distributional assumptions, were carried out with the Kruskal–Wallis nonparametric tests (Zar, 2010). For all statistical tests, the significance level was set at p-value < 0.05 and results were expressed as mean ± standard error of the mean (SEM). The IC_{50} was calculated from nonlinear regression analysis

using GraphPad Prism software through the equation: $Y = 100/(1 + 10^{(X-LogIC50)})$. All calculations were performed on GraphPad InStat v. 3.5 (GraphPad Software, La Jolla, CA, USA).

3.3. Results

3.3.1. Human pathogenic microorganisms' growth inhibition

To understand the antimicrobial potential of *S. muticum* and *A. armata* crude extracts and fractions, all the samples were tested at 1 mg/mL against five pathogenic microorganisms, namely *C. albicans*, *S. aureus*, *P. aeruginosa*, *E. coli,* and *S. enteritidis*, and the ones revealing more than 50% inhibition were also subjected to a dose-response analysis and the IC_{50} was determined. The antimicrobial potential of *S. muticum* at 1 mg/mL and the respective dose-response analysis are summarized in Tables 3.1 and 3.2, respectively.

Table 3.1. Antimicrobial activity of *Sargassum muticum* VLC fractions and crude extracts at 1 mg/mL against *Candida albicans, Staphylococcus aureus, Pseudomonas aeruginosa Escherichia coli* and *Salmonella enteritidis.* The values in each column represent the mean ± standard error of the mean from three independent experiments.

Fractions	Microorganisms growth inhibition (% of control)				
	C. albicans	*S. aureus*	*P. aeruginosa*	*E. coli*	*S. enteritidis*
SM M crude	25.1 ± 3.9	48.1 ± 3.8	31.5 ± 2.8	25.5 ± 1.8	41.3 ± 4.9
SM MF2	43.1 ± 4.6	100.0 ± 7.7	44.5 ± 1.5	100.0 ± 2.6	15.2 ± 3.7
SM MF3	12.1 ± 3.2	100.0 ± 3.1	63.3 ± 2.3	88.3 ± 3.7	47.6 ± 5.6
SM MF4	5.6 ± 2.7	100.0 ± 3.4	30.6 ± 2.4	100.0 ± 2.7	27.8 ± 5.5
SM MF5	5.7 ± 4.4	100.0 ± 7.6	83.5 ± 1.2	100.0 ± 2.5	22.1 ± 0.6
SM MF6	9.0 ± 4.5	100.0 ± 7.5	59.2 ± 0.9	47.2 ± 1.2	51.2 ± 0.5
SM MF7	35.8 ± 3.0	34.6 ± 2.7	51.9 ± 2.5	18.4 ± 5.2	47.1 ± 2.7
SM MF8	72.0 ± 4.7	00.0 ± 3.0	29.8 ± 1.5	2.3 ± 3.6	13.1 ± 1.8
SM MD crude	33.2 ± 4.0	43.4 ± 6.7	49.1 ± 1.6	6.4 ± 2.0	0.0 ± 3.7
SM MDF1	35.1 ± 3.5	32.8 ± 0.4	20.4 ± 3.4	31.3 ± 7.0	47.3 ± 5.6
SM MDF2	39.2 ± 6.3	84.7 ± 4.0	29.5 ± 5.5	6.0 ± 4.9	48.9 ± 5.7
SM MDF3	27.0 ± 2.5	100.0 ± 2.6	24.1 ± 2.9	22.8 ± 3.6	32.5 ± 2.5
SM MDF4	14.5 ± 2.9	77.3 ± 2.8	50.9 ± 1.1	12.7 ± 8.8	37.9 ± 4.5
SM MDF5	0.0 ± 5.6	74.8 ± 3.8	29.6 ± 2.4	10.6 ± 7.1	46.1 ± 4.2
SM MDF6	0.0 ± 4.3	48.7 ± 4.3	20.2 ± 1.2	15.4 ± 2.9	31.1 ± 0.3
SM MDF7	0.0 ± 3.6	27.9 ± 2.0	26.1 ± 2.7	0.0 ± 5.1	16.2 ± 1.7
SM MDF8	0.0 ± 9.9	0.0 ± 1.3	26.2 ± 4.6	0.0 ± 4.5	20.6 ± 4.2

SM MF_x- *Sargassum muticum* VLC fractions derived from the methanolic crude extract; SM MDF_x - *Sargassum muticum* VLC fractions derived from the dichloromethane:methanol crude extract; AAF_x – *A. armata* VLC fractions.

As shown in Table 3.1, *S. muticum* presents the highest antimicrobial potential against the Gram positive bacteria *S. aureus*. In addition, *S. muticum* also evidenced a great inhibitory potential against the Gram negative bacteria *E. coli,* and a lower inhibitory potential against the fungus *C. albicans*. The VLC fractions resulting from the methanolic extraction revealed the highest inhibitory

potential against *S. aureus* with 5 fractions (MF2-MF6) totally inhibiting this microorganism growth. A total inhibition against *E. coli* (100% inhibition) was also recorded for fractions MF2, MF4, and MF5, followed by fraction F3 with an inhibitory activity of 88.3%. Against *S. enteritidis*, the most active fractions were MF6, MF3, and MF7, with 51.2, 47.6 and 47.1% inhibition, respectively. Against *P. aeruginosa*, fractions MF5 presented the highest activity with 83.5% inhibition, followed by fraction MF3 with 63.33% inhibition. As regards to the antifungal activity, fractions MF8 and MF2 presented the highest inhibitory activity with 72.0 and 43.1% inhibition of *C. albicans* growth.
Concerning the VLC fractions resulting from the methanol:dichloromethane extraction, the highest inhibitory potential was exhibited by fraction MDF3 which totally inhibited the growth of *S. aureus,* followed by fraction MDF2, MDF4, and MDF5, with 84.7, 77.3, and 74.8% inhibition, respectively. Fraction MDF4 presented the highest inhibitory potential against *P. aeruginosa* (50.93%) and, as respect to *C. albicans,* the highest inhibitory potential was exhibited by MDF2 with an inhibition of 39.23%. Against *E. coli* the highest inhibitory activity was evidenced by fraction MDF2 with 31.3%. Regarding *S. enteritidis*, the most potent fractions were MDF3, MDF2, and MDF5, which showed 48.9, 47.3, and 46.1% inhibition, respectively.

Table 3.2. Antimicrobial activity of *Sargassum muticum* crude extracts and VLC fractions - dose response analysis, against the human pathogens *Candida albicans, Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*. IC_{50} values (µg/mL) were determined for a 95% confidence interval.

Fractions	Microorganisms			
	C. albicans	*S. aureus*	*P. aeruginosa*	*E. coli*
MF2	> 1000	116.3 (91.9 – 147.3)	> 1000	297.9 (197.6 – 449.1)
MF3	> 1000	46.2 (35.5 – 60.2)	602.9 (514.0-707.1)	244.2 (194.9 – 305.9)
MF4	> 1000	82.8 (69.0 – 99.5)	> 1000	269.8 (202.4 – 359.6)
MF5	> 1000	77.2 (62.3 – 95.9)	461.6 (362.2-588.6)	282.2 (204.7 – 389.0)
MF6	> 1000	> 1000	> 1000	> 1000
MF7	> 1000	> 1000	> 1000	> 1000
MF8	246.9 (185.2 – 329.0)	> 1000	> 1000	> 1000
MDF1	>1000	>1000	> 1000	>1000
MDF2	>1000	145.4 (118.4-178.6)	>1000	>1000
MDF3	>1000	365.9 (252.4-530.2)	> 1000	> 1000
MDF4	> 1000	92.4 (74.2-115.2)	>1000	>1000
MDF5	>1000	660.0 (557.4 -781.4)	> 1000	>1000
MDF6	>1000	>1000	> 1000	>1000
Streptomycin	-	19.0 (13.5 - 26.7)	11.8 (9.7 - 14.5)	36.9 (27.4 - 49.8)
Amphotericin B	0.2 (0.2 - 0.3)	-	-	-

MF_x- *Sargassum muticum* VLC fractions derived from the methanolic crude extract; MDF_x - *Sargassum muticum* VLC fractions derived from the dichloromethane:methanol crude extract.

In Table 3.2 it can be observed that the VLC fractions obtained with the methanolic extract have a higher antimicrobial potential, especially against *S. aureus* with the highest antimicrobial activity being exhibited by fraction MF3 with an IC_{50} = 46.2 µg/mL. Fractions MF4, MF5, and MF2 also exhibited strong antimicrobial activities against this microorganism with the IC_{50} varying between 77.2 and 116.3 µg/mL. Against *E. coli*, MF2, MF3, MF4, and MF5 presented similar results, with the IC_{50} varying between 244.2 and 297.9 µg/mL. Fraction MF4 presented the highest inhibitory potential against *P. aeruginosa,* and MF8 against *C. albicans,* with 461.6 and 246.9 µg/mL, respectively. All the fractions presented an IC_{50} superior to 1000 µg/mL against *S. enteritidis*.

Regarding the VLC fractions obtained with the methanol:dichloromethane extraction, the highest potential was exhibited against *S. aureus* with the fraction MDF2 scoring the lowest IC_{50}, namely 145.4 µg/mL. All the fractions presented an IC_{50} > 1000 µg/mL for *C. albicans*, *P. aeruginosa* and *S. enteritidis*.

In Table 3.3 the antimicrobial potential of *A. armata* fractions and crude extract at 1 mg/mL are shown.

Table 3.3. Antimicrobial activity of *Asparagopsis armata* VLC fractions and crude extracts at 1 mg/mL against *Candida albicans, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Salmonella enteritidis.* The values in each column represent the mean ± standard error of the mean from three independent experiments.

Fractions	Microorganisms growth inhibition (% of control)				
	C. albicans	*S. aureus*	*P. aeruginosa*	*E. coli*	*S. enteritidis*
crude	100.0 ± 5.0	100.0 ± 2.3	100.0 ± 2.7	3.4 ± 1.3	100.0 ± 5.6
F1	90.7 ± 1.4	100.0 ± 0.9	58.2 ± 1.8	23.0 ± 0.7	22.6 ± 1.4
F2	100.0 ± 2.4	100.0 ± 2.1	100.0 ± 6.7	98.6 ± 1.2	85.2 ± 2.5
F3	100.0 1.8	100.0 ± 1.3	100.0 ± 1.3	71.0 ± 1.2	100.0 ± 2.4
F4	100.0 ± 1.1	100.0 ± 2.7	100.0 ± 1.4	82.9 ± 1.9	99.2 ± 0.7
F5	97.0 ± 2.0	100.0 ± 2.8	100.0 ± 2.3	100.0 ± 1.7	71.3 ± 9.9
F6	100.0 ± 2.8	30.4 ± 3.0	100.0 ± 2.7	79.1 ± 3.0	36.2 ± 5.7
F7	20.3 ± 0.6	8.4 ± 2.8	28.0 ± 2.9	0.0 ± 0.5	6.9 ± 0.5
F8	6.0 ± 1.1	6.9 ± 2.4	28.5 ± 1.4	0.0 ± 0.7	1.7 ± 0.6

Asparagopsis armata fractions (F2-F5) and crude extract presented a great antimicrobial potential especially against *S. aureus* and *P. aeruginosa,* totally inhibiting the growth of these microorganisms. Additionally, fraction F1 and F6 also fully inhibited *S. aureus* and *P. aeruginosa* growth, respectively. Fractions F2-F4 and F6 were also the most potent inhibiting *C. albicans*, scoring 100% inhibition, followed by fractions F5 and F1 with 97.0 and 90.7% inhibition,

respectively. Against *E. coli*, fractions F5, F2, and F4 presented the highest inhibitory activity, namely, 100%, 98.6% and 82.9%, respectively. On the other hand, the fractions with the highest potential against *S. enteritidis* were F3 and F4, with 100% and 99.2% inhibition, respectively. The crude extract also revealed a high inhibitory potential against *S. enteritidis,* scoring 100% inhibition. For the most active fractions (inhibitory potential > 50% at 1 mg/mL), a dose-response analysis was performed and the IC_{50} determined. The results are summarized in Table 3.4.

Table 3.4. Antimicrobial activity of *Asparagopsis armata* VLC fractions – dose-response analysis, against *Candida albicans, Staphylococcus aureus, Pseudomonas aeruginosa Escherichia coli* and *Salmonella enteritidis*. IC_{50} values (µg/mL) were determined for a 95% confidence interval.

Fractions	Microorganisms				
	C. albicans	*S. aureus*	*P. aeruginosa*	*E. coli*	*S. enteritidis*
F1	169.1 (133.3-214.4)	84.3 (60.3-117.7)	297.5 (205.9 - 429.9)	>1000	>1000
F2	40.2 (31.0-52.1)	13.3 (8.4-20.9)	50.9 (30.2- 5.7)	480.8 (334.6-691.0)	432.8 (350.7-534.0)
F3	52.8 (38.6-72.1)	20.2 (14.9-27.5)	28.1 (19.1-41.3)	908.0 (586.7-1405)	161.4 (121.5-214.5)
F4	108.2 (85.7-136.6)	31.2 (24.4-39.9)	75.3 (52.6-107.8)	659.4 (477.1-911.3)	193.8 (152.5-246.2)
F5	176.4 (139.1-223.6)	42.5 (32.9-54.8)	53.7 (32.9-87.7)	410.1 (296.5-567.3)	531.9 (425.2-665.4)
F6	125.7 (68.7-230.0)	> 1000	57.6 (32.6- 101.7)	655.8 (510.8-868.0)	>1000
Crude	18.3 (14.6-22.8)	63.6 (53.6-75.5)	103.3 (83.2-124.4)	>1000	149.5 (115.3-193.8)

The results show that this seaweed produce compounds with high antimicrobial potential against all the studied microorganisms. Nevertheless, the most potent results were obtained against *S. aureus* with the fractions F2 and F3 presenting an IC_{50} of 13.3 and 20.2 µg/mL, respectively. These fractions were also the most potent against *C. albicans* and *P. aeruginosa,* with IC_{50} of 40.2, 52.8, 50.9, and 28.1 µg/mL, respectively. Against *E. coli*, the most active fractions were F5 and F2, with IC_{50} of 410.1 and 480.8 µg/mL, and against *S. enteritidis* fractions F3 and F4 were the most potent, with IC_{50} of 161.4 and 193.8 µg/mL, respectively.

3.3.2. Membrane permeability analysis

To understand the mechanisms behind the antimicrobial effects observed by seaweeds fractions, a membrane permeability analysis was accessed for the fractions that presented an IC_{50} lower than 100 µg/mL. The fractions were evaluated in a dose-dependent manner at the concentrations of IC_{50}, two times IC_{50} and half the IC_{50}. The results are summarized in Figure 3.2.

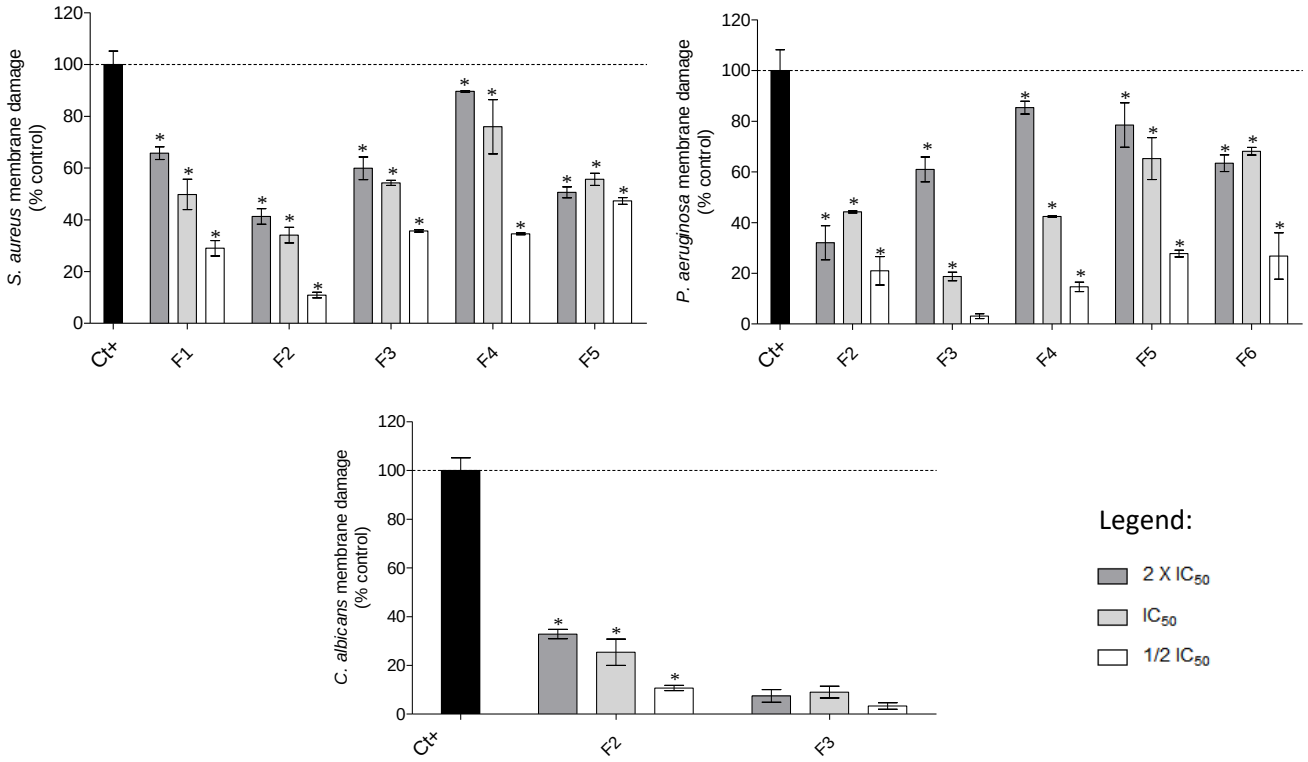

Figure 3.2. *Staphylococcus aureus (*A), *Pseudomonas aeruginosa* (B), and *Candida albicans* (C) membrane permeability analysis in the presence of *Asparagopsis armata* VLC fractions. Each column represents the mean ± standard error of the mean of at least two independent experiments. Symbols (*) represent statistically significant differences (ANOVA, Dunett's test, $p < 0.05$) when compared to control.

A. armata fractions induced significant membrane damage in *S. aureus* and *P. aeruginosa* and less on *C. albicans*. On *S. aureus*, fraction F4 imposed the higher damage percentage at the concentration of 2 x IC_{50} (89.6%) and IC_{50} (75.9%). This fraction was also the one that mediated the highest damage percentage on *P. aeruginosa* (85.3%), nevertheless at IC_{50} concentrations, fractions F6 and F5 induced the highest damage on *P. aeruginosa* (68.2 and 65.2%, respectively). Regarding *C. albicans*, fraction F2 produced damaging effects ranging from 10.7 to 32.8%, while fraction F3 did not induced membrane damage.

In this study *Sargassum muticum* fractions did not promote membrane damage in none of the studied microorganisms.

3.3.3. DNA damaging capacity

Since DNA is one of the targets of antimicrobial drugs, it was tested the possibility of the seaweed samples, which presented the highest antimicrobial activity (IC_{50} < 100 µg/mL), to bind and break DNA. The results are shown in Figure 3.3 and 3.4 for *A. armata* and *S. muticum*, respectively.

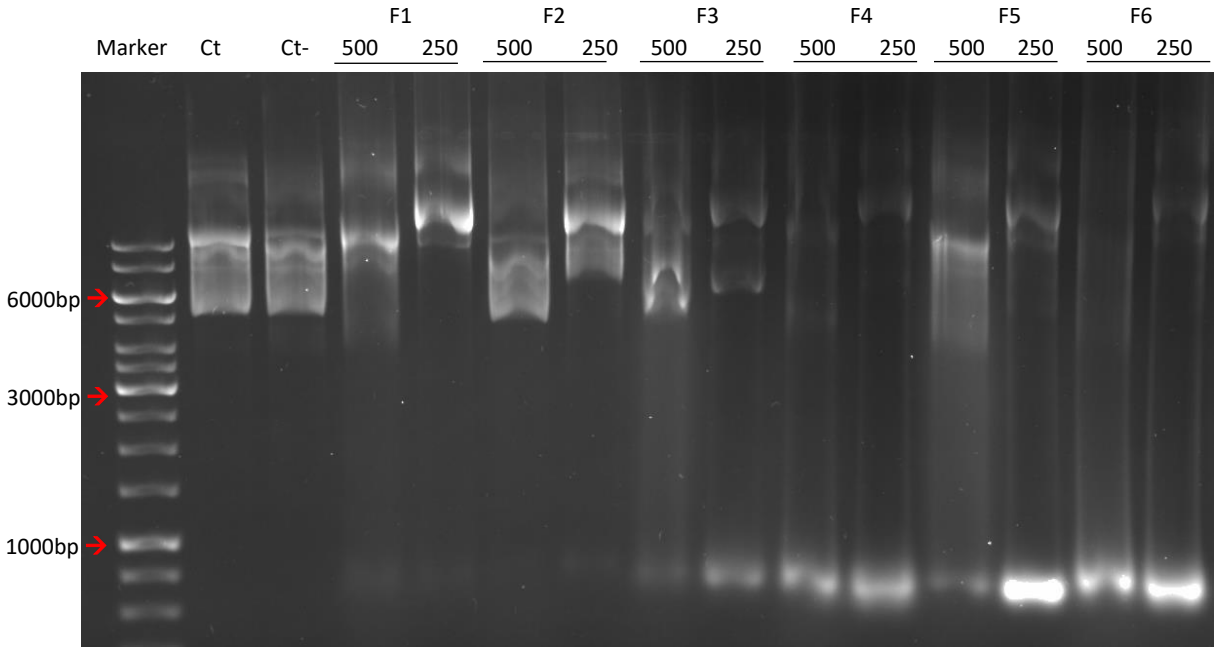

Figure 3.3. Agarose-gel electrophoresis of plasmid DNA exposed to *Asparagopsis armata* fractions at 500 and 250 µg/mL. The gel was visualized after gel-red staining and UV irradiation. Lanes Ct, control consisting of plasmid DNA only; Lane Ct-, consisting of plasmid DNA exposed to DMSO. The figure is representative of three independent experiments.

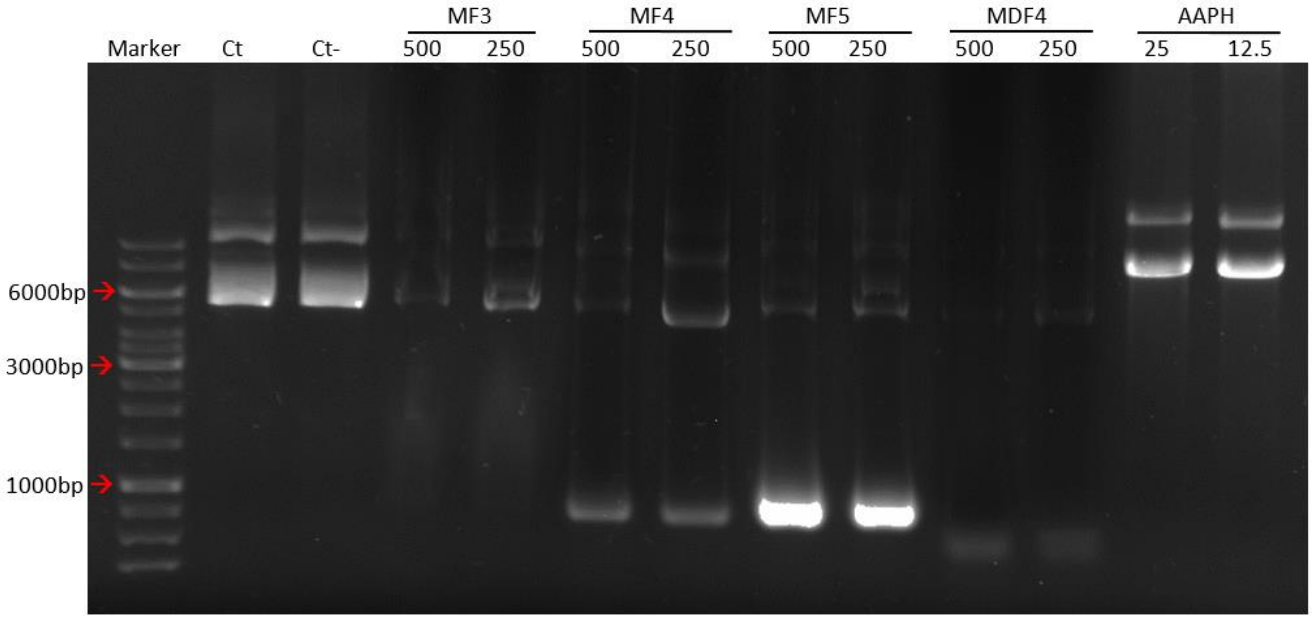

Figure 3.4. Agarose-gel electrophoresis of plasmid DNA (pGADT7 – 7987bp) exposed to *Sargassum muticum* VLC fractions at 500 and 250 µg/mL and AAPH at 25 and 12.5 mM. The gel was visualized after gel-red staining and UV irradiation. Lanes Ct, control consisting of plasmid DNA only; Lane Ct- consisting of plasmid DNA exposed to DMSO. The figure is representative of three independent experiments.

Figure 3.3 shows the DNA damage induced by *A. armata* most potent fractions (IC_{50} < 100 µg/mL), namely F1, F2, F3, F4, F5, and F6 at concentrations of 500 µg/mL and 250 µg/mL. Figure 3.4 shows the DNA damage induced by the most active fractions of *S. muticum* (IC_{50} < 100 µg/mL), namely MF3, MF4, MF5, and MDF4. AAPH (25 and 12.5 mM) was used as positive control since it is able to induce strong oxidative damage through hydroxyl radicals or peroxyl radicals (Hu et al. 2017). DMSO was used as vehicle.

All the fractions affected the DNA integrity. The disappearance of the supercoiled and circular forms, and the appearance of low molecular weight bands, that are not visible in the untreated DNA and in the treated with DMSO, suggest that the DNA was nicked and/or cut, leading to DNA degradation. The presence of smear also suggests DNA degradation, as can be seen in the lanes with treated DNA with each fraction.

So, in a first approach, it seems that *A. armata* fractions were able to damage the DNA. However, the efficiency between them was not the same. F3, F4, F5, and F6 were more effective than F1 and F2, even in the lower concentration, as can be seen by the disappearance of some supercoiled and by the high intensity of the low molecular weight bands. In addition, the smear is more intense in the F3, F4, F5, and F6 fractions. As a control, the untreated DNA, and the DNA treated with DMSO,

show the same molecular weight corresponding to the circular form and the various supercoiled forms, suggesting that DMSO did not affect the DNA integrity. These results suggest that molecules with high capacity to degrade the DNA are present in fractions F3, F4, F5, and F6.

Concerning *S. muticum* fractions (Figure 3.4) the MDF4 fraction seems to be the most potent one, since it completely degraded DNA, being visible only traces of small fragments. Nevertheless, the remaining fractions also exhibited potent damaging effects on DNA.

3.3.4. Biofilm formation inhibitory capacity

One of the main mechanisms associated with the development of microbial resistance to antimicrobial substances is related with the ability of certain microorganisms to develop biofilms. Accordingly, *S. muticum* and *A. armata* fractions were tested for their biofilm formation inhibitory potential on *P. aeruginosa*. The results are summarized in Figure 3.5.

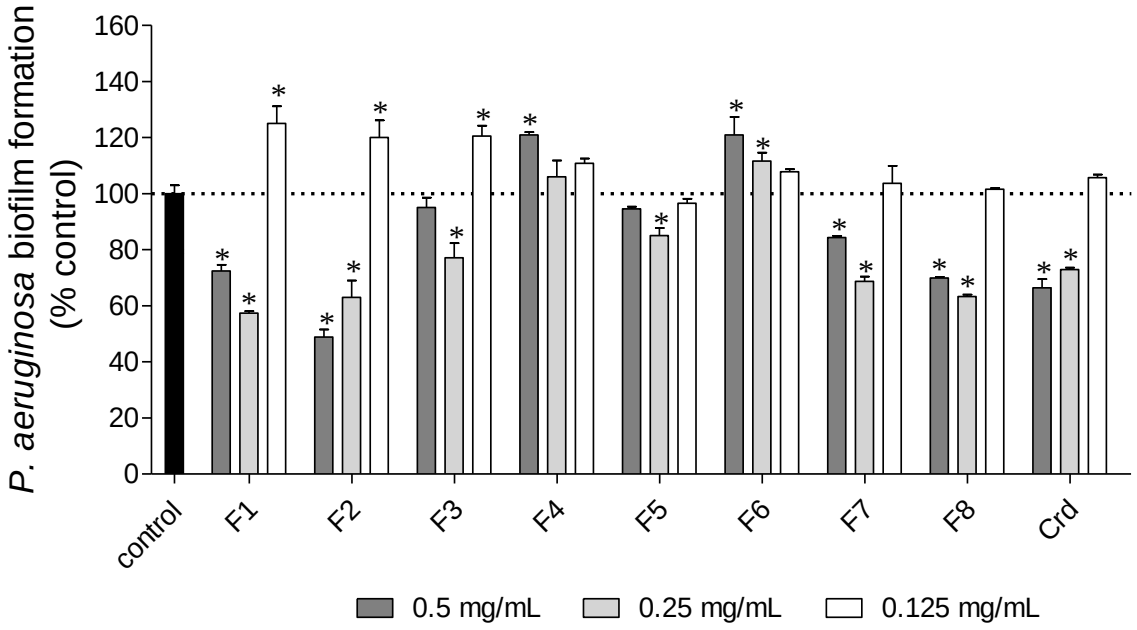

Figure 3.5. *Asparagopsis armata* VLC fractions potential to inhibit *Pseudomonas aeruginosa* biofilm formation. Each column represents the mean ± standard error of the mean of at least two independent experiments. Symbols (*) represents statistically significant differences when compared with the control (100%) (ANOVA, Dunett's test, $p < 0.05$).

Through Figure 3.5 it is possible to verify that at 0.5 mg/mL fractions F1, F2, F7, F8 and crude inhibited *Pseudomonas aeruginosa* biofilm production by 28, 51, 16, 31 and 34%, respectively, and at 0.250 mg/mL these fractions inhibited 43, 37, 32, 37 and 28%, respectively. At 0.250 mg/mL fractions F3 and F5 also revealed biofilm formation inhibitory potential by 23 and 15%, respectively.

At the lowest concentration (0.125 mg/mL), none of the fractions revealed capacity to inhibit *P. aeruginosa* biofilm formation.

S. muticum did not exhibited capacity to inhibit *P. aeruginosa* biofilm formation.

3.3.5. Identification of constituents present on the most active fractions

Fractions F2, F3, F4, F5, and F6 of *S. muticum* were the ones with the highest antimicrobial potential, and therefore were subjected to further analysis by GC-FID and by GC-MS. The main volatile metabolites were identified by comparing their mass fragmentation patterns with those available MS databases and its relative composition by GC-FID. The results are compiled on Tables 3.5 and 3.6.

Table 3.5. *Sargassum muticum* GC-FID and GC-MS analysis of VLC fractions.

Fractions	Major compounds	Retention Time (min)		GC-FID Relative quantity (%)
		GC-FID	GC-MS	
MF2	Hexadecanoic Acid	20.7	9.1	29.8
	Octadecanoic Acid	26.2	9.7	13.2
MF3	Hexadecanoic Acid	20.6	9.1	40.7
	Octadecanoic Acid	26.3	9.7	9.9
MF4	Hexadecanoic Acid	20.7	9.1	32.8
	Octadecanoic Acid	26.2	9.8	5.6
MF5	Hexadecanoic Acid	20.8	9.1	16.8
	Octadecanoic Acid	26.2	9.8	7.9
MF6	Hexadecanoic Acid	20.7	9.1	16.1
	Octadecanoic Acid	26.2	9.8	-
MDF2	Hexadecanoic acid	20.7	9.1	24.1
MDF3	Hexadecanoic Acid	20.7	9.1	22.2
MDF4	Octadecanoic Acid	26.2	9.8	8.5
MDF5	Hexadecanoic acid	20.8	9.1	15.2
MDF6	Hexadecanoic acid	20.7	9.1	28.1

- Not determined

The GC-MS analyses of *Sargassum muticum* suggest that the major compounds present in the fractions are hexadecanoic acid, commonly known as palmitic acid, and octadecanoic acid, commonly known as stearic acid, which are present in variable amounts. Fractions MF3, MF4 and MF2 scored the highest percentage of hexadecenoic acid, 40.70%, 32.84% and 29.79%, respectively, whereas fraction MF2 scored the highest amount of octadecanoic acid (13.23%) followed by fraction MF3 (9.87%).

Due to low mass availability, only fraction MDF2 was subjected to further purification by column chromatography. The resulting subfractions were further characterized by GC-MS and tested for their antimicrobial activity against *S. aureus*. The results are presented in Table 3.6.

Table 3.6. GC-MS analysis of *Sargassum muticum* subfractions resulting from fraction MDF2.

MDF2 Sub-fractions	Major Compounds	Retention time (min)	MW (g/mol)
2-19	6,10,14-Trimethylpentadecan-2-one	8.9	269
	Hexadecanoic acid	9.1	256
	bis(2-ethylhexyl) hexanedioate	10.6	371
	bis(2-ethylhexyl) decanedioate	12.3	427
37	Hexadecanoic Acid	9.2	256
	Octadecanoic Acid	9.9	284
39-42	7,9-ditert-butyl-1-oxaspiro[4,5]deca-6,9-diene-2,8-dione	9.2	276
	Hexadecanoic Acid	9.2	256
	Octadecanoic Acid	9.9	284
52-59	Hexadecanoic Acid	9.2	256
	Octadecanoic Acid	9.9	284
64-70	Hexadecanoic Acid	9.2	256
	Octadecanoic Acid	9.9	284
85-86	Hexadecanoic Acid	9.2	256
	Octadecanoic Acid	9.9	284
94-95	Hexadecanoic Acid	9.2	256
	Octadecanoic Acid	9.9	284
118	Hexadecanoic Acid	9.2	256
	Octadecanoic Acid	9.9	284
119-126	Hexadecanoic Acid	9.2	256
	Octadecanoic Acid	9.9	284

*None of the fractions presented antimicrobial potential against *S. aureus* (100 µg/mL).

Besides the previously identified fatty acids, the purification of the MDF2 fraction allowed the detection of the ketone 6,10,14-Trimethylpentadecan-2-one, the esters bis(2-ethylhexyl) hexanedioate and bis(2-ethylhexyl) decanedioate, and the flavonoid 7,9-ditert-butyl-1-oxaspiro[4,5]deca-6,9-diene-2,8-dione. None of the MDF2 subfractions (100 µg/mL) revealed antimicrobial potential against *S. aureus*.

Concerning *A. armata* fractions F1, F2, F3, F4, F5, and F6 revealed high antimicrobial potential, and therefore they were subjected to further analysis by GC-FID and by GC-MS. The main volatile metabolites were identified by comparing their mass fragmentation patterns with those available MS databases and its relative composition by GC-FID. The results are compiled on Tables 3.7 - 3.10.

Table 3.7. *Asparagopsis armata* GC-FID and GC-MS analysis of VLC fractions.

Fractions	Major compounds	Retention Time (min)		GC-FID Relative quantity (%)
		GC-FID	GC-MS	
F1	Hexadecanoic Acid	20.7	9.3	26.1
	Octadecanoic Acid	26.2	9.9	17.1
F2	Hexadecanoic Acid	20.8	9.1	41.3
F3	Hexadecanoic Acid	20.9	9.1	44.9
F4	Hexadecanoic Acid	20.9	9.1	35.3
	Octadecanoic Acid	26.0	9.8	25.5
F5	Hexadecanoic Acid	20.9	9.1	25.3
	Octadecanoic Acid	26.0	9.8	12.4
F6	Hexadecanoic Acid	20.7	9.1	33.2
	Octadecanoic Acid	26.0	9.8	16.8

Table 3.7 shows the major volatile compounds present in the most active VLC fractions of *A. armata*. Accordingly, the hexadecanoic acid and octadecanoic acid were the main detected constituents in all samples.

To concentrate and purify the compounds present in the most active fractions (F1-F6), several chromatographic steps were conducted. For fractions F2 and F4, since enough biomass was available, it was possible to proceed with a preparative column chromatography, and the resulting sub-fractions tested for their antimicrobial potential against *S. aureus*, *P. aeruginosa* and *C. albicans*. The results are summarized in Tables 3.8 and 3.9, respectively.

Table 3.8. GC-MS analysis of *Asparagopsis armata* F2 sub-fractions and respective growth inhibition (100 µg/mL) against *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Candida albicans*. Results are presented as the mean ± standard error of the mean of two independent experiments.

F2 Sub-fractions	Major compounds	Retention time (min)	MW (g/mol)	*S. aureus*	*P. aeruginosa*	*C. albicans*
25	Hexadecanoic Acid	9.2	256	39.4 ± 3.2%	6.7 ± 3.3%	0.0 ± 1.1%
	Mixture of fatty acids	22.5	647			
27	Tribromomethanol	7.7	269	89.5 ± 6.8%	93.8 ± 2.9%	47.5 ± 3.6%
	Hexadecanoic Acid	9.2	256			
28	Tribromoethanol	7.6	280	80.5 ± 4.1%	99.0 ± 2.4%	69.7 ± 1.1%
	Tribromomethanol	7.7	269			
	Tribromobutane	8.1	292			
	Hexadecanoic Acid	9.2	256			
44-47	2(Dibromomethyl)tetrahydrofuran	6.3	242	86.8 ± 6.9%	28.0 ± 2.5%	0.0 ± 1.3%
	Tetradecanoic Acid	8.6	228			
	Hexadecanoic Acid	9.2	256			
	Octadecyl bromoacetate	10.6	390			
48-51	Dodecanoic Acid	7.6	200	89.1 ± 9.2%	16.9 ± 3.0%	18.6 ± 4.5%
	Tetradecanoic Acid	8.5	228			
	Hexadecanoic Acid	9.3	256			
	Octadecanoic Acid	9.9	284			
52-59	Dodecanoic Acid	7.6	200	85.8 ± 6.2%	8.7 ± 2.9%	14.0 ± 2.7%
	Tetradecanoic Acid	8.5	228			
	Hexadecanoic Acid	9.3	256			
60	Dodecanoic Acid	7.6	200	94.3 ± 0.6%	51.6 ± 3.5%	9.2 ± 2.5%
	Tetradecanoic Acid	8.5	228			
	Hexadecanoic Acid	9.3	256			
61-65	Dodecanoic Acid	7.6	200	95.6 ± 0.5%	14.4 ± 1.8%	12.4 ± 4.1%
	Tetradecanoic Acid	8.5	228			
	Hexadecanoic Acid	9.3	256			

Table 3.8 shows the major volatile compounds present in the *A. armata* VLC fraction F2, identified through GC-MS. Accordingly, it is possible to verify that the main constituents are hexadecanoic acid (palmitic acid), tribromomethanol, tribromoethanol, 2-(dibromomethyl)tetrahydrofuran, octadecyl bromoacetate, dodecanoic acid (lauric acid), tetradecanoic acid (myristic acid), and octadecanoic acid (stearic acid). Subfractions 27 and 28, containing tribromomethanol were the most active against *P. aeruginosa* (93.8 and 99.0%, respectively), and against *C. albicans* (47.5 and 69.7%, respectively). Sub-fractions 60 and 61-65 containing dodecanoic acid, tetradecanoic acid, and hexadecanoic acid were the most active against *S. aureus* (94.3 and 95.6%, respectively), followed by sub-fractions 27 (89.5 ± 6.8%), 48-51 (89.1 ± 9.2%) and 44-47 (86.8 ± 6.9%).

Table 3.9. GC-MS analysis of *Asparagopsis armata* F4 sub-fractions resulting from fraction F4 and respective growth inhibition (100 µg/mL) against *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Candida albicans*. Results are presented as the mean ± standard error of the mean of two independent experiments.

F4 Sub-fractions	Major compounds	Retention time (min)	MW (g/mol)	*S. aureus*	*P. aeruginosa*	*C. albicans*
17-25	10,18-Bisnorabieta-8,11,13-triene	9.7	242	46.5 ± 0.9%	0.0 ± 3.5%	0.0 ± 1.1%
57-59	Cholesta-3,5-dien-7-one	16.0	382	23.6 ± 4.6%	0.0 ± 2.3%	0.0 ± 2.3%
60-63	Tribromoethanol	7.7	280	69.9 ± 0.7%	89.1 ± 2.7%	23.7 ± 7.2%
	1,1,4,4-tetrabromobut-3-on-2-ol	9.0	262			
64-65	Tribromoethanol	7.6	280	81.4 ± 1.3%	84.6 ± 1.5%	19.5 ± 0.8%
	Hexadecanoic Acid	9.3	256			
66-68	Tetradecanoic Acid	8.6	228	24.9 ± 0.4%	19.9 ± 2.8%	0.0 ± 1.0%
	Hexadecanoic Acid	9.3	256			
	Octadecanoic Acid	9.9	284			
80-86	2,5-dibromo-3-acetamidothiophene	6.8	172	86.1 ± 1.0%	68.8 ± 2.9%	0.0 ± 1.2%
	Dodecanoic Acid	7.8	200			
	Tetradecanoic Acid	8.6	228			
	Hexadecanoic Acid	9.3	256			
	Octadecanoic Acid	9.9	284			

Table 3.9 shows that the main constituents of F4 sub-fractions are 10,18-bisnorabieta-8,11,13-triene, tribromoethanol, 1,1,4,4-tetrabromobut-3-on-2-ol, tetradecanoic acid (myristic acid), hexadecanoic acid (palmitic acid), octadecanoic acid (stearic acid), 2,5-dibromo-3-acetamidothiophene and dodecanoic acid (lauric acid). Sub-fractions 80-86, 60-63 and 64-65, containing brominated compounds, revealed the highest antimicrobial potential.

Due to low biomass availability, fractions F3 and F5 were only possible to be analysed by semi-preparative HPLC. The major peaks were identified, collected and evaluated for their antimicrobial potential against *S. aureu*s (Table 3.10).

Table 3.10. Antimicrobial activity of *Asparagopsis armata* compounds identified by semi-preparative HPLC. The antimicrobial activity is expressed as the mean ± standard error of the mean of two independent experiments.

HPLC peak	Compounds	Retention Time (min)	MW (g/mol)	*S. aureus* growth inhibition at 100 µg/mL
F3_p20[a]	Hexadecanoic Acid	9.1	9.1	55.0 ± 2.1%
F5_p11[b]	Hexadecanoic Acid	9.1	9.1	26.0 ± 1.3%

[a] – major peak obtained from fraction F3; [b] – major peak obtained from fraction F5.

Table 3.10 shows the main constituents of two subfractions obtained by semi-preparative HPLC, from fractions F3 and F5. Accordingly, hexadecanoic acid (palmitic acid) is pointed as the major constituent of both subfractions, however, the antimicrobial activity against *S. aureus* was higher in the subfraction (p20) obtained from fraction F3 (55%). It was not possible to further investigate F1 and F6 fractions due to the low biomass availability.

Since the major fatty acids identified in *Sargassum muticum* and *A. armata* samples were octadecanoid and hexadecanoic acids, the antimicrobial activity of their commercial version was evaluated on *S. aureus* at 100 µg/mL.

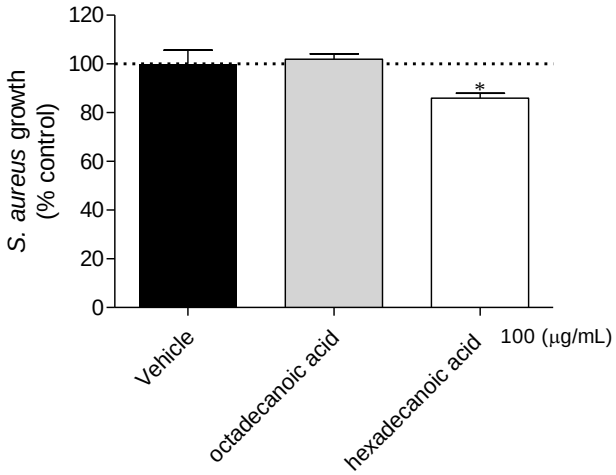

Figure 3.6. Antimicrobial activity of commercial octadecanoic acid (stearic acid) and hexadecanoic acid (palmitic acid) against *Staphylococcus aureus* at 100 µg/mL. Columns represent the mean standard error of the mean of three independent experiments. Symbols (*) represents statistically significant differences when compared with the vehicle (100%) (ANOVA, Dunett's test, $p < 0.05$).

Octadecanoic acid had no effect on the microorganism growth, while hexadecanoic acid inhibited the growth of *S. aureus* in about 15%.

3.4. Discussion and conclusion

In modern societies, some of the most concerning health issues are related with infection diseases, which continues to affect millions of people all over the world. Although major efforts are being done to develop more effective antimicrobials, the high adaptation capacity of microorganisms and development of resistant strains, imposes a permanent search for new compounds with antimicrobial properties (WHO, 2017; Balsalobre et al., 2014).

In the last decades, seaweeds have been widely studied for their ability to produce bioactive structures. Nevertheless, due to the scarcity of some marine species and their seasonality, allied to the negative impacts resulting from their collection, these are not viable options for industrial purposes. In line with this view, as invasive and highly impacting species, *S. muticum* and *A. armata* represent a great opportunity for new antimicrobials discovery and ultimate industrial production, transforming a serious problem into a major opportunity (Pinteus et al., 2018). Accordingly, in this work, *S. muticum* and *A. armata* were evaluated as potential sources of antimicrobial compounds against important human pathogens, namely *S. aureus* (Gram positive), *P. aeruginosa*, *E. coli*, *S. enteritidis* (Gram negative), and *C. albicans* (fungus). The fractionation of the bioactive compounds was achieved through column chromatography and the most active fractions were further investigated for the possible mechanisms of action involved in the antimicrobial effects, by testing membrane and DNA damages. For *S. muticum*, two extraction methods and subsequent sub-fractions were tested for their antimicrobial potential. It is possible to verify that the methanolic extraction afforded compounds with higher antimicrobial potential than the methanol:dichloromethane extraction. This was especially visible against *E. coli*, since 4 fractions inhibited this microorganism growth in more than 50%, and none of the methanol:dichloromethane fractions presented this potential. These results suggest that the vacuum liquid chromatography was efficient in concentrating antimicrobial compounds, since the crude extract presented a maximum inhibitory potential of about 48% against *S. aureus*, in contrast with fractions F2-F6, which totally inhibited this microorganism growth. The same enhancement was also verified against the other microorganisms. In addition, the methanolic fractions were also the ones presenting the lower IC_{50}, and therefore, the highest potency, especially fractions MF2-MF5 against *S. aureus* and *E. coli*, fraction MF5 against *P. aeruginosa,* and fraction MF8 against *C. albicans*.

The high antimicrobial potential of *S. muticum* extracts with polar characteristics is in agreement with several authors. Accordingly, although with other *Sargassum* species, Horie and co-workers (2008) used the same extraction approach to obtain antimicrobial compounds (methanol:dichloromethane extraction followed by a chromatography with a stepwise gradient of

ethyl acetate:hexane) and also verified strong inhibitory potential against *S. aureus*. Morales and co-workers (2006) screened the antimicrobial and antifungal potential of an ethyl acetate fraction obtained from a methanol crude extract and verified antibacterial activity against *S. aureus*, *Bacillus subtilis*, and against the fungus *Trichophyton mentagrophytes*, yet with no activity against gram negative bacteria. Tanniou and co-workers (2014) screened several samples of *S. muticum* collected in Portugal and Norway and verified that a water:acetone crude extract, and subsequent fractions, presented growth inhibitory potential >75% against *E. coli*, *S. aureus* and *B. subtilis* at 50 µg/mL.

In the present work, it can be verified that the extraction of *S. muticum* with a mixture of methanol:dichloromethane has also resulted in bioactive compounds with antimicrobial effects, especially visible against *S. aureus* and *E. coli.* In agreement with these results, Villarreal-Gómez and co-workers (2010) also identified antimicrobial activity against *Proteus mirabilis* in *S. muticum* extracts obtained with a dichloromethane:methanol mixture (3:7), recording an IC_{50} of 140.1 µg/mL.

Regarding *A. armata,* it was verified that this seaweed presents a broad-spectrum antimicrobial activity, since it exhibited potential against all microorganisms. The efficiency of the chromatography to concentrate the bioactive compounds is particularly visible in the results obtained against *E. coli*, since the crude extract did not reveal capacity to inhibit this microorganism growth, but fractions F2, F3, F4, F5, and F6 exhibit strong antimicrobial potential.

To understand the true potency of the extracts and fractions, these were subjected to a dose-response analysis and the IC_{50} determined. The results shown strong inhibitory effects, especially against *S. aureus*, *P. aeruginosa*, and *C. albicans*, with several fractions presenting an IC_{50} lower than 100 µg/mL. In agreement, previous studies suggested this seaweed extracts have growth inhibitory potential against *P. aeruginosa*, *C. albicans, S. aureus,* and *E. coli* (Pinteus et al., 2015; Oumaskour et al., 2013; Salvador et al., 2007; Paul et al., 2006), however, since their analysis was conducted by the disk diffusion method, it is difficult to compare the effects with the results obtained in the present work. No reports were found for the antimicrobial potential of *A. armata* against *Salmonella enteritidis*.

Although some works have been developed to understand the potential of *A. armata* and *S. muticum* as a source of antimicrobial compounds, to the present knowledge, few or none have been developed to understand the mechanisms behind the observed growth inhibitory effects. In line with this, the cytoplasmatic membrane and DNA integrity were evaluated. Accordingly, microorganisms were exposed to the most active fractions (IC_{50} < 100 µg/mL).

The membrane integrity analysis suggests that *A. armata* produces compounds with ability to induce significant damage in the cytoplasmatic membrane of *S. aureus* and *P. aeruginosa*, however few damaging effects were observed against *C. albicans*. The low membrane damage observed in *C. albicans* can be related with the additional nucleus protection that these microorganisms, as eukaryotic cells, possess (Legrand et al., 2019). *Candida albicans* are yeast (a type of fungus) that produce infections ranging from non-life threatening to serious invasive infections, especially in immune-compromised patients. These microorganisms are a real challenge for medicine. Due to the high similarity of some biological processes between humans and fungi, drugs that are toxic for fungi are also toxic for humans, consequently the therapeutic options for fungal infections are much more limited than for bacterial infections (Roemer and Krysan, 2014). The search for compounds to counteract persistent infections by targeting microbial membranes, is a recent approach in the drugs discovery pipeline. Most of the antimicrobials currently used are the evolution of the ones discovered between 1940 and 1980. These have the ability to kill or to inhibit microorganisms growth during cellular division (logarithmic growth) mainly targeting the biosynthesis of proteins, peptidoglycan, folic acid, DNA or RNA (Aminov, 2010; Fair and Tor, 2014; Powers, 2004). Nevertheless, it is known that in unfavorable environments, bacteria can remain in a quiescent state until the drug concentration is no longer effective and restart their growth promoting disease again. This is one of the major problems associated to persistent infections treatment, such as tuberculosis, since bacteria have a characteristic low growth with the ability to remain in a dormant state, and therefore not respond to antimicrobial drugs (Cohen et al., 2013). Although not much work can be found with marine natural products regarding antibacterial mechanisms of action, it was shown that essential oils from *Enteromorpha linza* were able to induce changes in *Listeria monocytogenes* cytoplasmatic membrane (Patra and Baek, 2016). Also, Wei and co-workers (2016) evaluated the antimicrobial potential of phlorotannins enriched extracts against *Vibrio parahaemolyticus* and verified growth inhibition, probably due to membrane damage. With the barrier function of the cell compromised, DNA is exposed to foreign aggressors prompting cell death. This possibility was also explored, and the most active fractions of *A. armata* (F1-F6) presented capacity to bind with plasmidic DNA decomposing it into smaller fragments. Thus, it is plausible to assume that the mechanisms of action behind the growth inhibition promoted by *A. armata* compounds may be associated with membrane and DNA damage, which represent a great opportunity for the development of new antimicrobial drugs of natural origins, especially due its dual targeting properties.

Although *S. muticum* did not exhibit membrane damaging potential, the fractions with the highest antimicrobial potential showed ability to break DNA. There are some mechanisms for compounds to reach DNA without compromising bacterial membranes' viability. These may include changes in microbial membrane permeability, transfer by diffusion or self-uptake (Kapoor et al., 2017). In agreement with these results, El Shafay and co-workers (2016) also verified a great antimicrobial activity of *Sargassum* species, linked to the ability to interfere with DNA constituents.

One of the main problems associated with the occurrence of resistant bacteria is their capacity to develop biofilms. In the present work, *P. aeruginosa* biofilm production was evaluated when exposed to both seaweed fractions. This species is known to produce strong biofilms, being associated with hospital infection outbreaks which treatments are difficult and sometimes fruitless (Taylor et al., 2014). The present results show that, *A. armata* extracts contain substances with capacity to inhibit *P. aeruginosa* biofilm production. Similarly, Hentzer and co-workers (2002) showed that an halogenated furanone compound derived from the seaweed *Delisea pulchra* had a potent inhibitory effect on *P. aeruginosa* biofilm production. One relevant aspect to note is that fractions F7 and F8 did not reveal a strong growth inhibition of *P. aeruginosa*, but revealed noteworthy biofilm inhibitory effects, suggesting that a different mechanism of action may be involved. In line with these results, Benneche and co-workers (2011) also verified that several natural derived compounds presented a higher biofilm reduction than inhibition of bacterial planktonic growth.

The results here reported reveal the biotechnological potential of *S. muticum* and *A. armata* as a source of antimicrobial compounds. Nevertheless, for new drugs development it is crucial to unveil the chemical structure of the bioactive compounds. The use of methanol and dichloromethane are quite common in natural products research, since these have potential to extract a wide myriad of compounds. However, since the resulting extracts are complex mixtures of compounds, the isolation and identification of the bioactive molecules are a very challenging task.

To investigate which compounds were possibly involved in the observed antimicrobial activities, several chromatographic and analytical steps were undertaken (preparative column chromatography, TLC, HPLC, GC-FID, GC-MS, and NMR). *S. muticum* F2- F6 fractions (from both extractions) and particularly *A. armata* F1 - F6 fractions revealed a high antimicrobial activity, however, the low extraction yields were limiting for more detailed chemical analysis. The most abundant constituents were screened by NMR, and the results revealed a profile characteristic of fatty acids, and therefore, further analyses were conducted through GC-MS for both seaweeds.

In the present work it was only possible to detect fatty acids as major compounds in *S. muticum* fractions. In addition, a deeper analysis on MDF2 constituents also pointed fatty acids as the major constituents, and these did not reveal antimicrobial properties, suggesting the presence of minor compounds which can be responsible for the antimicrobial effects previously exhibited by MDF2 fraction. Although several phlorotannins have been already identified in *S. muticum* extracts (Glombitza et al., 1978; Montero et al., 2016) and one tetraprenyltoluquinol chromane meroterpenoid (Balboa et al., 2015), there is an evident lack of knowledge concerning the chemical structures produced by this seaweed. Nevertheless, several studies on other *Sargassum* species reveal that these seaweeds are rich in unique chemical structures that can be related with the observed bioactivities, such as quinone derivatives (Horie et al., 2008), meroterpenoids (Iwashima et al., 2008), diterpenes (Ayyad et al., 2001),and glycolipids (Wu et al., 2009), among others (Liu et al., 2012).

Concerning *A. armata*, it was possible to identify several fatty acids commonly found in nature, such as palmitic, stearic, myristic, and lauric acids. Interestingly, it was verified that the mixture of these three fatty acids exhibit relevant antimicrobial activity (> 90% at 100 µg/mL) (Table 3.8). Since palmitic and stearic acids were the main fatty acids identified in *A. armata* and *S. muticum*, it was decided to test their commercial versions on *S. aureus* growth. Accordingly, Figure 3.6 shows that octadecanoic acid (stearic acid) did not exhibited any effect on *S. aureus* growth and hexadecanoic acid (palmitic acid) presented an inhibitory effect in about 15%. These results show that these fatty acids were not responsible for the antimicrobial effects detected on *A. armata* samples, since the effects were much potent. In agreement, Plaza and coworkers (2010) stated that it is more common to verify antimicrobial activity by long-chain unsaturated fatty acids, then by long-chain fatty acids such as palmitic acid and stearic acid. On the other hand, Bazes and collaborators (2009) investigated the antibacterial potential of palmitic acid against *Rhodobacteraceae bacterium* and verified an IC_{50} of 44 µg/mL, suggesting that this fatty acid may have a selective antimicrobial potential. As a result, the verified antimicrobial activity is probably due to the presence of minor compounds that were not possible to isolate and identify. Nevertheless, *A. armata* sub-fractions GC-MS analyses also revealed the presence of several brominated compounds, which can be involved in the observed antimicrobial effects. In agreement with these results, Paul and collaborators (2006) could verify that *A. armata* produce bromoterpenes and dibromoterpenes with antimicrobial potential against *E. coli*, *P. aeruginosa* and *S. aureus*. Additionally, several research works conducted on the Bonnemaisoniaceae family, have reported the presence of brominated compounds with antimicrobial activity (Pérez et al., 2016). Nylund and co-workers

(2008) showed that the red alga *Bonnemaisonia hamifera* is less fouled by marine bacteria due to the production of the 1,1,3,3-tetrabromo-2-heptanone compound. These works strengthen the possibility of the antimicrobial activity detected on *A. armata* fractions and sub-fractions, be due to the presence of brominated compounds. Thus *A. armata* derived brominated compounds should be considered as promising antimicrobial compounds aiming new drugs development.

The combat of infectious diseases will be the hardest challenge to overcome in developed societies in a near future. The World Health Organization (WHO) estimated that, in 2050, infectious diseases will cause more deaths than cancer, affecting more than 10 million people all over the world (O'Neill, 2014). Therefore, it is of utmost importance to investigate new possibilities to combat and to prevent multidrug resistant microorganisms.

The discovery of new compounds is only the very first step of the long, expensive, and risky drug discovery pipeline, which requires large supply of the metabolite of interest. In line with this view, *Asparagopsis armata* can be a valuable source of antimicrobial compounds having revealed a high growth inhibitory potential against different pathogens, including Gram-positive, Gram-negative bacteria, and fungi. Moreover, the verified inhibitory effects show signs of relevant mechanisms of action being involved, and also potential to inhibit microbial biofilms production. These characteristics, together with the highly abundance of this seaweed in many coastlines all over the world, point out *A. armata* as a valuable resource for compounds extraction aiming the development of new antimicrobial therapeutics targeting human challenging pathogens.

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Chapter 4

The antifouling potential of the marine invaders *Asparagopsis armata* and *Sargassum muticum*

This chapter is based and includes the following manuscripts:

Pinteus, S., Lemos, M. F., Alves, C., Silva, J., Pedrosa, R. 2021. The marine invasive seaweeds *Asparagopsis armata* and *Sargassum muticum* as targets for greener antifouling solutions. Science of the Total Environment 750, 141372.

Pinteus, S., Lemos, M. F., Freitas, R., Duarte, I. M., Alves, C., Silva, J., Marques, S.C., Pedrosa, R. 2020. Medusa polyps adherence inhibition: A novel experimental model for antifouling assays. Science of the Total Environment 136796.

4.1. Introduction

Submerged surfaces are ideal for biofouling communities to stablish, giving rise to one of the biggest problems faced by maritime industries (Almeida and Vasconcelos, 2015; Bixler and Bhushan, 2012; Yebra et al., 2004). The negative impacts are related with the increased costs of ships maintenance, and to the increase of fuel consumption due to frictional resistance, which in turn increases the emission of environmentally hazardous gases (Dobretsov, 2009; Saha et al., 2018; Townsin, 2003). In addition, biofouling is also one of the main vehicles for alien species spreading (Davidson et al., 2009; Piola et al., 2009).

For centuries, strategies have been developed to combat biofouling occurrence. The first coating to be patented goes back to 1625, consisting of copper sheathing with heavy metal-based coatings. The addition of organotin compounds, such as TBT (Tributyltin), occurred between 1950-1960 (Almeida et al., 2007; Yebra el al., 2004). Although being efficient antifouling substances, soon it would be discovered that organotin compounds induce highly negative effects in aquatic ecosystems, due to high toxicity towards non target species, imposex effects, persistence in the environment and bioaccumulation potential (Cardwell et al., 1999; Santillo et al. 2001), being prohibited worldwide by the International Maritime Organization (IMO, 2001; Commission Directive, 2002; Sarti et al., 2011).

Knowing that global maritime traffic showed a dramatic fourfold increase between 1990 and 2014 (Tournadre, 2014) and that in 2017 the number of registered merchant ships only, exceeded 50 000 (Statistas, 2018), it is imperative to find sustainable environmental friendly antifouling solutions. Biofouling involves a series of complex mechanisms not completely understood. Nevertheless, it is recognized to have four distinct stages (Amara et al., 2017) (Figure 4.1).

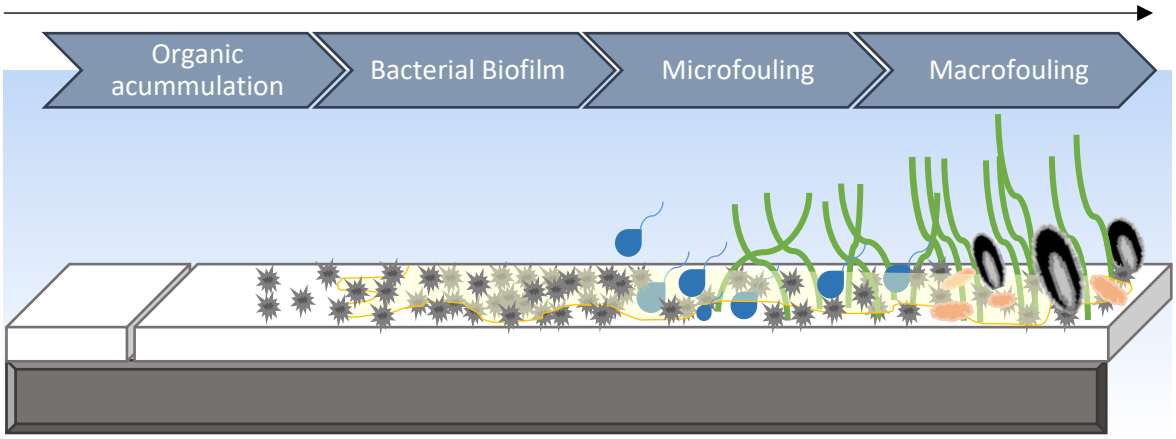

Figure 4.1. Schematic representation of fouling development.

Whenever a structure is submerged, macromolecules such as polysaccharides and proteins, together with inorganic ions adhere, changing electrostatic properties and wettability of the structure. These processes are regulated mainly by hydrodynamic features, mass transport events and electrostatic phenomena. This first stage, also called conditioning film, occurs in a very short period of time (about 1 min) and is essential for the subsequent stages to occur (Arunachalam and Bavya, 2010; Terlizzi et al., 2001). Fouling bacteria have now all the conditions to adhere and colonize the surface (Stage II) (Lehaitre et al., 2008; Martín-Rodríguez et al., 2015).

It is known that some species, such as *Bacillus* sp. and *Vibrio* sp., have the ability to produce strong biofilms (Mielich-Süss and Lopez, 2015; Wadood et al., 2015). These biofilms are mainly constituted by polysaccharides developing polymeric bridging between the cell and the substrate (Lehaitre et al., 2008). The mechanisms by which bacteria colonize immerged structures is not fully understood, nevertheless, bacterial cell-to-cell communication, mediated by *quorum sensing* seems to be a crucial feature (Fusetani, 2011). *Quorum sensing* can be defined as a set of chemical signs that allow bacteria to "communicate" within the same species or even between different species. These chemical signs include molecules that function as autoinducers (acylated homoserine lactones in Gram-negative bacteria and processed oligopeptides in Gram-positive bacteria), that promote different genetic responses depending on their concentration (Miller and Bassler, 2001). These genetic responses are involved in the production of extracellular polymers (polysaccharides and proteins) necessary for biofilm formation as well as to regulate the retention of nutrients, organic and inorganic substances in the environment (Miller and Bassler, 2001).

Therefore, if physico-chemical conditions at the surface are adequate, bacteria will develop colonies involved by strong biofilms. These biofilms will, in turn, favor the adherence of other microorganisms such as microalgae (specially diatoms), cyanobacteria, and fungi (stage III) (Amara et al., 2017; Lehaitre et al., 2008). In this stage, all the attached microorganisms will grow forming an irregular structure composed mainly by exopolymeric substances, gradually trapping more particles and organisms, allowing the subsequent adherence of macroorganisms such as algae, barnacles, polyps, tunicates, mussels, corals, sponges, anemones that will finally evolve into complex biological communities (stage IV). After this process, the biofouling process is considered irreversible and therefore, the prevention of biofouling is easier in the first stages (Amara et al., 2017; Lehaitre et al., 2008; Qian et al., 2009).

Biofouling can, therefore, be divided into microfouling - biofilm formation and bacterial adhesion and macrofouling - attachment of larger organisms phenomena. While microfouling tests are relatively simple, fast, with high reproducibility, *in-vivo* studies involving macroinvertebrates are

much more delicate and time consuming. The main species used as models include, barnacles, tubeworms, and mussels larvae (Almeida et al., 2015). Commonly, these organisms are collected from their natural environment and subjected to physical and or/ chemical stresses, in artificial controlled conditions, to induce larvae release (Briand, 2009; Karande, 1999; Rittschof et al., 1992; Tighe-Ford et al., 1970). Then, larvae have to overcome several metamorphic stages, until settlement, which, depending on the species, can take several days, normally with high mortality rates. On the other hand, *in vitro* molecular tests include the evaluation of the potential to block neurotransmission (such as acetylcholinesterase inhibitors) and the inhibition of enzymes involved in the polymerization of secreted proteins essential to settlement, such as phenol oxidases (Almeida et al., 2015; Kristensen et al., 2008). Although relatively simple, molecular *in vitro* tests have the limitation of targeting isolated metabolic pathways, disregarding the organism's own defenses as a whole. Therefore, *in vivo* tests are fundamental for a more realistic assessment of the antifouling potential of a determined compound/formulation (Almeida and Vasconcelos, 2015).

As a result, a question raises: are there other species with a sessile life cycle stage, more resilient, easy to work and abundant, that could be used as model for *in-vivo* anti-adherence tests? Within this framework, jellyfish were addressed.

Although without the same popularity as barnacles or mussels, Hydrozoa species (jellyfish) are also important and successful biofoulers (Graham and Bayha, 2007). These animals belong to the Cnidaria phylum and consists in more than 3000 species, the majority found in marine ecosystems. Most scyphozoans have a biphasic life cycle, involving a benthic stage (polyps and strobila) and a pelagic stage (ephyrae and medusa) (Astorga et al., 2012; Prieto et al., 2010; Graham and Bayha, 2007; Bouillon et al., 2006). The medusa are the dispersal and sexual stage of the life-cycle. When mature, they release eggs or sperm to the surrounding waters for fertilization, giving rise to the planula larvae, which settles down in the substrata and undergo metamorphosis to form young polyps, attached by a peduncle (Holst et al., 2007) (Figure 4.2).

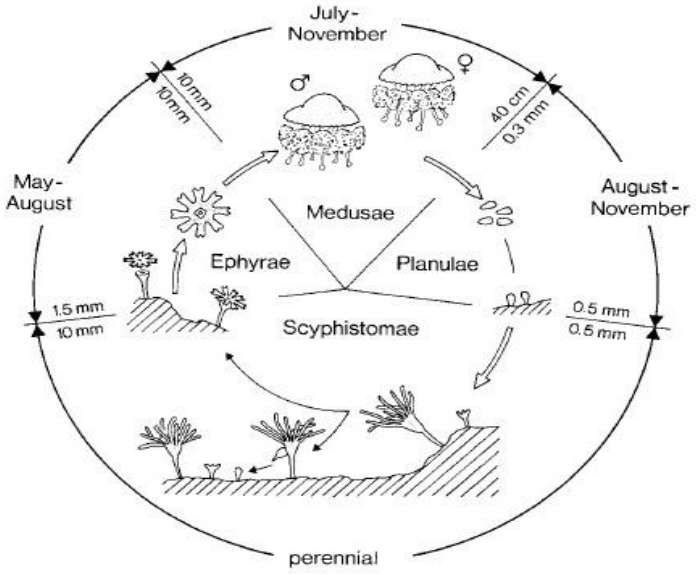

Figure 4.2. Scheme of the life-cycle of the scyphomedusae *Cotylorhiza tuberculata*, which is typical for most scyphozoan species (Kikinger 2008).

With appropriate conditions, these organisms can grow colonies of high number organisms (Schiariti et al., 2008; Holst et al., 2007; Lotan et al., 1994) presenting a higher life period than the pelagic phase, due to being less sensitive to environmental changes as temperature, salinity and dissolved oxygen (Prieto et al., 2010). Population size and dynamics of jellyfish species depend on the survival and reproduction of the sessile phase, determining the subsequent abundance of Medusae (Fu et al., 2014)

Due to their physiological, ecological and life-history traits, such as rapid growth, sexual and asexual reproduction, intensive predation and morphological plasticity, these organisms have all the characteristics to successfully spread and invade non-native areas, having capacity to disperse up to 1000 km owing to ocean currents and active swimming (Graham and Bayha, 2007). In addition, polyps resilience in estuarine and coastal environments allows them to survive worldwide voyages adhered to the hull of vessels, also providing habitats and chemical cues for other fouling organisms such as polychaetes and amphipods (Graham and Bayha, 2007; Xie et al., 2015). Furthermore, Scyphozoan polyps are known to be successful biofoulers of harbours and aquaculture facilities (Hoover and Purcell, 2009; Purcell et al., 2007) and new ephyrae released can easily pass through nets and cages, causing numerous problems (e.g. gill damage) and high mortality in farmed fish (Purcell et al., 2013).

Altogether, these characteristics pointed out medusa polyp as excellent candidates for biofouling assays.

In the quest for new antifouling substances from natural sources, seaweeds are strong candidates for antifouling compounds prospecting. In fact, studies have shown that seaweed surfaces present a selective microbiota, mainly constituted by species that are important for the seaweed development and defense, through mutualistic associations (Dahms and Dobretsov, 2017; Hollants et al., 2013). Therefore, a selective antifouling strategy is believed to exist behind this evolutionary process, which can be related with the production of bioactive secondary metabolites. Within this framework, in this chapter, *Asparagopsis armata* and *Sargassum muticum*, will be evaluated through different *in vitro* and *in vivo* methodologies on microfouling organisms, namely through the analysis of aquatic bacteria growth inhibition, biofilm formation inhibition, microalgae growth inhibition and acetylcholinesterase inhibitory activity, which are key steps for biofouling formation and development (chapter 4A). In addition, a new *in vivo* method on macrofouling organisms was developed, which was based on the use of Medusae polyp. Due to the novelty of this *in vivo* antifouling methodology it was decided to dedicate one exclusive sub-chapter to this issue (4B) (Figure 4.3).

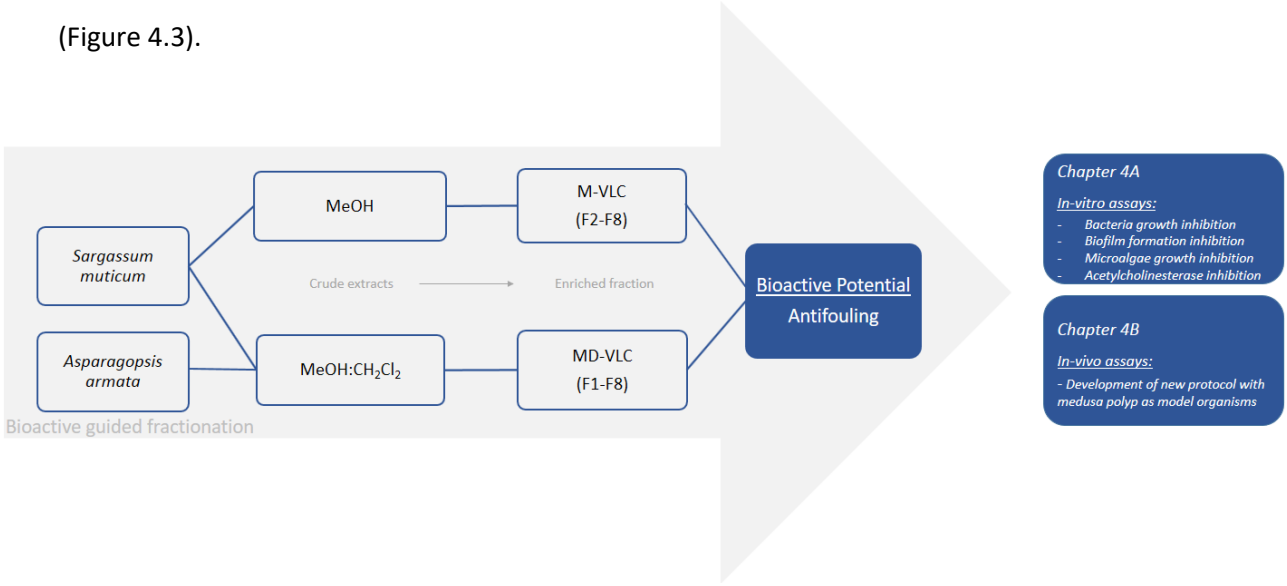

Figure 4.3. Overview of chapter 4 design experiments. MeOH – methanol; CH_2Cl_2 – dichloromethane; M-VLC – vacuum liquid chromatography of the methanolic extract; MD-VLC – vacuum liquid chromatography of the methanol:dichloromethane extract.

Chapter 4A

The antifouling potential of the marine invaders *Asparagopsis armata* and *Sargassum muticum* – targeting primary biofouling

A.1 – *Asparagopsis armata* and *Sargassum muticum* – an antifouling survey

Microfouling is the first step of biofouling phenomena, essential for the recruitment of benthic organisms by providing substrata for larval adherence. Thus, in the quest for new antifouling substances, targeting the first steps of biofouling (primary biofouling) may result in effective antifouling solutions.

Although with recognized antifouling properties, the available antifouling coatings are known to induce negative impacts in aquatic ecosystems. Therefore, greener alternatives are urgently required. Marine organisms are prone to biofouling and thus, developed strategies to defend themselves against undesirable species, which include the production of bioactive substances with antifouling properties.

Within this framework, the marine invasive seaweeds *Sargassum muticum* and *Asparagopsis armata* were addressed for antifouling compounds biodiscovery.

4A.2. Material and methods

4A.2.1. Microorganisms and culture media

The microorganisms *Edwardsiella tarda* (DSM-30052), *Aeromonas aquariorum* (DSM-17689), *Aeromonas hydrophila* (DSM-30016), *Photobacterium damselae* ssp. *piscicida* (DSM-22834), and *Vibrio anguillarum* (DSM- 21597) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). The biofilm producer's *Vibrio parahaemolyticus* and *Bacillus subtilis* were obtained from the marine biobank from MARE-IPLeiria, Peniche, Portugal. Microalgae, *Tetraselmis suecica, Tetraselmis chui, Thalassiosira weissflogii, Chaetoceros calcitrans*, *Cylindrotheca closterium, Nannochloropsis oculata, Phaeodactylum tricornutum, Isocrysis galbana* and *Nannochloropsis gaditana,* were obtained from Cetemares microalgae culture labs, Peniche, Portugal. Trypticase soy broth (TSB), Guillard's (F/2) Marine Water Enrichment Solution and NaCl were obtained from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany).

4A.2.2. Chemicals and reagents

Reagents and solvents of analytical grade were obtained from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany).

4A.2.3. Aquatic bacteria growth inhibitory potential

The growth inhibitory potential of *S. muticum* and *A. armata* VLC fractions was tested on five microorganisms, namely *E. tarda, A. hydrophila* (mainly freshwater), *A. aquariorum* (fresh-water), *P. damselae* ssp. *piscicida* and *V. anguillarum* (marine).

Marine organisms were grown in TSB with 1.5% NaCl, and the others in normal TSB in the presence/absence of extracts (1 mg/mL) at 28 °C. Negative controls were conducted with DMSO. Microbial growth was verified by optical density (600 nm) in a microplate reader (Synergy H1 Multi-Mode Microplate Reader, BioTek® Instruments, Winooski, USA). The growth inhibitory capacity was determined as percentage of growth relative to control. For extracts presenting an inhibitory capacity >50% at 1 mg/mL, a dose response analysis (1000-10 µg/mL) was conducted to determine the IC_{50} (concentration which inhibits 50% microorganism's growth).

4A.2.4. Biofilm inhibitory potential

The biofilm formation inhibitory potential was evaluated according to O'Toole (2011) as follows: in a microplate 10 µL of previously grown bacteria , 88 µL of fresh medium and 2 µL of test sample or DMSO (control) was added followed by incubation at 28 °C for 72 h. Blanks were performed with culture medium and test sample without bacteria. The medium was then removed, and wells washed with water and left to dry. Following, 125 µL of a 0.1% solution of crystal violet was added and the plate incubated at room temperature for 15 min.

After this period, the crystal violet was discarded, the wells washed 3 times with water and left to dry at 37 °C. The crystal violet was then solubilized by adding 150 µL of 30% acetic acid, incubated for 15 min and transferred to a new microplate. The absorbance was then read at 550 nm and the results presented as percentage of control.

4A.2.5. Microalgae growth inhibitory potential

Microalgae, namely *Tetraselmis suecica, Tetraselmis chui, Thalassiosira weissflogii, Chaetoceros calcitrans*, *Cylindrotheca closterium, Nannochloropsis oculata, Phaeodactylum tricornutum, Isocrysis galbana* and *Nannochloropsis gaditana,* were cultured aseptically in filtered seawater enriched with f/2 medium (Guillard, 1975) without silica for non-diatom species, at 20 °C with aeration and a constant photoperiod of 16/8 h.

The evaluation of the growth inhibitory potential was performed by the disk diffusion method. Briefly, 1 mL of microalgae culture with approximately 1x 10^9 cells was seeded in Petri dishes containing f/2 medium with 1.5% agar. Sterile paper disks with 6 mm diameter were embedded with 10 µL of sample and transferred to seeded plates. DMSO and $CuSO_4$ were used as negative and

positive controls, respectively. The plates were maintained at 20 °C, on a constant photoperiod of 16/8 h for 10 days.

4A.2.6 Acetylcholinesterase inhibitory activity

The acetylcholinesterase (AChE) inhibitory activity was determined according to the method described by Sharififar and co-workers (2012) with minor modifications. Briefly, to 173 µL of phosphate buffer 200 mM, pH 7.7, was added 2 µL of seaweed samples, 10 µL of 5,50-dithio-bis(2-nitrobenzoic acid) (DTNB) (3.96 mg of DTNB and 1.5 mg sodium bicarbonate dissolved in 10 mL phosphate buffer, pH 7.7), and 5 µL of enzyme (2 U/mL). Blanks were conducted for all samples without the addition of the enzyme. The mixture was incubated at 25 °C for 5 min, added to 10 µL of acetylthiocholine iodide (ATCI) (ATCI: 10.85 mg in 5 mL of phosphate buffer) and incubated again for 10 min at 25 °C. The reaction was read at 412 nm (Synergy H1 Multi-Mode Microplate Reader, BioTek® Instruments, Winooski, VT, USA) and the results determined as follows:

$$Inhibition\ (\%) = \left[1 - \left(\frac{(Ac - Abc) - (As - Ab)}{(Ac - Abc)}\right) x\ 100\right]$$

Where *Ac* is the absorbance of the control; A*bc* is the absorbance relative to the blank of the control; *As* is the absorbance of the test sample and *Ab* is the absorbance of respective sample blank. Donepezil, an acetylcholinesterase inhibitor, was used as positive control.

4A.2.7. Statistical analysis

The IC_{50} concentration was calculated from nonlinear regression analysis using GraphPad Prism software by means of the equation: $Y = 100/(1 + 10^{(X-Log\ IC50)})$. All data were checked for normality and homoscedasticity using the Shapiro–Wilk and Levene's test, respectively. Comparisons concerning variables, which did not meet variance or distributional assumptions, were carried out with the Kruskal–Wallis nonparametric tests (Zar, 2010). The statistical comparisons among the groups were performed with the Newman–Keuls multiple comparison test (Zar, 2010). For all statistical tests, the significance level was set at p-value < 0.05, and results were expressed as mean ± standard error of the mean (SEM). All calculations were performed on GraphPad InStat v.5.1 (GraphPad Software, La Jolla, CA, USA).

4A.3. Results

As mentioned above, the biofouling process is a complex phenomenon involving different mechanisms and different organisms. The biofouling process is considered to have four main stages, the adsorption of organic material (conditioning film); the adherence of bacteria (bacterial film); the adherence of microalgae; and the last stage involving the settlement of invertebrate larvae and macroalgae (Figure 4.1). Accordingly, in this chapter the potential of *A. armata* and *S. muticum* extracts to interfere in the three last stages of the biofouling process was investigated:

- the capacity to inhibit the growth of aquatic bacteria (stage II)
- the capacity to inhibit the production of bacterial biofilms (stage II)
- the potential to inhibit microalgae growth (stage III)
- the potential to inhibit the settlement of macrofouling organisms by neurotransmission disruption (acetylcholinesterase activity inhibition) (stage IV).

4A.3.1. Evaluation of aquatic bacteria growth inhibitory potential (stage II).

Immersed structures bacterial colonization is fundamental in biofouling processes. Accordingly, in this chapter, it was evaluated the growth inhibitory potential of both, marine and freshwater bacterial species, namely *A. hydrophila*, *A. aquariorum* and *E. tarda* (mainly fresh environments) and *V. anguillarum* and *P. damselae* subsp. *piscicida* (mainly marine environments) to have an indication of the potential of both seaweeds' fractions and crude extracts as antifouling additives. Table 4.1 shows the capacity of *A. armata* to inhibit different aquatic microorganism's growth at 1 mg/mL.

Table 4.1. *Asparagopsis armata* crude extract and VLC fractions growth inhibitory potential against marine and fresh-water microorganisms (1mg/mL). The results are expressed as percentage of control (%) ± standard error of the mean (SEM) determined from three independent experiments.

Fractions	Microorganisms growth inhibition (% of control)				
(1 mg/mL)	*A. hydrophila*	*A. aquariorum*	*E. tarda*	*V. anguillarum*	*P. damselae*
F1	64.1 ± 1.6	39.2 ± 3.1	86.0 ± 8.3	100.0 ± 1.8	96.8 ± 1.1
F2	87.0 ± 5.7	100.0 ± 5.1	100.0 ± 8.4	100.0 ± 3.6	97.5 ± 1.0
F3	100.0 ± 5.0	100.0 ± 7.3	100.0 ± 6.6	100.0 ± 7.0	99.2 ± 0.8
F4	100.0 ± 3.8	100.0 ± 5.9	100.0 ± 7.5	100.0 ± 4.3	100.0 ± 5.8
F5	100.0 ± 5.5	100.0 ± 4.4	100.0 ± 6.5	100.0 ± 2.7	84.1 ± 4.0
F6	100.0 ± 2.6	77.1 ± 4.5	100.0 ± 2.9	100.0 ± 3.5	100.0 ± 1.9
F7	10.8 ± 1.5	13.5 ± 1.8	25.0 ± 0.5	39.5 ± 3.1	6.0 ± 1.5
F8	18.7 ± 1.1	12.0 ± 5.1	19.2 ± 0.6	31. ± 6.1	0.0 ± 1.4
crude	5.70 ± 0.1	23.52 ± 0.6	37.0 ± 3.4	35.1 ± 5.66	0.0 ± 4.1

Fractions F1 – F6 revealed a high inhibitory potential against all microorganisms, the majority of them totally blunting these microorganism's growth. Fractions F7, F8 and crude extract revealed the lowest potential against all microorganisms.

For the most active fractions (inhibition >50%, at 1 mg/mL) a dose-response analysis was conducted and the IC_{50} was determined (Table 4.2).

Table 4.2. *Asparagopsis armata* VLC fractions capacity to inhibit the growth of marine and fresh-water bacteria – dose-response analysis (IC_{50} µg/mL). The values in parentheses represent the confidence intervals for 95%.

Fractions	Microorganisms growth inhibition (IC_{50} µg/mL)				
	A. hydrophyla	*A. aquariorum*	*E. tarda*	*V. anguillarum*	*P. damselae*
F1	836.1 (681.6 – 1026)	>1000	414.1 (319.5–536.7)	41.1 (34.5-49.0)	18.9 (16.5-21.8)
F2	293.5 (213.9-402.8)	588.1 (332.6-1040)	152.7 (121.4-192.0)	19.6 (16.1-23.8)	14.5 (11.8-17.7)
F3	162.9 (119.6-222.0)	443.8 (282.5-697.1)	54.4 (45.0-65.8)	41.7 (25.4-68.6)	13.6 (9.3-19.7)
F4	191.9 (145.6-253.1)	437.0 (281.1-679.5)	75.1 (60.1-93.8)	60.4 (45.0-81.1)	20.0 (16.5-24.4)
F5	265.1 (201.2-349.4)	421.1 (282.3-628.1)	83.8 (54.2-129.4)	52.5 (31.8-86.8)	11.7 (9.8-14.1)
F6	379.0 (275.5-521.4)	872.4 (521.6-1459)	244.5 (192.6-310.6)	89.9 (57.4-140.7)	17.3 (14.5-20.8)
$CuSO_4$	467.8 (326.3-670.6)	31.12 (26.9-35.9)	185.4 (118.6-289.7)	172.9 (140.5-212.7)	414.3 (305.0-562.7)

Fraction F3 exhibited the highest potency against three microorganisms, namely, *P. damselae*, *E. tarda* and *A. hydrophyla* with IC_{50} of 13.6, 54.4, and 162.9 µg/mL, respectively.

The potential of *S. muticum* crude extract and VLC fractions to inhibit the growth of marine and fresh-water microorganisms is shown Table 4.3.

Table 4.3. *Sargassum muticum* crude extracts and VLC fractions capacity to inhibit the growth of marine and fresh-water bacteria (1 mg/mL). The results are expressed as percentage of control (%) ± standard error of the mean (SEM) determined from three independent experiments.

Fractions (1 mg/mL)	Microorganisms growth inhibition (% of control)				
	A. hydrophila	*A. aquariorum*	*E. tarda*	*V. anguillarum*	*P. damselae*
MF2	0.0 ± 0.7	5.9 ± 4.4	59.9 ± 0.8	100.0 ± 2.3	18.8 ± 0.6
MF3	32.6 ± 1.1	14.6 ± 0.8	53.7 ± 0.9	100.0 ± 2.2	29.5 ± 0.6
MF4	2.8 ± 2.5	7.67 ± 1.1	33.0 ± 3.1	75.7 ± 3.9	22.4 ± 1.5
MF5	22.5 ± 2.7	9.70 ± 0.8	50.1 ± 2.3	92.0 ± 2.0	28.6 ± 1.7
MF6	9.8 ± 2.2	2.56 ± 1.3	51.1 ± 0.8	73.5 ± 1.8	14.8 ± 0.6
MF7	0.0 ± 2.6	5.04 ± 2.1	41.9 ± 3.5	21.0 ± 5.5	23.9 ± 1.2
MF8	0.0 ± 0.8	0.00 ± 2.0	42.4 ± 5.2	45.4 ± 1.8	6.3 ± 1.9
Mcrude	0.0 ± 5.4	3.48 ± 1.1	38.4 ± 1.2	15.9 ± 2.1	5.3 ± 0.6
MDF1	29.2 ± 3.5	48.0 ± 8.8	96.5 ± 7.5	43.7 ± 4.0	14.6 ± 2.0
MDF2	38.2 ± 2.5	43.4 ± 2.6	60.0 ± 5.8	54.5 ± 6.6	13.3 ± 1.7
MDF3	49.4 ± 1.2	19.5 ± 1.3	37.1 ± 3.2	67.0 ± 2.9	13.2 ± 0.7
MDF4	58.8 ± 1.6	59.9 ± 7.4	60.7 ± 1.7	88.7 ± 2.2	19.1 ± 1.8
MDF5	47.4 ± 1.6	57.0 ± 2.3	100.0 ± 5.3	100.0 ± 2.7	25.2 ± 1.3
MDF6	52.7 ± 0.5	69.0 ± 2.1	99.7 ± 0.9	91.9 ± 4.3	34.0 ± 7.7
MDF7	1.9 ± 1.4	22.1 ± 3.9	43.8 ± 9.0	0.0 ± 3.6	7.7 ± 1.1
MDF8	10.3 ± 2.5	45.2 ± 11.8	49.7 ± 10.3	18.0 ± 7.0	9.2 ± 1.1
MDCrude	4.2 ± 1.7	15.8 ± 6.5	76.2 ± 2.6	24.1 ± 1.7	18.8 ± 1.7

*M – methanolic fraction; MD – methanol:dichloromethane fraction

Concerning the methanolic VLC (MVLC fractions), the highest potential was exhibited by fractions MF2 and MF3 against *V. anguillarum* with 100% inhibition, followed by fractions MF5, MF4, and MF6 with 92.0, 75.7, and 73.5% inhibition, respectively. MF2, MF3, MF6, and MF5 fractions also revealed potential against *E. tarda* growth with 59.9, 53.7, 51.5, and 50.1% inhibition, respectively. No relevant activity was registered against *A. hydrophila, A. aquariorum*, and *P. damselae*. Concerning MDVLC fractions, the highest inhibitory potential was also verified against *E. tarda* and *V. anguillarum* with fractions MDF5 and MDF6 totally inhibiting these microorganisms' growth. Fraction MDF1 also presented a high inhibitory activity against *E. tarda* inhibiting its growth by 96.5%. Against *A. hydrophila*, MDF4 scored the highest inhibition (58.8%) while against *A. aquariorum*, MDF6 was the most potent, with an inhibitory potential of 69.0%. No relevant activity was detected with the crude extracts, from both extractions, excepting with MDcrude against *E. tarda*, which revealed an inhibitory potential of 76.2%.

For the most potent fractions (inhibition >50% at 1 mg/mL), a dose-response analysis was conducted and the IC_{50} determined (Table 4.4).

Table 4.4. *Sargassum muticum* crude extracts and VLC fractions capacity to inhibit the growth of marine and fresh-water microorganisms – dose-response analysis (IC_{50} µg/mL). The values in parentheses represent the confidence intervals for 95%.

Fractions	Microorganisms growth inhibition (IC_{50} µg/mL)			
	A. hydrophila	*A. aquariorum*	*E. tarda*	*V. anguillarum*
MF2	>1000	>1000	347.1 (278.6-431.9)	176.3 (141.9-219.0)
MF3	>1000	>1000	>1000	165.4 (118.5-231.0)
MF4	>1000	>1000	>1000	418.8 (329.6-532.1)
MF5	>1000	>1000	>1000	170.5 (128.5-226.1)
MF6	>1000	>1000	>1000	396.2 (341.7-459.4)
MDF1	>1000	>1000	315.5 (211.1-471.5)	>1000
MDF2	>1000	>1000	405.3 (318.3-516.1)	509.2 (336.7-707.0)
MDF3	>1000	>1000	>1000	141.9 (111.9-179.9)
MDF4	309.7 (251.7-308.9)	661.9 (541.2-809.5)	570.4 (474.8-685.4)	54.5 (45.1-65.8)
MDF5	>1000	1011 (876.7-1165)	115.9 (83.0-161.9)	97.8 (84.9-112.6)
MDF6	543.3 (455.5-648.1)	813.7 (653.7-1013)	162.9 (110.7-212.1)	197.0 (165.9-234.0)
MDF7	>1000	>1000	>1000	>1000
MDCrude	>1000	>1000	233.0 (162.0-334.9)	>1000
$CuSO_4$	467.8 (236.3-670.6)	31.1 (26.9-35.9)	185.4 (118.6-289.7)	172.9 (140.5-212.7)

The MVLC fractions were the most potent against *V. anguillarum*, with the lowest IC_{50} exhibited by fractions MF3, MF5, and MF1 with 165.4, 170.5, and 176.3 µg/mL, respectively, followed by fractions MF6 and MF4. Against *E. tarda,* fraction MF2 revealed the highest potential with an IC_{50} of 347.1 µg/mL.

Concerning the MDVLC fractions, fraction MDF4 revealed the highest potency against *V. anguillarum* with an IC_{50} of 54.5 µg/mL, followed by fraction MDF5 with an IC_{50} of 97.8 µg/mL. This fraction was also the most potent against *E. tarda*, with an IC_{50} of 115.9 µg/mL. Concerning *A. hydrophila* two fractions are worthy of note, namely MDF4 and MDF6 with IC_{50} of 309.7 and 543.3 µg/mL, respectively. Against *A. aquariorum*, fraction MDF4 was the most potent with IC_{50} of 664.9 µg/mL, followed by MDF6 and MDF5 with IC_{50} of 813.7 and 1011 µg/mL, respectively.

4A.3.2. Evaluation of bacteria biofilm formation inhibitory potential (stage II)

As mentioned above, bacterial biofilm formation is an essential step on biofouling processes. Therefore, in this chapter, the biofilm formation of marine bacteria *Vibrio parahaemolyticus* and *Bacillus subtilis* was evaluated in the presence of both seaweed fractions and crude extracts. The results are shown in Figures 4.4 and 4.5 for *Asparagopsis armata* and 4.6, 4.7 (methanolic extraction), 4.8 and 4.9 (methanol:dichloromethane extraction) for *Sargassum muticum*.

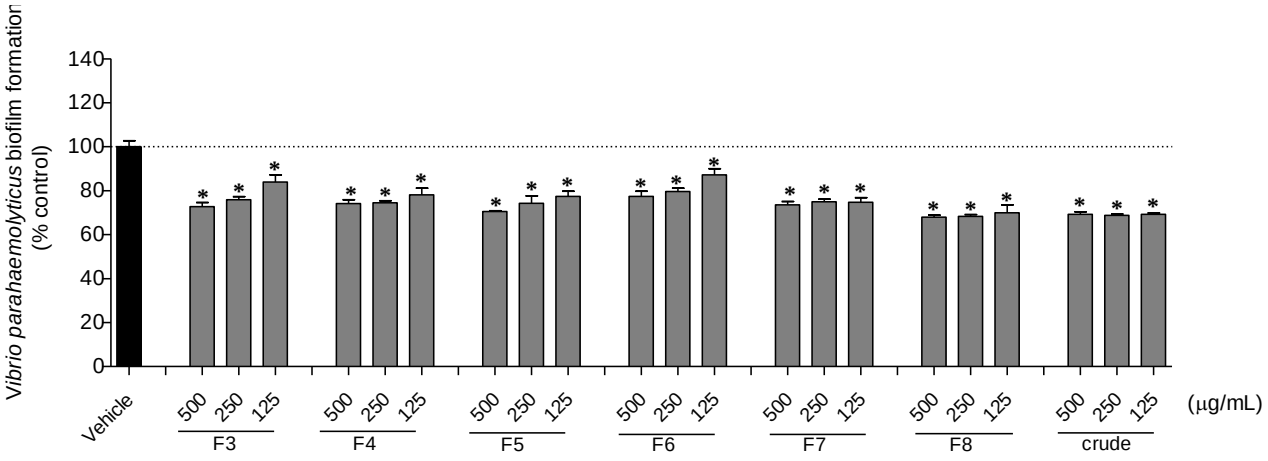

Figure 4.4. *Asparagopsis armata* crude extract and VLC fractions capacity to inhibit *Vibrio parahaemolyticus* biofilm production. The columns represent the mean ± standard error of the mean (SEM) from three independent experiments. Symbols (*) represent statistically significant differences (ANOVA, Dunett's test, $p < 0.05$) when compared to control.

Excepting fractions F1 and F2, all *A. armata* fractions, and the crude extract, presented capacity to inhibit *V. parahaemolyticus* biofilm formation (Figure 4.4). The inhibitory activity ranged between 20 and 30% excepting for F6 at 125 μg/mL, which inhibited 13%.

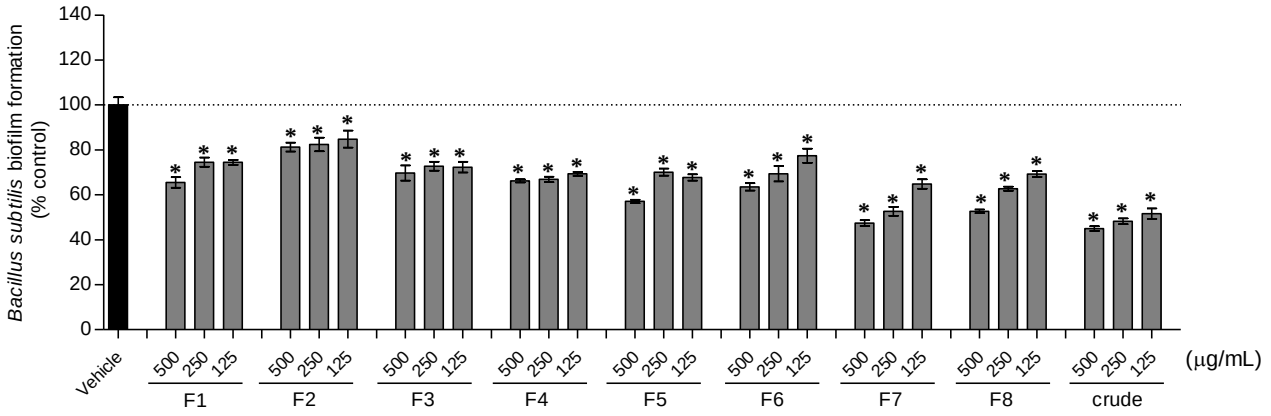

Figure 4.5. *Asparagopsis armata* crude extract and VLC fractions capacity to inhibit *Bacillus subtilis* biofilm production. The columns represent the mean ± standard error of the mean (SEM) from three independent experiments. Symbols (*) represent statistically significant differences (ANOVA, Dunett's test, $p < 0.05$) when compared to control.

All *A. armata* fractions, and the crude extract, presented capacity to inhibit *B. subtilis* biofilm formation (Figure 4.5). The highest inhibitory activity was exhibited by the crude extract ranging between 49 and 36% inhibition.

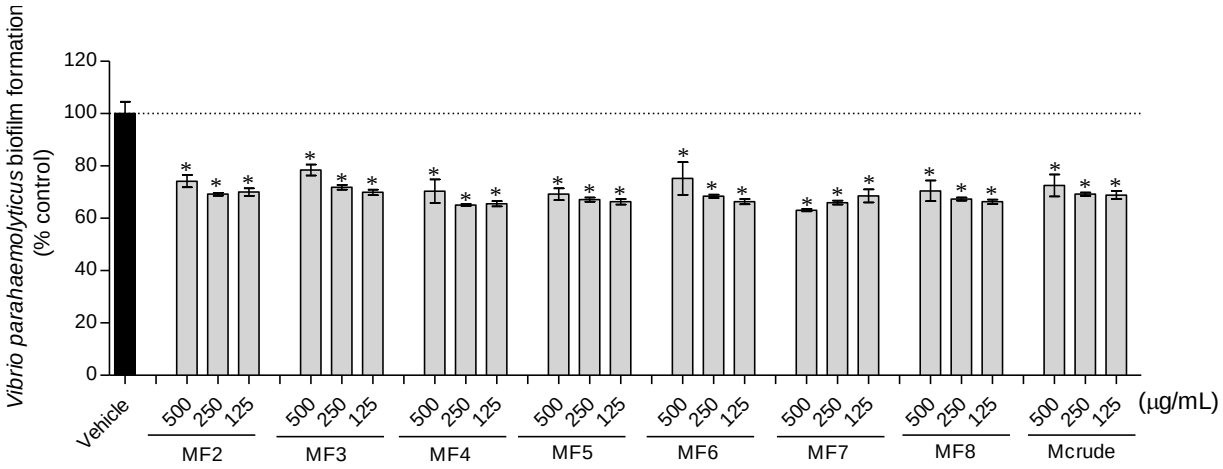

Figure 4.6. *Sargassum muticum* crude methanol extract and VLC fractions capacity to inhibit *Vibrio parahaemolyticus* biofilm production. The columns represent the mean ± standard error of the mean (SEM) from three independent experiments. Symbols (*) represent statistically significant differences (ANOVA, Dunett's test, $p < 0.05$) when compared to control.

The biofilm inhibitory activity of *S. muticum* (methanolic extraction) on *V. parahaemolyticus* ranged between 22 and 37% (Figure 4.6).

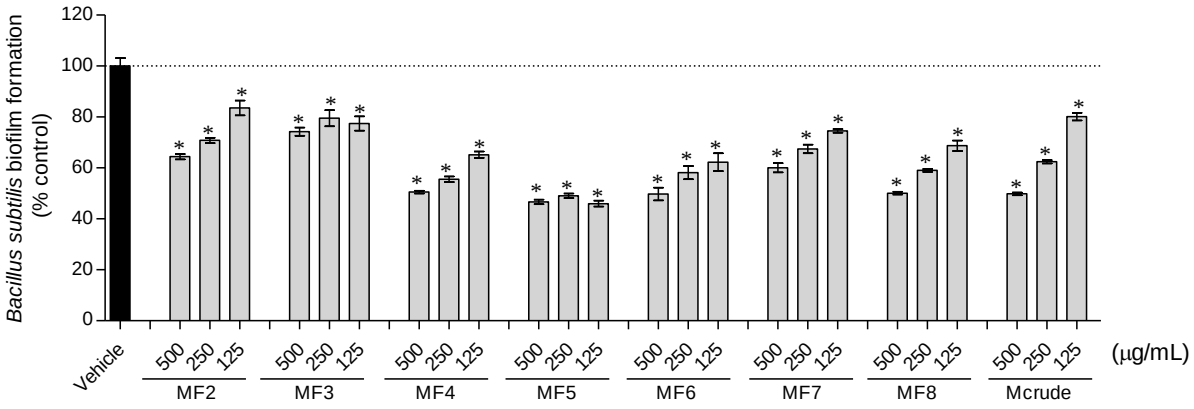

Figure 4.7. *Sargassum muticum* crude methanol extract and VLC fractions capacity to inhibit *Bacillus subtilis* biofilm production. The columns represent the mean ± standard error of the mean (SEM) from three independent experiments. Symbols (*) represent statistically significant differences (ANOVA, Dunett's test, $p < 0.05$) when compared to control.

The biofilm inhibitory activity of *S. muticum* (methanolic extraction) on *B. subtilis* is shown on Figure 4.7. All the fractions, excepting MF1 exhibited inhibitory activity ranging between 20 and 57%, with the MF5 fraction revealing the highest potential (50-57% biofilm inhibition).

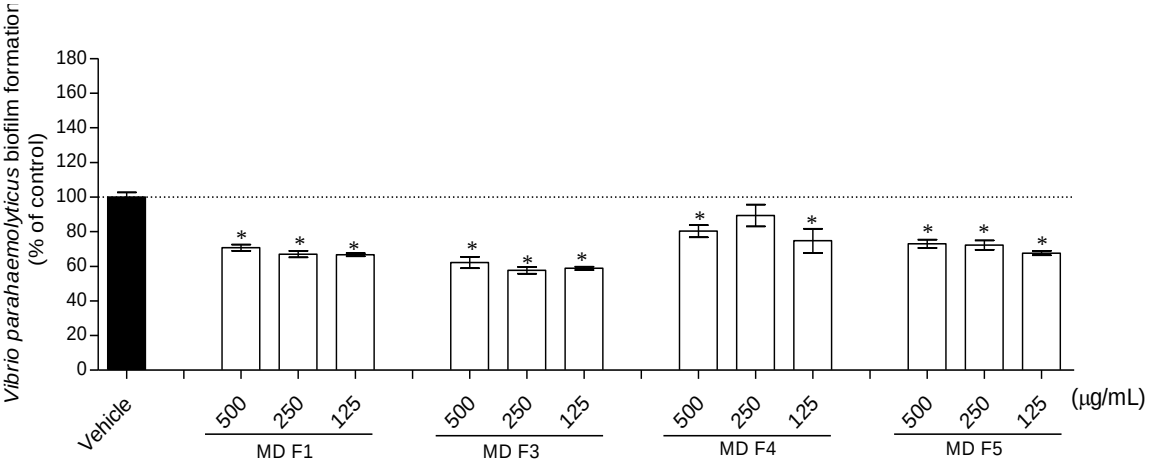

Figure 4.8. *Sargassum muticum* crude methanol:dichloromethane extract and VLC fractions capacity to inhibit *Vibrio parahaemolyticus* biofilm production. The columns represent the mean ± standard error of the mean (SEM) from three independent experiments. Symbols (*) represent statistically significant differences (ANOVA, Dunett's test, $p < 0.05$) when compared to control.

In Figure 4.8 is shown the *V. parahaemolyticus* biofilm production in the presence of *S. muticum* fractions and crude extract (methanol:dichloromethane extraction). Fractions MDF2, MDF6-MDF8 and the crude extract produced no effects on *V. parahaemolyticus* biofilm development. Fractions MDF1, MDF3 and MDF5 produced no effects at 1000 μg/mL, however when decreasing the concentrations, the biofilm formation was inhibited ranging 30-40%.

The *B. subtilis* biofilm production in the presence of *S. muticum* MDVLC fractions and crude extract is shown in Figure 4.9.

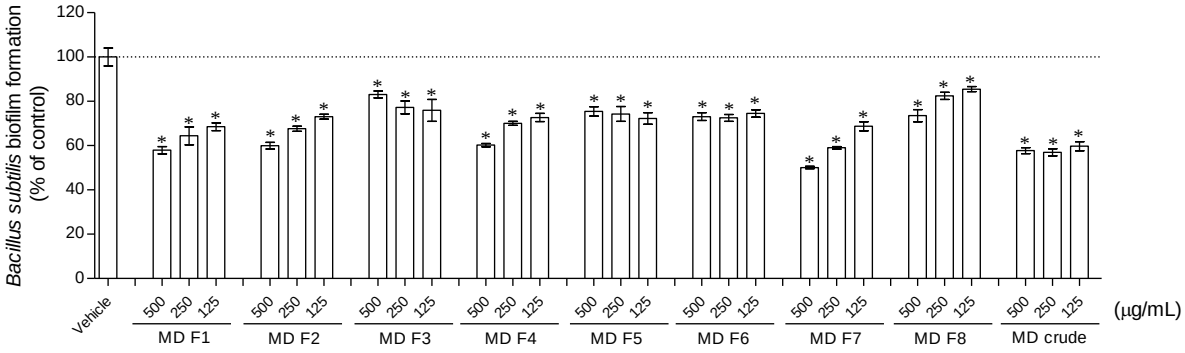

Figure 4.9. *Sargassum muticum* crude methanol:dichloromethane extract and VLC fractions capacity to inhibit *Bacillus subtilis* biofilm production. The columns represent the mean ± standard error of the mean (SEM) from three independent experiments. Symbols (*) represent statistically significant differences (ANOVA, Dunett's test, $p < 0.05$) when compared to control.

All the fractions revealed to contain compounds with capacity to inhibit biofilm formation, being the crude extract the most potent exhibiting 40% inhibition at 125 mg/mL.

4A.3.3. Evaluation of microalgae growth inhibitory potential (stage III)

Following bacterial colonization, microalgae populations are the next organisms to settle, stablishing a complex network with pre-existing organisms, contributing for the following organisms to settle, by producing organic material and changing surface matrices. Accordingly, in this chapter the potential of both seaweeds fractions and crude extracts to inhibit the growth of common microalgae, including diatoms was evaluated and the results are presented in Table 4.5.

Table 4.5. *Asparagopsis armata* crude extract and VLC fractions potential to inhibit marine microalgae growth. Numbers correspond to inhibition zone in millimeters, including the diameter of the paper disk (6 mm); mean value of at least two independent experiment ± SEM.

Fractions (1 mg/disk)	Microalgae growth inhibition (non Diatoms)					Diatoms growth inhibition			
	T. suecica	*N. occulata*	*T. chui*	*I. galbana*	*N. gaditana*	*C. closterium*	*C. calcitrans*	*T. weissflogi*	*P. tricornutum*
F1	19.5 ± 1.2	13.0 ± 0.6	15.3 ± 0.3	10.0 ± 0.8	9.7 ± 1.2	16.7 ± 0.7	15.5 ± 0.4	8.5 ± 0.1	13.3 ± 1.4
F2	24.0 ± 0.0	18.0 ± 0.6	28.3 ± 1.2	19.0 ± 0.8	14.7 ± 1.4	28.7 ± 0.7	14.0 ± 0.0	7.0 ± 0.0	25.7 ± 0.9
F3	28.0 ± 0.0	23.0 ± 1.2	32.3 ± 0.3	16.5 ± 2.0	14.0 ± 0.6	30.7 ± 0.7	11.5 ± 0.4	9.5 ± 0.4	31.3 ± 0.3
F4	24.5 ± 0.4	14.3 ± 1.2	20.3 ± 0.3	12.5 ± 0.4	11.7 ± 0.3	29.3 ± 0.7	12.5 ± 0.4	10.5 ± 0.4	23.3 ± 1.2
F5	22.8 ± 0.6	17.0 ± 0.3	20.3 ± 0.3	21.0 ± 0.8	11.3 ± 0.3	19.0 ± 1.6	26.0 ± 1.0	27.3 ± 1.4	19.7 ± 0.9
F6	19.0 ± 0.0	13.3 ± 0.3	13.0 ± 0.0	17.5 ± 0.4	13.3 ± 0.7	20.0 ± 1.2	20.7 ± 0.7	31.7 ± 1.7	20.0 ± 1.6
F7	0.0 ± 0.0	7.7 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	9.0 ± 0.6	11.3 ± 0.9	19.7 ± 0.9	12.0 ± 0.0	8.0 ± 0.8
F8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	10.7 ± 0.9	16.3 ± 0.9	12.0 ± 1.0	0.0 ± 0.0
Crude	10.0 ± 0.0	12.3 ± 0.3	14.3 ± 0.7	13.3 ± 0.3	14.0 ± 0.0	16.6 ± 0.9	15.7 ± 0.7	11.3 ± 0.7	19.3 ± 0.3

*DMSO was used as negative control (0.0 ± 0.0 mm for all microorganisms) and $CuSO_4$ (1 mg/disk) as positive (> 30 mm, for all studied microorganisms).

Generally, fractions F1-F6 exhibited the higher inhibitory activity against all species, nevertheless fraction F3 should be highlighted since it exhibited the highest inhibitory potential against five species, including two diatoms, namely *T. suecica, N. occulata, T. chui* and the diatoms *C. closterium* and *P. tricornutum,* with 28.0, 23.0, 32.3, 30.7, and 31.3 mm inhibition, respectively. The crude extract also revealed high potential to inhibit all the studied microorganisms with the inhibitory halos ranging from 10 mm (against *T. suecia*) to 19.3 mm (against *P. tricornutum*).

Table 4.6 shows the microalgae growth inhibitory potential of *S. muticum* crude extracts and VLC fractions.

Table 4.6. *Sargassum muticum* crude extracts and VLC fractions potential to inhibit marine microalgae growth. Numbers correspond to inhibition zone in millimeters, including the diameter of the paper disk (6 mm); mean value of at least two independent experiments ± SEM.

Fractions (1 mg/disk)	Microalgae growth inhibition (non Diatoms)					Diatoms growth inhibition			
	T. suecica	*N. occulata*	*T. chui*	*I. galbana*	*N. gaditana*	*C. closterium*	*C. calcitrans*	*T. weiss*	*P. tricornutum*
MF2	0.0 ± 0.0	0.0 ± 0.0	8.3 ± 0.0	17.0 ± 0.8	0.0 ± 0.0	8.0 ± 0.1	19.7 ± 0.9	0.0 ± 0.0	10.5 ± 0.4
MF3	16.0 ± 0.8	0.0 ± 0.0	8.3 ± 0.4	9.0 ± 0.4	0.0 ± 0.0	9.5 ± 0.1	14.0 ± 0.6	0.0 ± 0.0	12.5 ± 0.4
MF4	10.5 ± 0.4	0.0 ± 0.0	8.3 ± 0.4	7.3 ± 0.4	0.0 ± 0.0	9 ± 0.1	13.7 ± 0.7	0.0 ± 0.0	7.5 ± 0.1
MF5	11.5 ± 0.4	0.0 ± 0.0	10.7 ± 0.3	8.0 ± 0.4	0.0 ± 0.0	12.5 ± 0.4	10.3 ± 0.3	11.5 ± 0.4	12.5 ± 0.4
MF6	9.0 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	9.0 ± 0.1	0.0 ± 0.0	13.5 ± 0.4	10.5 ± 0.4
MF7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	7.0 ± 0.0
MF8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
MCrude	12.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	12.7 ± 0.7	0.0 ± 0.0	13.6 ± 0.4
MDF1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	9.5 ± 0.4	21.7 ± 0.3	0.0 ± 0.0	0.0 ± 0.0
MDF2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	9.5 ± 0.4	19.7 ± 0.3	0.0 ± 0.0	8.0 ± 0.1
MDF3	8.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	8.5 ± 0.4	16.7 ± 0.3	0.0 ± 0.0	8.0 ± 0.1
MDF4	10.5 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	11.5 ± 0.4	12.7 ± 0.3	0.0 ± 0.0	9.0 ± 0.0
MDF5	16.5 ± 0.4	9.0 ± 0.6	14.7 ± 0.0	9.5 ± 0.4	18.5 ± 1.2	21.0 ± 0.8	28.7 ± 0.3	12.5 ± 0.4	0.0 ± 0.0
MDF6	8.0 ± 0.4	0.0 ± 0.0	9.3 ± 1.2	0.0 ± 0.0	11.5 ± 0.4	17.0 ± 0.8	17.7 ± 0.3	0.0 ± 0.0	19.0 ± 0.8
MDF7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	8.5 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	12.5 ± 0.4
MDF8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
MDCrude	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	14.3 ± 0.7	0.0 ± 0.0	10.0 ± 0.6

*DMSO was used as negative control (0.0 ± 0.0 mm for all microorganisms) and $CuSO_4$ (1 mg/disk) as positive (> 30 mm, for all studied microorganisms).

Concerning the MVLC fractions, the highest inhibition was shown by MF2 fraction against *C. calcitrans* and *I. galbana with* 19.7 and 17.0 mm of inhibition halo, respectively, followed by fraction MF3 against *T. suecica* with 16.0 mm. Less inhibitory activity was recorded against the remaining species with MF6 fraction revealing the larger inhibition halos against *T. Weiss* (13.5 mm), and MF5 against *C. closterium* (12.5 mm) and *T. chui* (10.7 mm). The crude extract also revealed inhibitory capacity against *P. tricornutum* (13.6 mm), *C. calcitrans* (12.7 mm) and *T. suecica* (12.0 mm). There was no inhibitory activity against *N. occulata* and *N. gaditana.*

Regarding the MDVLC fractions, MDF5 stood out by revealing the highest inhibitory activity against 8 of the 9 studied species, with the halo's sizes varying between 9.0 mm (against *N. occulata*) and 28.7 mm (against *C. calcitrans*).

A4.3.4. Acetylcholinesterase inhibitory activity (stage IV)

Although not completely understood, it is known that specific cues are essential for invertebrate larvae to metamorphose and settle in a specific substratum. These cues (settlement signals) will trigger a cascade of signalling pathways in which are involved specific neurotransmitters, such as the enzyme acetylcholinesterase (AChE). This enzyme is responsible for modulating motor and cognitive functions, playing a fundamental role in the settlement sessile crustaceans such as barnacles. Accordingly, substances with capacity to inhibit AChE at low concentrations, are very promising antifouling compounds (Almeida et al., 2015; Chen and Qian, 2017).

In this chapter, the potential of both seaweeds fractions and crude extracts to inhibit AChE activity was evaluated, and the results are presented in Figure 4.10 and Table 4.9.

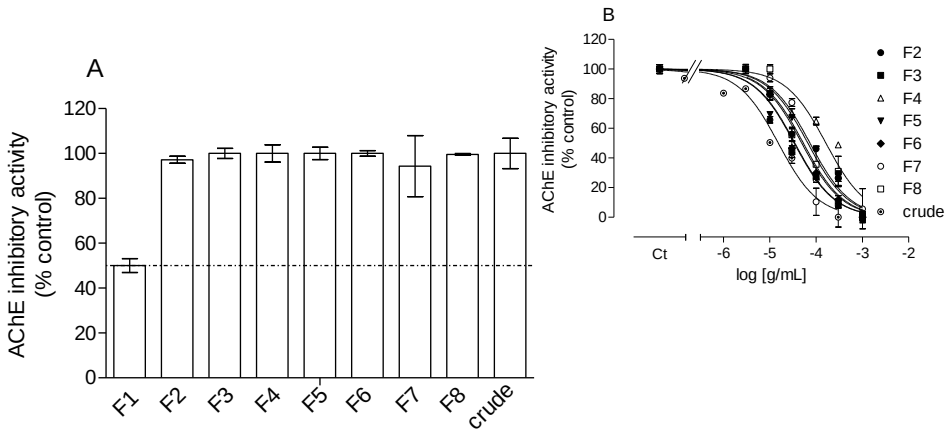

Figure 4.10. Acetylcholinesterase inhibitory activity by *Asparagopsis armata* fractions and crude extract (1 mg/mL) - (A) and respective dose-response analysis (1000-10 µg/mL) - (B). The columns represent the mean ± standard error of the mean (SEM) from three independent experiments. Horizontal line indicates 50% AChE inhibition.

According to Figure 4.10 it is possible to verify that *A. armata* fractions and crude extract have a high potential to inhibit AChE activity, with all fractions, excepting F1, totally inhibiting this enzyme activity (Figure 4.10 - A). On the other hand, none of the fractions or crude extracts obtained from *Sargassum muticum* inhibited AChE activity. For the most potent fractions (inhibition > 50%), a dose response analysis was conducted (Figure 4.10 – B) and the IC_{50} determined. The results are presented in Table 4.7.

Table 4.7. Acetylcholinesterase inhibitory activity by *A. armata* fractions and crude extract - IC_{50} determination. The values in parentheses represent the confidence intervals for 95%.

VLC fractions	IC_{50} µg/mL
F2	52.93 (39.58-70.77)
F3	33.62 (26.94-41.96)
F4	162.6 (118.00-224.10)
F5	73.67 (58.34-93.03)
F6	34.77 (28.12-43.00)
F7	48.90 (30.76-77.74)
F8	66.22 (43.85-99.99)
Crude extract	15.24 (11.09-19.86)
Donepezil	0.15 (0.11-0.19)

All *A. armata* fractions presented a high potential to inhibit AChE activity, with the crude extract showing the highest inhibitory potential (IC_{50} of 15.24 µg/mL) and fraction F4 the lowest (IC_{50} of 162.6 µg/mL).

4A.4. Discussion

Biofouling is part of a successful evolutionary process by foulant species, allowing them to survive and prosper in different environments, also increasing their resistance to antifouling chemicals. Biofouling processes involves complex mechanisms, and can be divided into microfouling - biofilm formation and microorganism adhesion and macrofouling - attachment of larger organisms phenomena. Antifouling paints are, therefore, used to prevent attachment of both micro and macroorganisms. The first organisms to settle in the biofouling process are bacteria and microalgae. These organisms are essential in biofouling expansion processes since they produce biofilms that are indispensable for the following invertebrate species to settle (Lehaitre et al., 2008).

Due to the increasing need to find new eco-friendly options for antifouling coatings, in the last decades marine organisms have been studied for their ability to produce compounds with antifouling properties. Owing to the competitive environment where they live in, sessile species developed strategies to overcome the fouling by foulant species. In fact, it has been reported that marine seaweeds contain less macro and microepibionts on their thalli compared to biofilms on inanimate substrata (Pérez et al., 2016). This can be linked with the production of secondary metabolites and explained as a defense mechanisms that prevent bacterial attachment and growth of undesired planktonic bacterial colonizers (El Bour et al., 2013), and often, the screenings for new antifouling substances, targets marine microorganisms, including bacteria and microalgae (Pinteus et al., 2018).

In this work the antifouling properties of *A. armata* and *S. muticum* crude extracts and fractions were evaluated in three of the four biofouling stages: the bacterial growth and biofilm formation inhibitory effects on aquatic bacteria; the growth inhibitory effects on marine microalgae (including diatoms); and the inhibition of acetylcholinesterase, a key enzyme in neurotransmission processes essential for settlement.

4A.4.1. Microorganisms growth inhibition

In a first approach, the microorganism's growth inhibitory activity was determined at 1 mg/mL of seaweed extract. It is possible to verify that *A. armata* exhibit the greatest inhibitory effects revealing capacity to inhibit all the studied microorganism's growth. Through dose-response analysis and IC_{50} determination it was possible to verify that fractions F2-F6 strongly inhibited all microorganisms' growth, especially *E. tarda* and *V. anguillarum*. *Vibrio anguillarum* and *Photobacterium damselae* are microorganisms widely present in marine environments, while *Aeromonas hydrophila, Aeromonas aquariorum* and *Edwarsiella tarda* are mainly found in fresh water environments. These results point out the relevance of *A. armata* as a source of natural biocide with broad spectrum activity, with potential to be used as an additive to coatings for marine and freshwater structures. Accordingly, Paul and co-workers (2006) showed that two compounds produced by *A. armata*, bromoform and dibromoacetic acid, have important antifouling properties, decreasing the bacterial density on the seaweed surface. On the other hand, Bansemir and co-workers (2006) and Pinteus and co-workers (2015) also disclosed the potential of *A. armata* crude dichloromethane extracts to inhibit the growth of several marine microorganisms, including *Vibrio anguillarum*, *Pseudomonas anguilliseptica*, *Aeromonas salmonicida*, *Aeromonas hydrophylla*, among others.

Regarding *S. muticum*, it is possible to verify that both extractions afforded compounds with antibacterial activity, especially against *E. tarda* and *V. anguillarum*. Nevertheless, through dose-response analysis it was observed that the MDF4, MDF5 and MDF6 fractions have potential to inhibit all the studied microorganisms, excepting *P. damselae*, therefore revealing to be more promising for further use as additives in antifoulant coatings. In agreement with these results, *S. muticum* was previously studied for its potential to inhibit the growth of several marine microorganisms, revealing interesting activity against *Cobetia marina*, *Polaribacter irgensii*, *Serratia marcescens*, *Shewanella putrefaciens* and different *Vibrio* species (Tanniou et al., 2014; Plouguerné et al., 2008; Plouguerné et al., 2010; Hellio et al., 2000). These works strengthen the idea of *S.*

muticum as source of antimicrobial substances with potential to be included in antifouling formulations.

4A.4.2. Biofilm formation inhibitory potential

Biofilms are complex matrices of polymeric substances that provide structure for bacteria to agglomerate and attach to different structures (Singh et al., 2017), being considered an essential step for the establishment of a fouling community.

Not all bacterial species can produce biofilms. Within the biofilm producers, *Vibrio* sp. and *Bacillus* sp. are some of the principal species found in fouled surfaces (Mielich-Süss and Lopez, 2015; Yuan et al., 2007; Santhakumari et al., 2017; Dhanasekaran et al., 2009; Revilla-Castellanos et al., 2015) being therefore selected for anti-biofilm assays.

The results herein obtained show that both seaweeds exhibited anti-biofilm effects on *V. parahaemolyticus.* Nevertheless, the effects were more marked on *B. subtilis*, specially by *S. muticum* MF5 fraction, the MDF7 fraction, and the crude extract obtained from *A. armata*. Despite, the lack of studies concerning the anti-biofilm properties of both species, other works with the same genus support the idea that *Sargassum* and *Asparagopsis* species produce anti-biofilm compounds. Accordingly, Prabhakaran and co-workers (2012) verified anti-biofilm properties on *Sargassum weii*. Also, Jha and co-workers (2013) verified that extracts obtained from *Asparagopsis taxiformis* inhibited quorum-sensing of biofilm producing bacteria, which is an essential mechanism for bacterial biofilms development.

Although not much information exists related directly with anti-biofilm properties of compounds produced by *S. muticum*, other studies concerning the antifouling properties of *S. muticum* can be found in literature. Bazes and co-workers (2009) developed an *in situ* investigation and verified that a surface coated with paint containing a dichloromethane extract of *S. muticum* revealed less fouling organisms, compared with paints containing only copper, after 2 months of immersion. The antifouling effects verified by these authors may be the result of anti-biofilm properties of the tested extract.

The mechanisms behind bacterial biofilm development are not fully understood, nevertheless, it is widely accepted that biofilm formation is a strategy that allows bacteria to survive in hostile environments. In bacterial colonization and biofilm development *quorum sensing* plays a key role by coordinating bacterial interactions through signaling molecules. As a result, seaweed extract may act as signals for *quorum sensing* activation leading to bacterial biofilms development (e.g. induction of attachment and triggering exopolysaccharides production, etc.) (Spoering and

Gilmore, 2006). In the presence of a stressor, bacteria may become dormant without growing until optimal conditions; on the other hand, if challenged with a non-bactericidal substance, bacteria may get opportunistic and rely on the new features for biofilm development. Studies have shown that some extracts may coat the surface of the culture container providing a better chemical environment, improving bacterial colonization and thus increasing the biofilm formation (Merritt et al., 2006). These adaptive strategies may be behind the increase on the biofilm formation observed for some extracts at higher concentrations. These effects were previously observed with plant extracts on *Pseudomonas aeruginosa* biofilms (Plyuta et al., 2013).

4A.4.3. Microalgae growth inhibition

The third step of the biofouling process is the settlement of microalgae. Therefore, 9 different microalgae, including 4 diatoms species, which are the most abundant group of unicellular protists in all aquatic environments, being presumed to be the major foulers of immerged surfaces (Molino and Wetherbee, 2008), were used in anti-algal assays.

The results show that *A. armata* was more effective than *S. muticum* inhibiting the studied microalgae. In fact, the Bonnesmaisoniae family have already shown to produce compounds with capacity to interfere with microbial communities that can be related with their ability to produce an array of volatile compounds. In the specific case of *A. armata,* this is a strong possibility, since this species is known to produce over 100 compounds including halogenated compounds such as bromoform, bromines, chlorine, iodinated methanes, ethanes, ethanols, acetaldehydes, acetones, 2-acetoxypropanes, propenes, epoxypropanes, acroleins, butenones, and several halogenated acetic and acrylic acids (Burreson et al., 1976; McConnell and Fenical, 1977; Woolard et al., 1976, 1979) which can be involved in antifouling activities (De Nys et al., 1995; Scardino and de Nys, 2011). On the other hand, previous studies showed that S*. muticum* also produces compounds with capacity to inhibit the growth of microalgae, including diatoms species (Hellio et al., 2002; Hellio et al., 2004; Silkina et al., 2012; Plouguerné et al., 2010), nevertheless the extraction strategy was different, which may justify the lower inhibitory activity shown in the present work.

Results showed strong inhibitory activities on microalgae growth, including against diatom species, especially by *A. armata* fractions and crude extract. These results are very relevant since during biofilm development, diatoms are considered as one of the most important contributors, being estimated that diatoms microfouling itself can increase fuel consumption by up to 18%, reducing navigability in more than 20% (Lewin, 1984). In addition, substances produced by these microorganisms promote the corrosion of immerged metallic materials (Rittschof, 1999).

Accordingly, the high microalgae growth inhibitory ability exhibited by *A. armata* fractions and crude extract suggests a great potential of this seaweed to be used as source of antifouling compounds.

4A.4.4. Acetylcholinesterase inhibitory activity

Due to the biocide effects of most of commercially available antifouling compounds, studies have focused in exploring the molecular strategies behind barnacle and mussels' larvae metamorphosis and settlement mechanisms. Therefore, instead of killing the target organisms, molecular-targeting antifouling compounds may act by inhibiting an essential metabolic via for setting and cementing, resulting in a more environmentally friendly solution.

The settlement of invertebrate organisms is a result of a cascade signaling pathways in which cholinesterases (ChEs) play a fundamental role. The cholinergic system, responsible for sensorial functions and motor modulation in synaptic neurons, is mostly regulated by the neurotransmitter acetylcholine, which is fundamental for mussels and barnacles' larvae to undergo metamorphosis and settle (Almeida et al., 2015; Faimali et al., 2003). Accordingly, several studies suggest that acetylcholinesterase exist in significant amounts in the cement gland of barnacle cyprids, and its complete inhibition results in the total inhibition of larval settlement, pointing out the importance of this neurotransmitter in the modulation of settlement and adhesion of these organisms (Almeida et al., 2015). In agreement, *S. muticum* and *A. armata* fractions and extracts were tested for their ability to inhibit AChE. While *S. muticum* did not reveal inhibitory activity, *A. armata* was able to strongly inhibit this enzyme, revealing therefore, potential to inhibit the adherence of invertebrates' larvae. Although not much information exists on the potential of seaweeds to prevent biofouling through ChEs inhibition, several natural compounds were already tested for their ChE inhibitory potential. A natural sponge-derived compound (poly-APS) was able to inhibit the settlement of barnacle's larvae via AchE inhibition (Turk et al., 2007). Also, Almeida and co-workers (2015) showed that several cyanobacteria extracts exhibited a strong AChE inhibition which could be related with the anti-settlement effects verified *in vivo* with the mussel *Mytilus galloprovinciallis*. Altogether, these results point out the importance of ChEs inhibitors as excellent tools for antifouling tests, with the marine invasive *A. amata* crude extract reveling a strong anti-AChE activity, and therefore potential to be incorporated in antifouling coating formulations, with less environmental impacts.

Chapter 4B

Medusa polyps adherence inhibition:

a novel model to test anti-adherence properties of antifouling formulations or compounds

4B.1. New antifouling assays – rational strategy

Although in the last decades significant advances have been made to improve antifouling formulations, the main current options continue to be highly toxic to marine environment, leading to an urgent need for new safer alternatives. For anti-adherence studies, barnacles and mussels are commonly the first choice for experimental purposes. However, the use of these organisms involves a series of laborious and time-consuming stages.

In the present work, a new approach for testing antifouling formulations was developed under know formulations and novel proposed options.

Due to their high resilience, ability of surviving in hostile environments and high abundance in different ecosystems, medusa polyps present themselves as prospect candidates for antifouling protocols. Within Medusae species, *Aurelia aurita* and *Phyllorhiza punctata* are successful marine invaders. Due to their high abundance and resilience (Graham and Bayha, 2007), these species were selected to be evaluated as new *in vivo* models for anti-adherence studies.

Thus, a complete protocol to test antifouling formulations using Medusa polyp is presented, while the antifouling properties of two invasive seaweeds, *Asparagopsis armata* and *Sargassum muticum* were evaluated within this new test model framework.

4B.2. Materials and Methods

4B.2.1. Chemicals and reagents

The chemicals tributyltin (TBT), 2-methylthio-4-tertiary-butylamino-6-cyclopropylamino-s-triazine (irgarol), 1-(3,4-dichlorophenyl)-3,3-dimethylurea (diuron), and copper sulfate ($CuSO_4$) were obtained from Sigma (Sigma-Aldrich GmbH, Steinheim, Germany). For all the assays, TBT, irgarol, and diuron were dissolved in dimethyl sulfoxide (DMSO) and $CuSO_4$ in water. A commercial antifouling ink, “Titan Special Pesqueros” (TITAN, Spain) was obtained in a local supply shop.

4B.2.2. Medusa Polyps maintenance

Aurelia aurita polyps and *Phyllorhiza punctata* were obtained from the Aquaculture Laboratory from CETEMARES building of the Polytechnic of Leiria, Peniche, Portugal. Both species were maintained in filtered (0.45 µm) natural seawater, 33-35‰ salinity at 20 °C, under artificial light conditions (14:10 L:D), weak aeration and fed daily with newly hatched *Artemia salina* nauplii.

4B.2.3. Experimental design

A first experiment was conducted to understand the ability of the polyps to adhere consistently to a gelatin base matrix. Medusa polyps were randomly collected from three different aquariums and placed on 24-well plates (seven per well) containing 200 µL of cooled agarose (1%) bottom and 1 mL of filtered (0.45 µm) and sterilized seawater (30-35‰ salinity) (Figure 4.11). The entire procedure was conducted in a laboratory with controlled temperature of 20 ± 1 °C, under artificial light conditions (14:10 L:D). After 10 days exposition time, the number of dead polyps and the non-adherent live polyps were observed in a magnifying glass for further evaluation. Polyps were considered dead when non-responsive to touch. After this assessment, the test plate was inverted to remove the water and unattached polyps and observed under a magnifying glass for live adherent polyps verification.

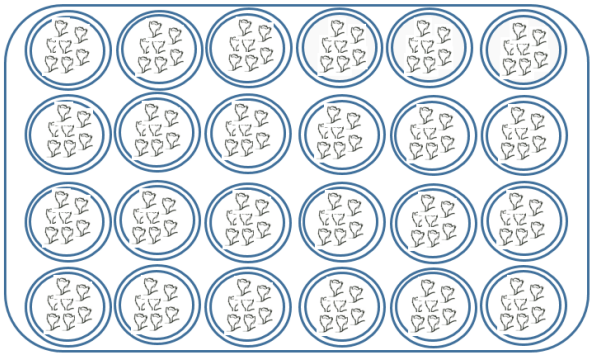

Figure 4.11. Schematic design of anti-adherence tests conducted on 24 well-plate with medusa polyp.

4B.2.4. Anti-adherence assays

To select the different concentrations to be tested, the commercial antifoulants (positive controls) TBT, diuron, irgarol, and $CuSO_4$ were tested for range-finding in a ten-fold dilution, to verify the minimal concentration which induced anti-adherence effects on live organisms (Nagata, 2008).

The anti-adherence tests were conducted by adding 10 µL of commercial antifoulants or seaweed extracts to a 24 well-plate well followed by the addition of 190 µL of agarose (1%, 40 °C) which was immediately shaken vigorously. After cooling, randomly collected polyps were transferred to each well (five to seven per well) and 1 mL of filtered (0.45 µm) and sterilized seawater (30-35‰ salinity) was added. DMSO (10 µL) instead of the samples was used as negative control. The antifoulants ink was swabbed in the bottom of the well prior to agarose addition.

The plates were maintained under artificial light (14:10 L:D) at 20 °C ± 1 °C, and the number of dead polyps and non-adherent live polyps was registered after 10 days. During the experiments the

polyps were kept in starvation. In all experiments only non-strobilating polyps (scyphistoma) were used (Fig. 4.12).

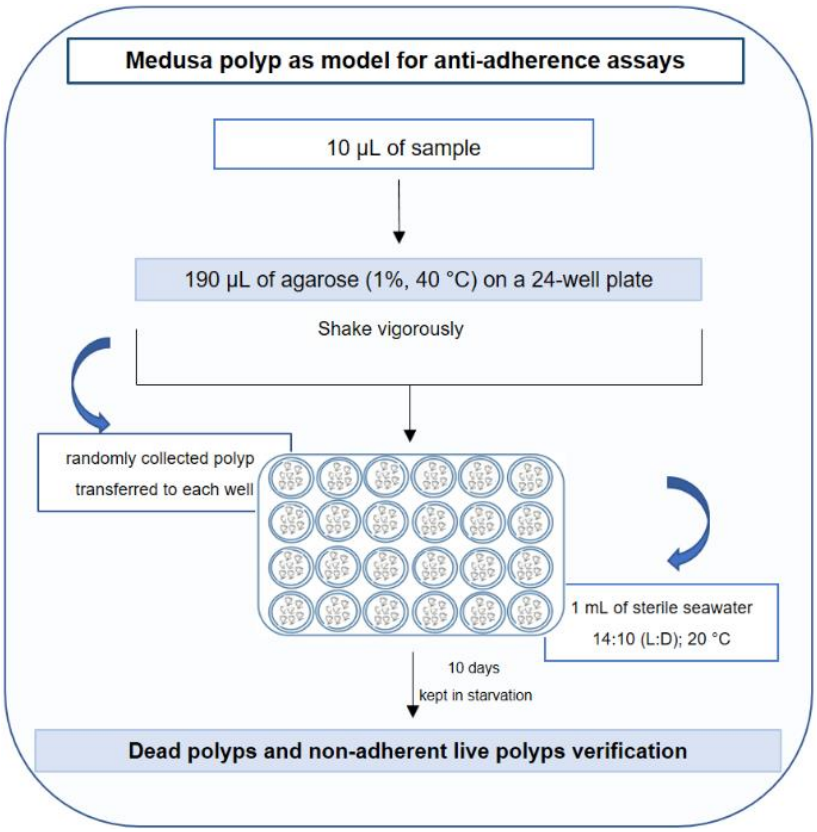

Figure 4.12. Medusa polyp as model for anti-adherence assays - protocol flowchart.

4B.2.5. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison of group means to determine significant differences relatively to control. The Tukey's test was applied for the remaining multiple comparisons. All data were checked for normality and homoscedasticity. Results are presented as mean ± standard error of the mean (SEM). Differences were considered statistically significant at level of 0.05 ($p< 0.05$). All experiments were conducted in triplicate. Calculations were performed using IBM SPSS Statistics 26 version 6.0 (IBM Corporation, Armonk, NY, USA) and GraphPad v5.1 (GraphPad Software, La Jolla, CA, USA) softwares (Zar, 2010).

4B.3. Results

In the first experiment to assess the suitability of the polyps to be applied in this method, all the polyps adhered to the gelatin based matrix in the 24-well plates. Those were further exposed to commercial antifoulants and seaweeds extracts. The results are organized by target organism.

4B.3.1. *Aurelia aurita* polyp as model to test antifouling formulations.

The behaviour of *Aurelia aurita* polyp when exposed to known antifouling chemicals is shown in Figure 4.13.

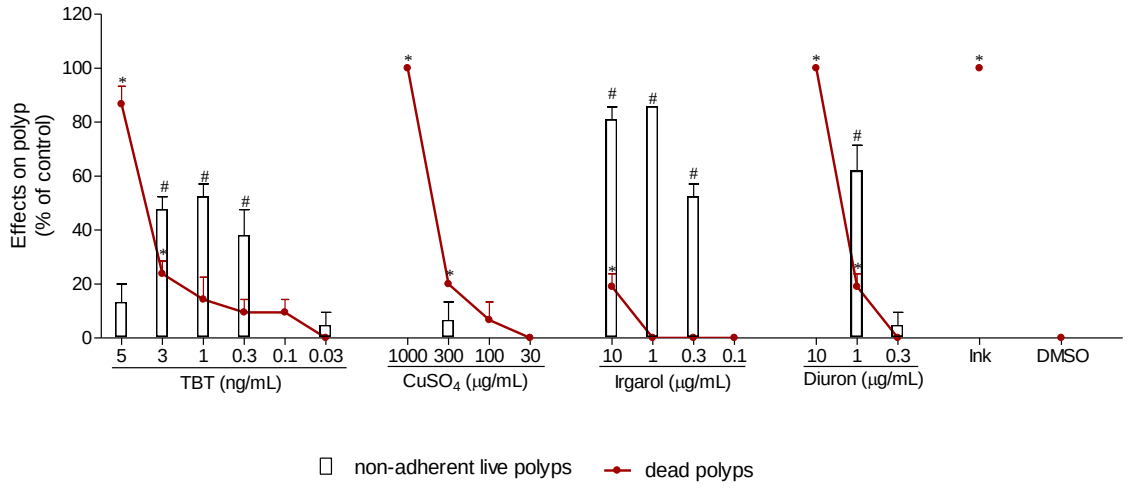

Figure 4.13. *Aurelia aurita* polyp's adherence inhibition by known antifouling compounds. Each column and each line represent the mean ± standard error of the mean of three independent experiments. Symbols represent statistically significant differences (p<0.05) when comparing non-adherent polyps (#) and dead polyps (*) to control situation (DMSO).

Antifouling ink was the most toxic substance inducing 100% death. TBT also induced significant toxicity promoting ≈ 85% polyp's death at 5 ng/mL, followed by diuron which revealed high toxicity at 10 µg/mL, inducing 100% death. Irgarol revealed high anti-adherence properties with no associated deaths at 1 and 0.3 µg/mL. DMSO produced no effects on the polyps.

4B.3.1.1. Evaluation of anti-adherence properties of *Asparagopsis armata* and *Sargassum muticum* on *Aurelia aurita* polyp

The results of the exposure of *A. aurita* polyp to seaweeds *A. armata* and *S. muticum* extracts are shown in Figures 4.14, 4.15 and 4.16.

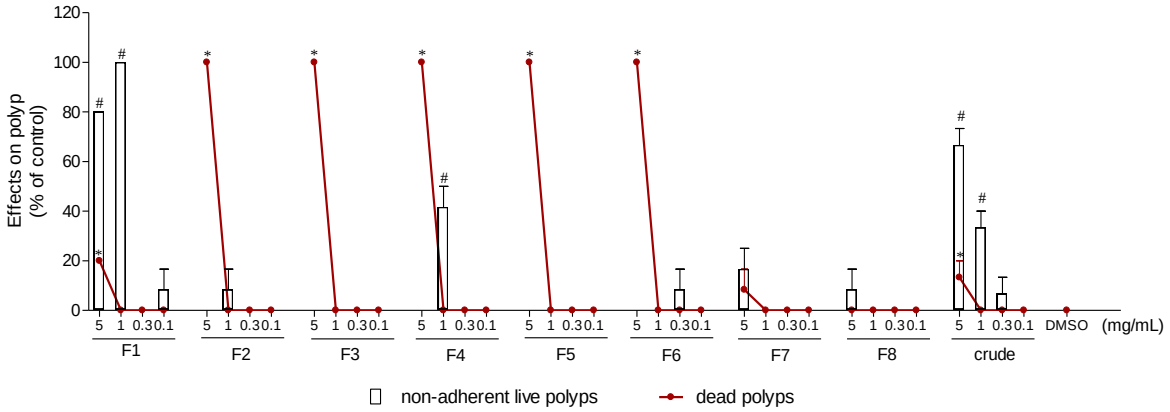

Figure 4.14. *Aurelia aurita* polyp's adherence inhibition by *Asparagopsis armata* crude and VLC fractions. Each column and each line represent the mean ± standard error of the mean of three independent experiments. Symbols represent statistically significant differences (p<0.05) when comparing non-adherent polyps (#) and dead polyps (*) to control situation (DMSO).

A. armata fractions F2-F6 revealed high toxicity at 5 mg/mL, inducing 100% deaths. When decreasing the concentration, these fractions did not exhibit any effects, excepting F4 fraction that revealed anti-adherence properties at 1 mg/mL (≈42%). Fraction F1 and crude extract revealed anti-adherence properties with low associated toxicity at 5 and 1 mg/mL (Fig. 4.14).

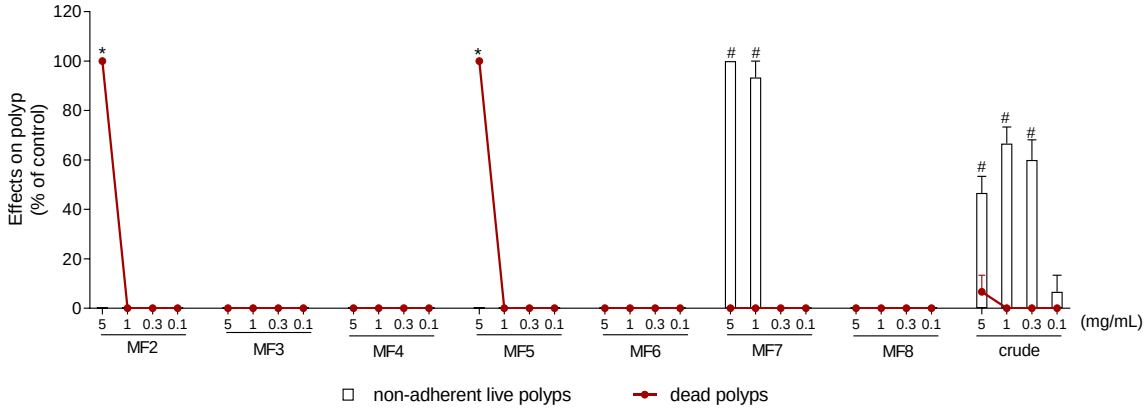

Figure 4.15. *Aurelia aurita* polyp's adherence inhibition by *Sargassum muticum* (methanolic extraction) crude and VLC fractions. Each column and each line represent the mean ± standard error of the mean of three independent experiments. Symbols represent statistically significant

differences (p<0.05) when comparing non-adherent polyps (#) and dead polyps (*) to control situation (DMSO).

S. muticum methanolic fractions MF3, MF4, MF6 and MF8 did not produced any effects on *A. aurita* polyps. Fractions MF2 and MF5 induced high toxicity promoting 100 % polyp's death at 5 mg/mL, and no effects at the other tested concentrations. Fraction MF7 and the crude extract revealed high anti-adherence properties. Fraction MF7 shown ≈100 % anti-adherence effects at 5 and 1 mg/mL, with no associated toxicity. The crude extract exhibited relevant anti-adherence effects between 5 and 0.3 mg/mL (47, 67 and 60% un-adherent polyps, respectively) with no associated deaths (Fig. 4.15).

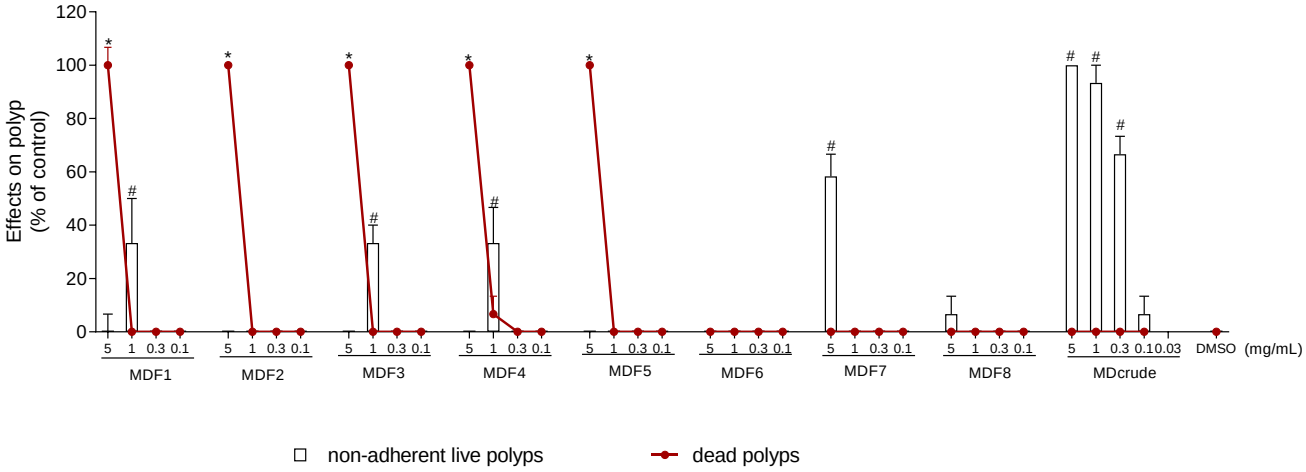

Figure 4.16. *Aurelia aurita* polyp's adherence inhibition by *Sargassum muticum* (methanol:dichloromethane extraction) crude and VLC fractions. Each column cand each line represent the mean ± standard error of the mean of three independent experiments. Symbols represent statistically significant differences (p<0.05) when comparing non-adherent polyps (#) and dead polyps (*) to control situation (DMSO).

S. muticum crude (methanol:dichloromethane) extract presented relevant anti-adherence properties with no toxicity. This extract totally inhibited polyp adherence at 5 and 1 mg/mL, inhibiting 93% at 0.3 mg/mL, and 67% at 0.1 mg/mL. Fraction MDF7 also revealed strong anti-adherence properties at 5 mg/mL (58% un-adherent polyp) however with no effects whatsoever at the other tested concentrations. Fractions MDF1 – F5 revealed high toxicity at 5 mg/mL (100% deaths) loosing toxicity at the other tested concentrations. Fractions MDF1, MDF3, and MDF4

revealed similar anti-adherence properties at 1 mg/mL (≈ 33%), with no effects at the lower tested concentrations (Fig. 4.16).

4B.3.2. *Phyllorhiza punctata* polyp as model to test antifouling formulations.

The behaviour of *P. punctata* polyps when exposed to known antifouling chemicals is shown in Figure 4.17.

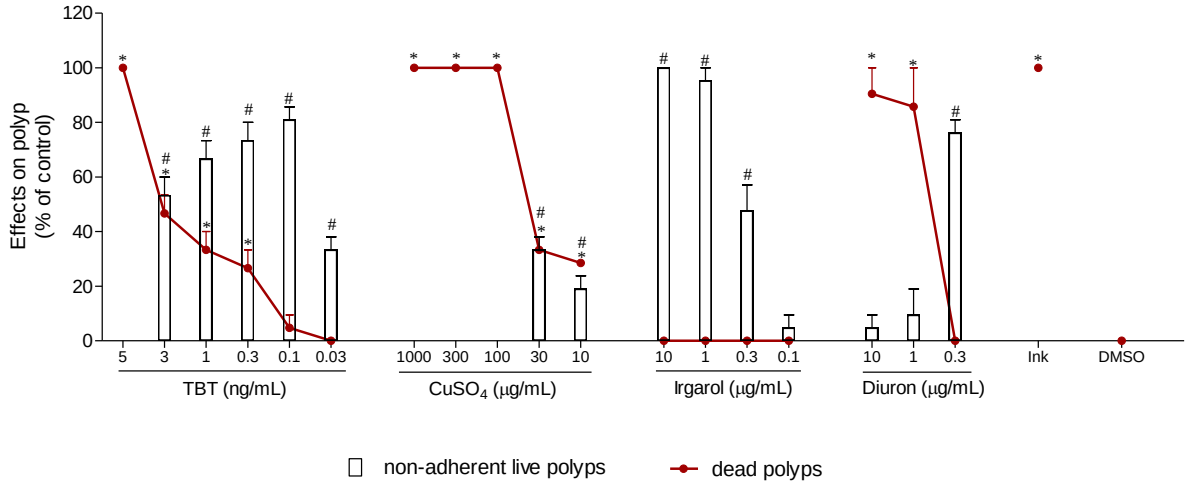

Figure 4.17. *Phyllorhiza punctata* polyp's adherence inhibition by known antifouling compounds. Each column and each line represent the mean ± standard error of the mean of three independent experiments. Symbols represent statistically significant differences (p<0.05) when comparing non-adherent polyps (#) and dead polyps (*) to control situation (DMSO).

All the antifouling chemicals revealed toxic effects on *P. punctata* excepting irgarol, which revealed anti-adherence properties with 100, 95, 48, and 5% adherence inhibition for the concentrations of 10, 1, 0.3, and 0.1 µg/mL, respectively, with no associated deaths (Figure 6). TBT induced high toxicity resulting in 100, 47, 33, and 26% deaths, at 5, 3, 1, and 0.3 ng/mL, respectively, revealing to be the most toxic compound for this species. Nevertheless, at 0.1 ng/mL, TBT revealed no toxicity and great anti-adherence properties (≈ 80% non-adherent live polyp). $CuSO_4$ revealed high toxicity at 1000, 300, and 100 µg/mL, although, at 30 µg/mL, $CuSO_4$ inhibited polyp adherence in about 30% also resulting in 30% deaths. Diuron exhibited maximum toxicity at 10 and 1 µg/mL (≈ 90 and 85% deaths, respectively). At 0.3 µg/mL, this chemical inhibited polyps' adherence in about 76% with no

associated deaths. The tested ink induced 100% deaths and DMSO produced no effects on the polyps (Figure 4.17).

4B.3.2.1 Evaluation of anti-adherence properties of *Asparagopsis armata* and *Sargassum muticum* on *Phyllorhisa punctata* polyp

The results of the exposure of *P. punctata* polyp to *A. armata* and *S. muticum* extracts are shown in Figures 4.18, 4.19 and 4.20.

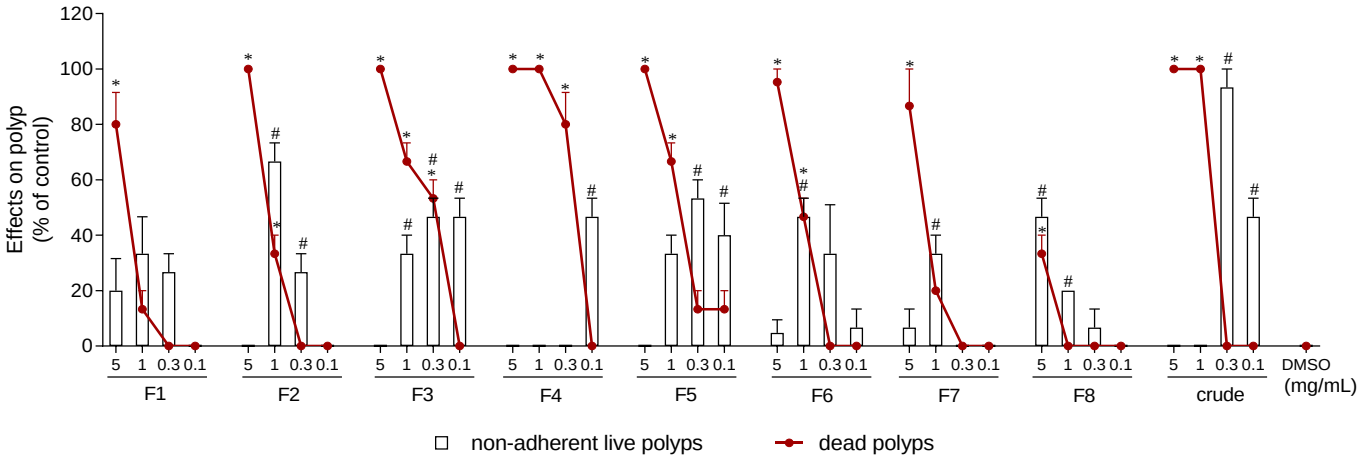

Figure 4.18. *Phyllorhiza punctata* polyp's adherence inhibition by *Asparagopsis armata* crude and VLC fractions. Each column and each line represent the mean ± standard error of the mean of three independent experiments. Symbols represent statistically significant differences (p<0.05) when comparing non-adherent polyps (#) and dead polyps (*) to control situation (DMSO).

Data gathered in Figure 4.18 shows that at the highest concentration (5 mg/mL), *A. armata* samples induced high toxicity resulting in 100% polyps death when exposed to F2-F5 fractions, followed by fractions F6-F8 which induced 95, 86 and 33% deaths, respectively. Fraction F4 and crude extract induced 100% deaths at 5 and 1 mg/mL, nevertheless, at 0.3 mg/mL the crude extract presented high anti-adherence properties (93%) with no associated deaths, whereas at the same concentration, fraction F4 induced 80% polyp death, revealing, therefore, to be the most toxic fraction.

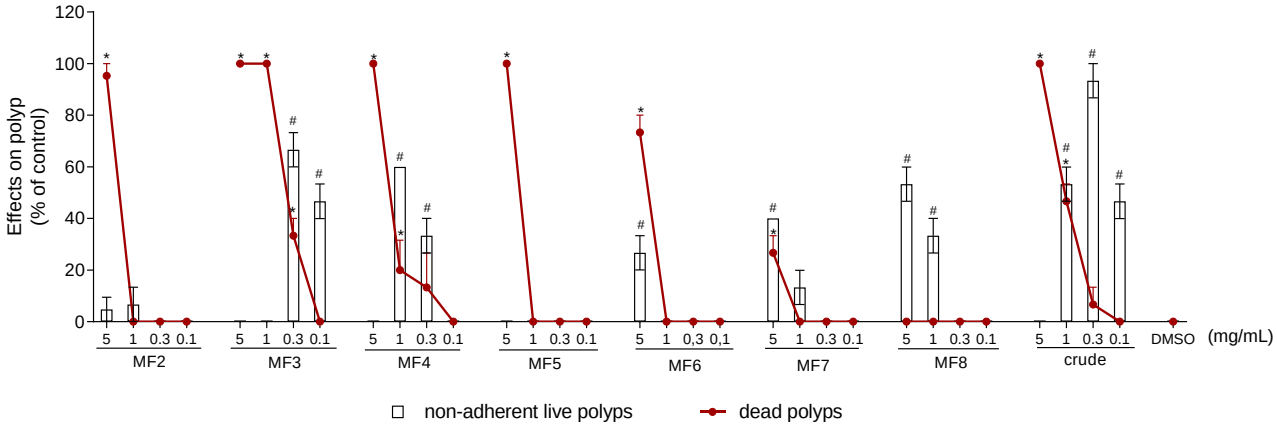

Figure 4.19. *Phyllorhiza punctata* polyp's adherence inhibition by *Sargassum muticum* (methanolic extraction) crude and VLC fractions. Each column and each line represent the mean ± standard error of the mean of three independent experiments. Symbols represent statistically significant differences (p<0.05) when comparing non-adherent polyps (#) and dead polyps (*) to control situation (DMSO).

Data gathered in Figure 4.19 show that the methanolic fractions MF2-MF5 and crude extract of *S. muticum* induced high toxicity at 5mg/mL, resulting in 100% deaths. Nevertheless, fraction MF3 seems to be the most toxic since at 1 mg/mL also induced 100% deaths.

At 5 mg/mL, fractions MF6 and MF7 induced 73 and 26% deaths, respectively. Fraction MF8 did not exhibited toxicity at all the tested concentrations yet showed anti-adherence properties at 5 and 1 mg/mL (53 and 33% un-adherent polyps, respectively). Fractions MF3 and the crude extract presented the highest anti-adherence properties at 0.3 mg/mL resulting in 67 and 93% live un-adherent polyps, respectively, however at 0.3 mg/mL, MF3 induced 33% deaths while crude extract presented only 6%. At 0.1 mg/mL, both samples presented 47% anti-adherence effects with no associated deaths. Fraction MF4 also showed anti-adherent properties at 1 and 0.3 mg/mL (60 and 33%, respectively) inducing 20 and 13% deaths, respectively.

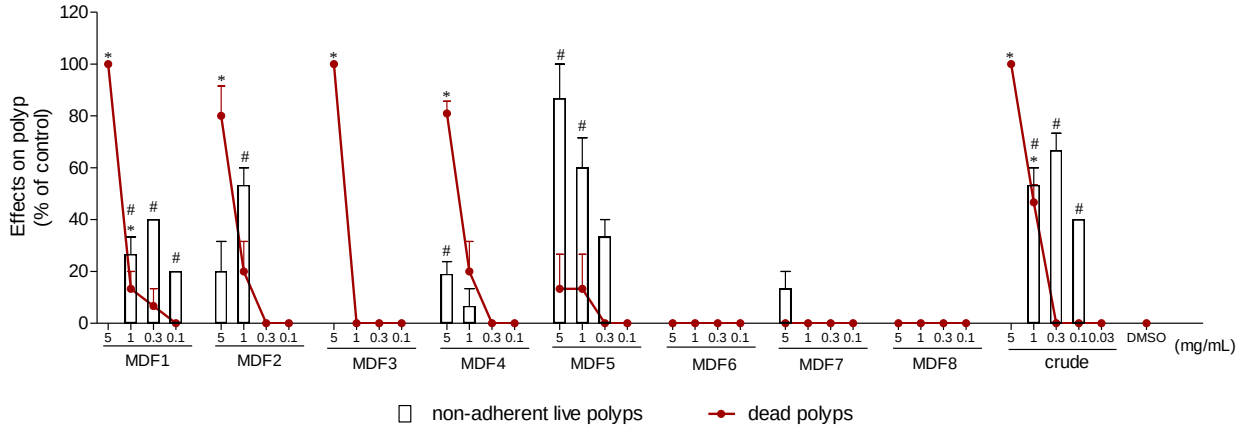

Figure 4.20. *Phyllorhiza punctata* polyp's adherence inhibition by *Sargassum muticum* (methanol:dichloromethane extraction) crude and VLC fractions. Each column and each line represent the mean ± standard error of the mean of three independent experiments. Symbols represent statistically significant differences (p<0.05) when comparing non-adherent polyps (#) and dead polyps (*) to control situation (DMSO).

Data gathered in Figure 4.20 reveal that the most promising fractions of *S. muticum* for further antifouling purposes are fractions MDF5 and the crude extract. Fraction MDF5 presented low toxicity with high anti-adherence properties at 5, 1, and 0.3 mg/mL with 87, 60, and 33% anti-adherence activity, respectively, exhibiting 13% toxicity at 5 and 1 mg/mL and no toxicity at 0.3 mg/mL. The crude extract revealed high anti-adherence properties at 1 mg/mL (53%) however inducing 47% deaths; nevertheless, this extract showed strong anti-adherence properties at 0.3 and 0.1 mg/mL, with no associated toxicity (67 and 40% un-adherent polyps). Fractions MDF6-F8 revealed no toxicity and no anti-adherence properties. Fractions F1-F4 induced 100% deaths at 5 mg/mL. At 1 mg/mL fractions MDF1 and MDF2 decreased substantially their toxicity (13 and 20%, respectively), presenting anti-adherence properties, namely 27 and 53%, respectively.

The effects of chemicals and seaweed extracts and fractions on both polyp species, according to EC_{50} and LC_{50} are shown in Table 4.8.

Table 4.8. LC_{50} and EC_{50} values of chemicals and seaweed extracts

	Sample	LC_{50}	EC_{50}
Aurelia aurita	TBT	3.48 (2.53 - 4.77) ng/mL	1.23 (0.80 -1.89) ng/mL
	$CuSO_4$	456.0 (325.5 – 639.0) µg/mL	-
	Irgarol	> 10 µg/mL	363.1 (273.1 – 482.7) ng/mL
	Diuron	4.78 (3.42-6.70) µg/mL	-
	AA Crude	> 5 mg/mL	2.39 (1.67-3.42) mg/mL
	SM MDCrude	> 5 mg/mL	0.24 (0.16-0.34) mg/mL
	SM MCrude	> 5 mg/mL	0.29 (0.17-0.48) mg/mL
Phyllorhiza punctata	TBT	1.56 (1.18-2.07) ng/mL	0.10 (0.06 – 0.16) ng/mL
	$CuSO_4$	33.67 (20.60 – 54.86) µg/mL	-
	Irgarol	> 10 µg/mL	319.7 (221.0-462.4) ng/mL
	SM_MDcrude	1.19 (0.85-1.68) mg/mL	0.25 (0.18-0.35) mg/mL
	SM_MDF5	> 5 mg/mL	0.70 (0.43-1.15) mg/mL
	SM_Mcrude	1.15 (0.83-1.60) mg/mL	0.22 (0.13-0.35) mg/mL
	SM_MF3	0.38 (0.28-0.53) mg/mL	-
	SM_MF4	1.66 (0.94-2.95) mg/mL	0.72 (0.56-0.93) mg/mL
	AA_F1	2.76 (1.76-4.35) mg/mL	-
	AA_F2	1.52 (1.12 – 2.06) mg/mL	0.71 (0.52-0.97) mg/mL
	AA_F3	0.43 (0.32-0.59) mg/mL	-
	AA_F4	2.02 (1.23 – 3.14) mg/mL	-
	AA_F5	0.71 (0.50 – 1.01) mg/mL	-
	AA_F6	1.30 (0.95-1.76) mg/mL	-
	AA_F7	2.23 (1.43 – 3.50) mg/mL	-

*AA – *Asparagopsis armata*; SM – *Sargassum muticum*; LC_{50} – concentration which promote 50% deaths; EC_{50} – concentration which promote 50% non-adhesion; - not determined (low activity, or insufficient data points for analysis mainly due to "all or nothing" effects).

It can be verified that TBT is by far the most toxic compound for both species, and irgarol the less, with anti-adherence effects similar on both species (EC_{50} of 363.1 (273.1 – 482.7) and 319.7 (221.0-462.4) ng/mL, for *A. aurita* and *P. punctata*, respectively). Both seaweeds crude extracts revealed anti-adherence effects on *A. aurita* polyps, nevertheless *S. muticum* exhibited ten times more potency. Against *P. punctata*, both seaweeds crude extracts presented anti-adherence effects, however *A. armata* crude extract revealed higher toxicity. Additionally, it can be verified that fraction MDF5 from *S. muticum* also produced high anti-adherence effects with low associated toxicity.

4B.4. Discussion

Underwater, any structure is a surface prone to biofouling. To reduce consequent impacts this fouling may represent in economic activities, paints and coatings are mandatory. Due to demonstrated deleterious effects on the environment elicited by classical antifouling compounds, greener alternatives are requested to counteract these phenomena.

While antifouling *in vitro* tests were already discussed above, it was relevant to understand the potential of both seaweeds on fouling organisms through *in vivo* tests.

The most commonly used organisms for *in vivo* tests are mussels and barnacles, which experimental protocols undergoes the collection of the target organisms which are further stressed (physically or chemically) for stimulating larvae release. These protocols are time consuming, most of the times comprising high mortality rates (Almeida and Vasconcelos, 2015). Therefore, a question was raised: are there other fouling species with potential to be used in *in vivo* antifouling experiments, which can result in less associated mortality rates, fast results with high reproducibility? In this quest, jellyfish species were addressed.

Jellyfish are capable of surviving in hostile environments and are highly abundant in different ecosystems. In addition, the possibility to either use from laboratorial culture, which can be maintained indefinitely, or collect these organisms from their natural environment without losing the capacity to settle again, without having to overcome metamorphosis, allied to their robustness, and capacity to adhere to different surfaces, make them exceptional organisms to be used as models for anti-settlement experiments (Graham and Bayha, 2007; Xie et al., 2015).

Within this framework, a simple experimental design was here presented. In a first approach, the polyps were placed in a gelatin-based membrane (agarose 1%) in a 24-well plate with 1 mL of sterilized seawater (30-35 ‰ salinity), under low aeration, and their settlement verified after 10 days. This experiment was repeated three times, and in all experiments, all the polyps adhered. The next step consisted in the same process but adding the sample to the gelatin matrix, having as negative control, the gelatin-based matrix with DMSO (the solvent in which the samples were dissolved). The rationale is to “trap” the bioactive compounds in the matrix preventing the immediate dissolution in the water.

In a second approach, to validate the model, several antifouling compounds, including commercial and non-commercial ones, were tested in a dose-dependent manner. To select the different concentrations to be tested, the compounds TBT, diuron, irgarol, and $CuSO_4$ were tested in a ten-fold dilution, to verify the minimal concentration which induced anti-adherence effects on live

organisms (Nagata, 2008). Having these results as reference, the concentrations were optimized for *A. aurita* and *P. punctata*.

TBT is by far the most toxic antifouling substance in the assay and worked perfectly as positive control. In both species, this organotin compound exhibited toxic effects in the order of nanograms. TBT became popular in the late 1950s revealing to be excellent in preventing antifouling. However, its proved toxicity led to new European regulations in 1989 restricting the use of this substance (Readman, 2006). With a restricted legislation concerning the use of TBT base coatings to prevent biofouling, antifouling industries developed alternatives, the majority based in biocide substances already used in agriculture. These new formulations resulted in 18 new compounds, among them, irgarol and diuron are two of the most commonly used antifoulants worldwide. On the other hand, copper-based formulations are still massively used in antifouling coatings (Readman, 2006).

In the present work was possible to determine that the toxicity of these compounds on the polyps was shown as follows: TBT>diuron>irgarol>$CuSO_4$. These results were in agreement with other investigations which showed that irgarol have strong anti-adherent properties with less toxicity than other antifoulants such as diuron and TBT (Bazes et al., 2009). $CuSO_4$ was the less toxic substance, nevertheless, was also the less efficient compound inhibiting the settlement of medusa polyps. Comparing the results obtained with antifouling chemicals in both species, *Phyllorhiza punctata* seems to be more susceptible to the toxicity exhibited by the tested antifouling chemicals, resulting in 100% deaths when exposed to TBT at 5 ng/mL, to $CuSO_4$ at 1000, 300 and 100 µg/mL, and ≈ 80% deaths when exposed to diuron at 10 and 1 µg/mL. In both species, irgarol revealed to be the most efficient antifouling compound, revealing high anti-adherence properties at subtoxic-concentrations. The non-adherence of the polyps may be the result of behaviour changes to avoid adverse substances or a result of more complex metabolic processes. Few is known concerning the mechanisms behind medusa polyp's settlement, nevertheless, several strategies are transversal to other macroinvertebrates. Thus, the anti-adherence effects may be the result of several metabolic changes, including, neurotransmission disorders, biofilm inhibition, adhesive production/release inhibition and oxidative stress (Chen and Qian, 2017).

The most desirable alternative for biocidal coatings is inert surfaces with antifouling characteristics, which inhibit organism's settlement through avoidance strategies. A successful example is the use of ethylene glycol in the biomedical area ($(EG)_x$ containing coatings)), however, experiments on aquatic environments proved it unfitting for long-term applications due to chemical degradation (Rosenhanhn and Sendra, 2012). These evidences reinforce the need for the search of new substances for the development of greener antifouling alternatives. Within this framework, the

marine environment has revealed to be a rich source of chemical structures with potent bioactive activities, upon which new antifouling formulations can be based on.

Within marine environment, seaweeds have revealed to be a great source of bioactive compounds with potent effects in numerous contexts (Pereira and Costa-Lotufo, 2012). These bioactive structures are assumed to be produced as part of an evolutionary process as a defence mechanism against physical, chemical and biological stressors. Underwater, all organisms are prone to biofouling. While some microbial relations benefit the hosts, most microorganisms are undesirable and therefore, marine organisms, such as seaweed, developed antifouling strategies to counteract colonization by detrimental microorganisms (Müller et al., 2013; Wahl, 1989).

Due to the high reproducibility of the proposed methodology, the model was used to test the antifouling potential of *A. armata* and *S. muticum* extracts and fractions.

A. armata fractions F2, F3, F4, F5 and F6 present high toxicity towards *A. aurita* polyp. On the other hand, fraction F1 and the crude extract presented low toxicity, also revealing capacity to inhibit the adherence of *A. aurita* polyp, especially fraction F1 which inhibited the adherence in 100% at 1 mg/mL. Concerning *P. punctata* the results were quite similar, with all *A. armata* fractions (excepting F8) revealing high toxicity at the higher tested concentration (5 mg/mL). However, when decreasing the concentrations, it was possible to register non-adherent live polyps. The crude extract revealed toxicity at 5 and 1 mg/mL for this species, which allied with the results obtained with the known antifouling chemicals, reinforce the idea of this species to be more sensible to the tested substances.

Although it was not possible to find studies directly related with anti-adherence properties of compounds or extracts obtained from *A. armata*, its antifouling potential was verified by Paul and co-workers (2006) which showed that two compounds produced by *A. armata*, bromoform and dibromoacetic acid, have the ability to decrease the bacterial density on the algae surface. On the other hand, the Bonnemaisoniaceae family, in which *A. armata* is included, it is known to produce a wide array of halogenated methanes, which are regarded as potent biocides. In addition, *Asparagopsis* species have also revealed to produce a large number of small molecules such as tetrabromoacetone, di-bromoacetic acids, chloroform and carbon tetrachloride (Fenical, 1982) which can be involved in the effects here verified. The results obtained with *S. muticum* on *A. aurita* polyps revealed that fraction MF7 and the crude extract from both extractions were the most effective inhibiting polyps' adherence with no toxic effects. On the other hand, against *Phyllorhiza punctata,* the most effective was fraction MDF5 since it revealed high anti-adherence effects without toxicity. The crude extracts from both extractions also revealed high anti-adherence

properties at 0.3 mg/mL with no toxicity. In agreement with the present results, Bazes and collaborators (2009) investigated the antifouling properties of a dichloromethane extract and verified that, after 2-months immersion, the treated surface presented less fouling organisms than the non-treated one, including no barnacles or mussels. Also, Maréchal and Hellio (2011) tested the effects of several extracts from *S. muticum* on *Amphibalannus amphitrite* (barnacles) cypris settlement and verified that an ethanol extract was highly effective inhibiting the settlement of these organisms. These authors also verified anti-settlement effects of dichloromethane and water extracts. Although the extracts were not obtained with the same solvents as in the present work, the characteristics of the solvents are very similar (e.g. methanol and ethanol) point out the abundance of polar constituents in both works.

In the present work a new *in vivo* model for testing antifouling substances was developed. Since medusa polyp do not have to overcome metamorphosis to be used test their adherence, the results are obtained faster when compared with tests conducted with barnacles or mussels, with extremely low mortality rates in controls. Although the data presented in this work was a result of 10 days of exposure, both medusa polyp were able to settle in 5-6 days. Since the polyps are highly resilient, with capacity to re-adhere, increasing the exposure time would increase data consistency. Yet, in future screenings, this time can be shortened. Both species afforded consistent data in the experiments; nevertheless, *Aurelia aurita* polyps, due to morphological features (e.g. peduncle size), can be easier to manipulate.

4.2. Conclusions

Due to the increasing accumulation of toxic substances in the marine environment, it is fundamental to find alternative environmentally friendly solutions for preventing biofouling events. One sustainable approach may rely on the exploration of marine natural sources, since they naturally developed antifouling strategies to prosper in marine environments. Accordingly, invasive species, as they occur in great amounts in many coastlines all over the world, offer an extremely treasured opportunity for new natural bioactive compounds discovery. In line with this view, in this work the possibility of *A. armata* and *S. muticum* as sources of antifouling compounds was explored and the findings suggest that the potential is enormous. Having as target, organisms which are fundamental for biofouling processes (marine bacteria and microalgae) as well biochemical mechanisms essential for invertebrate's adhesion (neurotransmission), both seaweeds showed potential to slowdown bacteria and microalgae growth also decreasing bacterial biofilms production. Specifically, *A. armata* revealed great potential in all the biofouling stages studied (II-IV), while *S. muticum* revealed high potential inhibiting bacterial biofilm production (stage II). It is important to stand out that both seaweeds revealed relevant bioactivity on their crude extracts. Therefore, the use of their crude extracts (specially obtained with methanol:dichloromethane) can be the best strategy for further antifouling products development, since it reduces significantly the time of the procedure and the need to use high quantities of organic solvents in further chromatographic steps. This strategy may streamline the scale-up process and reduce costs for industrial development with high revenues, resulting, therefore, in a more sustainable and greener product. On the other hand, since the resulting biofouling product may be a mixture of substances, it can be interesting to test the combination of *A. armata* and *S. muticum* crude methanol:dichloromethane extracts to enhance the antifouling properties.

In the present work was developed a new *in vivo* model for testing antifouling substances. The easy maintenance of medusa polyps in laboratorial conditions, their high propagation rates and high resilience makes them excellent candidates for antifouling *in vivo* tests. This theory was proved by the results here presented, since, with a simple protocol design, the method revealed to have high reproducibility. Since these organisms do not have to overcome metamorphosis to test their adherence, the results are obtained faster when compared with tests conducted with barnacles or mussels, with extremely low mortality rates. From a biotechnological point of view, it can also be said that *A. armata* and *S. muticum* are a rich source of natural antifouling compounds. The crude extracts here tested presented high anti-adherence properties, on both medusa species, with very low associated deaths.

These seaweeds represent, therefore, a great opportunity for antifouling industries to develop greener alternative formulations to counteract biofouling. Moreover, since the extraction procedures are simple with high extraction yields comparing with more complex extractions and fractionations (≈ 9% dry weight for *S. muticum* crude extract and ≈ 15% dry weight for *A. armata* crude extract, open the possibility to be implemented at industrial scale with high revenues. On the other hand, since these seaweeds occur in excessive amounts in several coasts around the world, the lack of biological material and the negative impacts on the marine ecosystem should not be a bottleneck, in a contrary, their collection from the ocean will certainly contribute for a more balanced ecosystem.

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Chapter 5

General conclusions and Final considerations

5.1. General conclusions and final considerations

Invasive species are a global threat for the ecosystem's equilibrium causing serious economic and environmental impacts. It is then imperative to find strategies to control their spread and success. *Sargassum muticum* and *Asparagopsis armata* are recognized for being highly successful marine invaders, occurring in great amounts in all Europe's coast lines (Streftaris and Zenetos, 2006); however, much less attention has been given to their use as possible sources of new natural high valuable compounds. In an Era where society is increasingly focused in a two-folded aim for marine drugs discovery and sustainable development, these two marine invaders present, in turn, promising bioactive sources with a wide range of sustainable possibilities and applications. This thesis focused on the screening of the antioxidant, antimicrobial and antifouling properties of both seaweeds, disclosing their value as a source of bioactive compounds with application on several industries such as food, cosmeceutical, pharmaceutical, paint and other chemical industries. Both revealed to produce molecules with high economic value, yet, *S. muticum* revealed the greatest potential as a source of antioxidant substances. The results presented in chapter 2 suggest that *Sargassum muticum* produce substances with the capacity to protect cells against oxidative stress, which is one of the main conditions enrolled in the genesis of innumerous diseases, including neurodegenerative disorders, cancer, diabetes, cardiovascular diseases, etc. This seaweed reveals, therefore, to be extremely interesting for further exploration for pharmaceutical purposes. On the other hand, and being an edible alga with strong antioxidant properties, its use in food industries as a food additive replacing synthetic antioxidants may be a strategy for the development of healthier formulations.

Concerning the antimicrobial potential, *A. armata* revealed to produce compounds with high antimicrobial potential against both Gram-positive and Gram-negative bacteria, and also against fungi, revealing therefore a broad-spectrum activity. These results are shown in chapter 3 in which the mode of action of the most active fractions was also discussed. Accordingly, the most potent *A. armata* fractions showed ability to disrupt membrane integrity, and break DNA, leading to bacterial death. In chapter 3 is also discussed the ability for both seaweeds to inhibit the development of bacterial biofilms. The production of biofilms by bacteria are one of the strategies for bacteria to survive in hostile environments, being widely related with the occurrence of resistant bacteria and persistent infections. *A. armata* showed capacity to inhibit *P. aeruginosa* biofilm production, thus revealing a high advantage for pharmaceutical purposes. The identification of the bioactive compounds is a crucial step for further pharmacological tests. Therefore, in chapter 3, several approaches were carried out for bioactive compounds identification. It was possible to verify that

all *A. armata* fractions were mainly constituted by fatty acids, being also possible to detect the presence of brominated compounds. The main detected constituents were tribromomethanol, hexadecanoic acid (palmitic acid), dodecanoic acid (lauric acid), tetradecanoic acid (myristic acid) and octadecanoic acid (stearic acid). These results suggest that the antimicrobial activities verified can be related with the presence of brominated compounds but also with minor compounds which were not possible to identify in this work.

Concerning the antifouling properties (chapter 4), in general *A. armata* presented the highest potential to inhibit the growth of marine and freshwater bacteria, and the highest potential to inhibit microalgae growth. On the other hand, both seaweeds revealed anti-biofilm properties. By inhibiting the growth of microfouling species, the biofouling process is compromised therefore hampering the subsequent adherence of macroinvertebrate fouling species. Both seaweeds were also screened for their ability to inhibit the settlement of macroinvertebrates by neurotransmission disruption. The settlement of invertebrate organisms is a result of a cascade signaling pathways in which, neurotransmitters such as cholinesterases (ChEs) play a fundamental role. Accordingly, seaweeds were tested for their ability to produce substances with anti-cholinesterase activity. The results shown in chapter 4A reveal *A. armata* as a source of substances, which strongly inhibit cholinesterases activity, specifically acetylcholinesterase activity. With this result, *A. armata* revealed potential to intervene in several stages of the biofouling process, reinforcing the high potential of *A. armata* extracts to be incorporated in new anti-fouling formulations safer for ecosystems.

In the present thesis, a new approach for testing anti-adherence properties of antifouling formulations is proposed in which medusa polyps are used as models (Chapter 4 - part B).

Medusae are abundant organisms which life cycle undergo a sessile stage, the polyps. Although with fragile appearance, the polyps are resilient organisms capable of surviving in hostile and demanding environments, with ability to settle nearly everywhere. On the other hand, these organisms have a high proliferation rate. These characteristics make polyps extremely interesting organisms to be used as models for anti-adherence tests. Accordingly, two species of medusa polyps, *Aurelia aurita* and *Phyllorhiza punctata*, were exposed to different commercial antifouling substances (TBT, Diuron, Irgarol and $CuSO_4$) and their settlement behavior observed. It was shown that medusa polyps are simple model that can be used in *in-vivo* antifouling screenings. In the presence of an effective antifouling substance, when exposed to non-lethal concentrations, polyps did not adhere. TBT exhibited the highest toxicity and $CuSO_4$ the lowest, for both species. When exposing polyps to *A. armata* and *S. muticum*, it was possible to verify that the crude extracts from

both seaweeds contain substances which exhibiting low toxicity, showed high anti-adherence properties. Therefore, and in line with the results obtained in Chapter 4 (part A), it can be concluded that a simple methanol:dichloromethane extraction of both seaweeds results in bioactive substances with antifouling properties that may be included in antifouling formulations. Since for antifouling formulations it is not required an isolated and pure substance, the use of crude extracts may result in a more sustainable and profitable product. Moreover, being natural substances, the negative impacts in aquatic ecosystems will be certainly diminished.

While cosmeceutical, food and antifouling industries do not demand a pure compound for new products development, for pharmaceutical purposes it is crucial to have an isolated compound for more specific tests and ultimately to enter the drug discovery and development pipeline. Even though many strategies were applied, in this work it was not possible to obtain pure compounds. In fact, despite the existence of innumerous works on the marine bioactive substances framework, few have resulted in pure compounds. This is related with the difficulty of the isolation and purification procedures, which can result in low extraction yields and in the loss of valuable compounds, as may have occurred in the present work. In addition, the bioactivities evidenced by some extracts may be the result of synergisms between molecules resulting in limitations for pharmacological applications where well-characterized compounds are required.

Within the available bibliographic research gathered on the publication of Pinteus and co-workers (2018), part of this thesis, it was also possible to verify the lack of standardized methodologies for specific targets. Depending on the characteristics of seaweed species and on the chemical nature of the desired compounds, the extraction methods should be optimized for maximum extraction yields, especially for industrial purposes. On the other hand, studies point out differences on the production of secondary metabolites related with the collection place and seasonality. Since seaweeds respond to physical stresses such as temperature, solar exposition, pH, oxygenation, predation, etc., depending on these inputs, the amount of bioactive compounds produced vary, and therefore, for industrial purposes it may be necessary to perform simple initial screenings for bioactives detection and quantification (Pereira and Costa-Lotufo, 2012). Consequently, more research is needed to understand the nature of the desired bioactive compounds and establish directed extraction approaches, for maximum extraction yields without bioactivity loss.

Despite these restrains and difficulties, seaweed extracts can be used for cosmetics, nutraceuticals, agriculture purposes, such as soils enrichment and animal feed, and as additives for antifouling purposes. In fact, an *A. armata* extract is already commercialized under the name Ysaline100® for cosmetic purposes being also included in skin products formulations and shampoo (Algues&Mer,

2017; Moigne, 1999). On the other hand, *S. muticum* has been used for centuries in Oriental traditional medicine for the treatment of different diseases such as arteriosclerosis, skin diseases, and acute bronchitis among others, and for food purposes. In Europe the main uses of *S. muticum* are limited to agriculture as fertilizer and for animal feed (McHugh, 2003; Pereira, 2010) and yet ethnopharmaceutical sustained studies ought to reveal greater potential for its biotechnological use (Liu et al., 2012).

The present work demonstrates the high potential of *S. muticum* as a source of antioxidant molecules with cytoprotective properties. Although more intensive work is needed for further pharmacological applications, having as background the results here presented, and the ethnopharmaceutical knowledge of Asian communities, the potential of *S. muticum* to be used as a healthy food ingredient, food additive or in the cosmeceutical industry is enormous.

The present work also shows that the crude extracts obtained from both seaweeds have high potential to be used as additives in antifouling paints, and therefore potential to respond to an emergent problem in the maritime industry: the use of toxic substances that impose huge negative impacts on aquatics environments, entering food chains and ultimately promote diseases in humans through seafood products consumption. This can be, ultimately, the fastest way to obtain a high valuable product from these seaweeds, reaching an industry of millions of dollars all over the world, and therefore justifying their collection from invaded areas. Moreover, the results here presented can be further enhanced, by optimization of solvents rate and extraction time, together with the possibility of a solvent recirculation system, for higher extraction yields, and thus, maximum extraction efficiency.

In a general way, the present work reveals the potential of both seaweeds to provide valuable compounds opening a new window for sustainable development with high economic benefits.

In marine natural products research and development, one of the main bottlenecks for industrial applications is the scarcity of the biological target and the negative impacts that are associated with its collection from the ecosystem. Since *A. armata* and *S. muticum* are successful invaders that occur in great amounts in many coastlines of the world, their collection could contribute for marine ecosystem restoration and equilibrium by means of profitable harvesting for a high revenue industry, thus, turning a real threat into a major opportunity.

5.2. References

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