



***The biotechnological potential of the seaweed
Gracilariopsis longissima (Rhodophyta, Gracilariales):
assessment of growth performance and nutraceutical
value of a natural resource***

Marta Alexandra dos Santos Veríssimo Silvestre de Freitas

2017



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value of a natural resource***

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Mouga and co-supervision of Doctor Clélia Correia Neves Afonso

2017

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Escola Superior de Turismo e Tecnologia do Mar

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Abstract

In recent times, humankind has increasingly explored the oceans in the search of novel, natural, and sustainable sources of bioactive compounds, such as those from macroalgae, which nowadays occupy a noticeable place within the multifaceted fields of Science and Healthcare. Seaweeds feature prominently in the history and tradition of many cultures, and are now harvested and farmed for many purposes, according to species and country, but especially for food and hydrocolloid production. The seaweed *Gracilariopsis longissima* (Rhodophyta, Gracilariales) belongs to a family of red seaweeds widely valued as agarophytes, while the species itself was recently acknowledged as an effective bioremediator in IMTA systems. The *G. longissima* seaweed from Lagoa de Óbidos was hereby the focus of the present study, being the whole work divided in two main chapters, concerning research with distinct objectives. Chapter One is mainly focused on the assessment of *G. longissima* growth rates according to salinity and under controlled conditions, where it was uncovered that this seaweed presents the best growth rate and performance at 35‰ salinity (with 1.611%.day⁻¹ of length increase, and the emergence of 16 new ramifications), while being unable to survive at salinities of 15‰ and below. Chapter Two establishes a nutritional profile for *G. longissima*, namely by defining seasonal variations of protein content and determining the fatty acids profile, as well as providing insights on seasonal variations of antioxidant activity. Seasonal variations of antimicrobial activity were also tested, against the pathogenic bacteria *Escherichia coli*, *Bacillus subtilis*, and *Vibrio alginolyticus*. Results show promise regarding the protein content (ranging from 11.19 to 27.04% of dry weight), and a fatty acid profile rich in arachidonic and palmitic acid (36.78 and 43.84% of total FAs, respectively), while presenting weak antioxidant activity (with total phenolic content ranging from 0.54 to 1.90 mg GAE.g⁻¹, and DPPH scavenging effect ranging from 3.93 to 8.92%). *G. longissima* also holds a remarkable antibacterial activity against the bacteria tested, with no seasonal significant differences detected. Overall, *G. longissima* stands as a potential natural source of biologically active compounds, being thus theoretically relevant for a wide range of biotechnological applications. Although being distinct works, the results obtained from Chapter One and Chapter Two must be considered synergistically, in the sense that they offer a contribution regarding the growth conditions for *G. longissima*, and also insights on interesting compounds the seaweed may provide, that can eventually justify future attempts to a large-scale and thriving cultivation system dedicated to this seaweed.

Keywords: salinity range, growth rate, nutritional value, antioxidant, antimicrobial.

Resumo

Recentemente, a humanidade tem sondado os oceanos na procura de fontes naturais de compostos bioativos, tais como os provenientes de macroalgas, que hoje em dia ocupam lugares de destaque em campos da Ciência e Medicina. As macroalgas figuram na história e tradição de várias culturas, sendo hoje em dia exploradas e cultivadas para diversos propósitos, dependendo da espécie e país, mas geralmente como alimento ou produção de ficocolóides. A alga *Gracilariopsis longissima* (Rhodophyta, Gracilariales) pertence a uma família de macroalgas vermelhas amplamente autenticadas pelo seu valor como agarófitas, sendo a espécie em si ultimamente reconhecida em sistemas IMTA como biorremediadora. A alga *G. longissima* da Lagoa de Óbidos foi assim o objeto do presente estudo, estando este dividido em dois capítulos principais, relativos a pesquisa com objetivos distintos. O Capítulo Um foca-se sobretudo na avaliação das taxas de crescimento de *G. longissima* em salinidades distintas e sob condições controladas, e onde se inferiu que a macroalga apresenta o melhor crescimento e performance a uma salinidade de 35‰ (com um aumento de comprimento de 1.611%.dia⁻¹, e 16 novas ramificações), não sobrevivendo contudo em salinidades de 15‰ e inferiores. O Capítulo Dois estabelece um perfil nutricional para *G. longissima*, nomeadamente deteção de sazonalidade no conteúdo em proteína e determinação do perfil lipídico, fornecendo também um parecer sobre variação sazonal na sua atividade antioxidante. Foi igualmente testada sazonalidade na atividade antimicrobiana, contra as bactérias *Escherichia coli*, *Bacillus subtilis* e *Vibrio alginolyticus*. Os resultados indicam um considerável conteúdo em proteína (desde 11.19 a 27.04% de peso seco), e um perfil de ácidos gordos rico em ácido araquidónico e ácido palmítico (36.78 e 43.84 % do total de ácidos gordos, respetivamente) mostrando, no entanto, uma fraca atividade antioxidante (conteúdo total em polifenóis desde 0.54 a 1.90 mg EAG.g⁻¹, e atividade sequestradora do DPPH desde 3.93 a 8.92%). A atividade antibacteriana de *G. longissima* é notável, não apresentando variação sazonal contra qualquer uma das bactérias testadas. De modo geral, *G. longissima* apresenta-se como uma potencial fonte de compostos biológicos ativos, com apreciável relevância em um vasto leque de aplicações biotecnológicas. Apesar de distintos, os resultados obtidos nos Capítulos Um e Dois devem ser considerados mutuamente, pois oferecem contribuições respeitantes a condições de crescimento para *G. longissima*, e um parecer sobre compostos fornecidos pela macroalga, que poderão eventualmente justificar o futuro cultivo em larga-escala dedicado a esta espécie.

Palavras-chave: salinidade, taxa de crescimento, valor nutricional, antioxidante, antimicrobiano.

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Introduction

The oceans are home to amazingly diverse and interesting life forms, providing home to nearly 90% of the organisms inhabiting Earth, yet surprisingly, and although covering more than two-thirds of the Earth's surface, its depths are still widely unknown. This is, in main part, due to the extreme environments that characterize the deep blue, rendering human life impossible and research a genuine challenge. It is generally known, however, that oceans provide many unique environments and resources, with plentiful and diverse marine organisms with great potential to produce bioactive compounds with applications in several fields of science and healthcare. Within this group, macroalgae stand prominently as one of the most interesting resources of such compounds (Ibañez and Cifuentes 2013; Andrade et al. 2013; Kim and Chojnacka 2015), as they are perfectly adapted to a wide range of ecological aquatic niches. Algae produce diverse and unique compounds and secondary metabolites in order to survive and compete successfully in their environment, having built perfectly fine-tuned defence strategies and metabolic pathways as a response to their perpetual adaptation and evolution over the last 2.45 billion years (Cardozo et al. 2007; Wang et al. 2015). Reflecting such potential, research has already chemically determined over 15.000 novel compounds from both macro and microalgae (Cardozo et al. 2007).

Macroalgae are, in the broadest definition, macroscopic and multicellular aquatic plants that lack the highly specialized structures of higher plants, such as true leaves, stems and roots. Also known as seaweeds, their modest structure consists of a thallus, sometimes provided with a leaf-like lamina or blade, and a holdfast to provide attachment to a surface (Gallardo 2015). Marine macroalgae can thrive in a great variety of ecological niches, namely intertidal, shallow, and deep sea areas up to 180 m depth, and also in estuaries, either freely floating or attached to a solid substrate (Vijayan et al. 2016). Macroalgae perform photosynthesis, and possess biological and ecological functions similar to those of higher plants. All seaweeds have the photosynthetic pigments chlorophyll *a*, carotenoids, and xanthophylls and are classified into three main groups, according to the visible colour conveyed by the nature and combination of their dominant pigments: the green algae Chlorophyta contain the chlorophylls *a* and *b*, and carotenoids; the brown algae Phaeophyceae (phylum Heterokontophyta) has the chlorophylls *a* and *c*, carotenoids, and xanthophylls with fucoxanthin as the dominant pigment, and the one responsible for their characteristic brown colour; and the red algae Rhodophyta contain the chlorophylls *a* and *d*, carotenoids, and a number of phycobilins such as phycoerythrin, which gives these seaweeds their typical reddish hues (Rowan 2011; Gallardo 2015; Pereira 2016). The selective presence in these pigments is related

to the sea habitat of the seaweed, due to their different light intensity requirements to perform photosynthesis. Green algae generally occupy niches with large amounts of sunlight energy available, such as coastal areas, whereas brown algae are mainly found in intermediate depths; red algae, however, are better adapted to greater depths or sheltered niches where sunlight radiance is limited (Barsanti and Gualtieri 2014).

Seaweed Industry and Biotechnological Applications

Historically, seaweeds are harvested from nature, yet since the early 20th century there has been a fast and significant growth of the seaweed cultivation, not only as a response to continuous and increasing demands in food and pharmaceutical industries (Yang et al. 2015), but also to counter the risk of resource exhaustion. The depletion of natural stocks is an issue especially pertinent to coastal regions still lacking proper management of the sustainable exploitation of seaweed natural stocks (Rebours et al. 2014). Forster and Radulovich (2015) list several species which currently are the selected targets of the existing seaweed farming industry, known to perform well in culture systems, namely the brown seaweeds *Laminaria japonica* and *Saccharina lattisima*, and various *Porphyra* (Rhodophyta) species for temperate waters; for tropical waters, farming industries prefer the red seaweeds *Kappaphycus alvarezii* and a number of *Euchema* species, being the latter grown mainly for hydrocolloid production. Other seaweed species are grown commonly for food, though in far smaller amounts, such as species belonging to the genus *Gracilaria* (Rhodophyta), *Ulva* and *Caulerpa* (Chlorophyta), and *Sargassum* (Phaeophyceae). The abovementioned authors call to attention, however, that it is too premature yet to settle on preferences from among this list.

Seaweeds have been part of the human diet for thousands of years, based on archaeological evidence in Chile dating back to 14 000 years before present, and early written records from China (300 A.D.) and Ireland (600 A.D.) (Wells et al. 2017). Seaweeds now currently hold worldwide recognition as an important living natural provider of various chemicals, being one of the richest and most promising source of bioactive primary and secondary metabolites. Phycocolloid compounds such as agar, carrageenan, and alginate are examples, being widely used in food, cosmetics, and biomedical industries as gelling, thickening, and stabilizing agents (Samaraweera et al. 2011; Kasanah et al. 2015; Pereira 2016). Polysulfated polysaccharides are other examples, namely galactosyl glycerol and fucoidan, to name a few, whose strength lies in their use as antioxidant, antiallergic, anti-HIV, anticancer, and anticoagulant agents (Jiao et al. 2011).

Many Asian countries highly regard seaweeds as a valuable resource, and use them as diet staples on a daily basis (Sahoo and Yarish 2005; Pereira 2011; Pereira 2016). Japan was the first country to coin the term “functional food” in the 1980s, to name food products fortified with components promoting the human health, and endorsing this way a further understanding of the relationship between nutrition and health which stands as the basis of this concept (Hamed et al. 2015). This traditional view seeped into the western culture and is now extensively supported by algal researchers worldwide, who brand seaweeds as potential candidates as an healthy food source, with low calorie content, rich in polysaccharides, minerals, vitamins, essential amino acids and dietary fibers (Mabeau and Fleurence 1993; Lordan et al. 2011; Syad et al. 2013; Carvalho and Pereira 2015; Debbarma et al. 2016; Wells et al. 2017). Macroalgae are acknowledged to fight obesity, tackle free radicals, reduce the incidence of cardiovascular diseases, and promote a healthy digestion (Plaza et al. 2008; Cardoso et al. 2015; Roohinejad et al. 2016). Moreover, recent studies also stress the potential value of seaweeds as therapeutic agents, with reportedly demonstrated biological properties such as antibacterial (Kasanah et al. 2015), antifungal (Peres et al. 2012), antiviral (Chen et al. 2013), antioxidative (Yang et al. 2012; Yeh et al. 2015; Raja et al. 2016; Pinteus et al. 2017), anti-inflammatory (D’Orazio et al. 2012; Lee et al. 2013), anti-tumour (Horta et al. 2014; Pádua et al. 2015; Rodrigues et al. 2015a), and antihypertensive activities (Suetsuna et al. 2004; Fitzgerald et al. 2012; Paiva et al. 2016). A detailed review by Vijayan et al. (2016) focus on seaweeds as a novel and eco-friendly source in bionanotechnology, relevant to the fields of medicine, environmental monitoring, and electronics.

Seaweeds even take part in human day-to-day life on a regular basis, in certain ways: soap, toothpaste, shampoo and air freshener, are just a small sample of products that often contain compounds of macroalgae origin. Recent awareness of the state of the planet, under anthropogenic pressure and depletion of its natural resources, as well as suspicion over products with chemical ingredients, has led most of the general public into heeding a more environmental-friendly lifestyle and preferring basic, natural and environmentally sustainable products. This relatively new tendency has promoted research into the potential of marine algae as cosmeceuticals (Wang et al. 2015), with the encouraging results driving cosmetic companies to increasingly advertising seaweed extract based products among others (Carvalho and Pereira 2015), soundly promoting their benefits to a progressively attentive audience.

Seaweeds play a critical role as ecosystem engineers (Jones et al. 1994), by protecting shorelines and shaping reefs, providing shelters and serving as feeding/nursery ground of marine organisms (Carvalho and Pereira 2015), ultimately creating habitats and

promoting species diversity (Haywood et al. 1995). Seaweeds are also known to reduce eutrophication, control harmful microalgal blooms, absorb nutrient excess, remediate contaminants and sequester CO₂ (Aresta et al. 2005; Singh et al. 2011; Chen et al. 2015; Cabral-Oliveira et al. 2016; Krause-Jensen and Duarte 2016), ultimately contributing to the improvement of coastal environments. Both wild and farmed seaweed communities can potentially mitigate global warming by dampening wave energy, buffering ocean acidification, and providing oxygen to the waters (Roleda and Hurd 2012; Castro and Huber 2016; Duarte et al. 2017). Yang et al. (2015) discuss in their review not only the aforementioned benefits, but also state that the large-scale cultivation of seaweed holds the key to eco-friendly water quality improvement in the coastal waters of the world. Algal pigments have been applied as trophodynamic indicators in biogeochemistry and ecology studies, with research focused mainly on chlorophylls and carotenoids (Kleppel 1988). Current research also investigates the applicability of algae as a potential biofuel source, shedding light upon standpoints on macroalgae-based biorefinery technology (Chen et al. 2015; Suutari et al. 2015; Kumar et al. 2016).

More recently, seaweeds are an increasingly valued component in Integrated Multi-Trophic Aquaculture (IMTA) systems (Abreu et al. 2011b; Carvalho and Pereira 2015; Samocha et al. 2015), in which two or more trophic levels grow in one farm, and the by-products provided by one species can feed another; fed aquaculture is combined with inorganic and organic extractive aquaculture to create a synergistically balanced system, as close as possible to natural schemes (Neori et al. 2004). Therefore, there is a growing tendency of the research related to the improvement of seaweed (Raikar et al. 2001; McHugh 2003; Reddy et al. 2008; Baweja et al. 2009), essential to the development of the commercial seaweed industry. However, to this date, merely about 5% of the roughly ten thousand identified algae species worldwide is being explored, especially as human food or animal feed. The highest contributor to this low percentage are the Asian countries, where seaweeds are used as sea vegetables, being an inherent part of their culture and routine (Schmid et al. 2014). Conversely, in Southern Europe edible seaweeds have still a meagre contribution to feeding habits and nearly no place in regional recipes; the assumption that seaweeds are unfit for consumption is still deeply rooted in the European mindset, and Portugal is no exception to this. As research is being released and updated, however, this trend is shifting into a slowly but steady recognition of the potential seaweeds present due to their nutritional, pharmaceutical, and cosmetic value (Pereira 2011).

The Red Seaweed *Gracilariopsis longissima*

Species Profile

The marine Rhodophyta *Gracilariopsis longissima* (S. G. Gmelin) (Steenft et al. 1995) is widely known by several common names: cabelo de velha in Portuguese; thin dragon beard plant and Ceylon moss in English; hai mien san, fen tsai, hunsai, hai tsai, and hoi tsoi in Chinese; ogo and ogo-nori in Japanese; nuoc-mam, rau-cau, and xoa xoa in Vietnamese (Pereira 2016; Guiry and Guiry 2017). Some of these common names may possibly derive from its exceptionally bushy appearance, given by the repeatedly division of the plump branches (up to 2 mm in width) which protrude from the cylindrical shaped terete thallus. Healthy specimens of *G. longissima* usually hold a deep dark red colour, being nearly black in the base of the thalli; similarly to many other seaweed species, the rich colour fades to a complete greyish white when the individual perishes. Colour variants such as green and yellow may also appear, and it is likely the result of ordinary genetic controls, as observed for the *Gracilaria* genus (Santelices and Doty 1989). The male and the female gametophytes can be identified by the presence of cystocarps in the latter, which are fruiting bodies which appear as distinct semi-circular dark lumps scattered all over the thalli (Sahoo and Yarish 2005; Pereira 2016). *G. longissima* occupies a variety of habitats both in tropical and temperate latitudes, on intertidal or shallow-subtidal surfaces with low water motion, and where the sediment is sandy or muddy. Having a worldwide distribution, *G. longissima* is found in SW and SE Atlantic, Indian Ocean, SW Asia in Iran, Israel and Sri Lanka, SE Asia in Vietnam, NE and E Pacific, Australia, and Pacific Isles in Hawaii, Polynesia and Samoa (Pereira 2016).

The current standoff in *Gracilariaceae* taxonomy

Extensive research, reports and reviews attest the genus *Gracilaria* exceptional value regarding several fields of health and industry (Santelices and Doty 1989), rendering the research and findings in *Gracilariopsis* genus very poor in comparison. Mendeley database currently includes 2.321 entries under the name *Gracilaria* and only 245 entries under the name *Gracilariopsis*; a quick NCBI research yields results filed under the name *Gracilaria* in 22 databases, whereas the name *Gracilariopsis* is presented in 15 databases, and in a comparatively lower number of entries in each database (for example, in the nucleotide database there are 3.480 sequences submitted for *Gracilaria*, and only 721 for *Gracilariopsis*) (NCBI Resource Coordinators 2017). The hypothesis of identification irregularities is put forward, where there is the possibility that all *Gracilariopsis* spp. might have been identified as *Gracilaria* spp. in several studies and even FAO reports, given not only the outstanding similarity between both genera, but also the fact that the genus *Gracilariopsis* was only recently reinstated by Fredericq and

Hommersand in 1990 (Guiry and Guiry 2017). Knowledge of the reproductive structures permits the trustworthy taxa identification of gracilarioids, however, some populations are sterile and grow merely by vegetative fragmentation (Santelices and Doty 1989). Morphologically barely indistinguishable from other *Gracilariopsis* species, and even from species belonging to the entire family, the identification of the species requires a well-trained eye. Consequently, the entire taxonomy of Gracilariaceae, especially between the *Gracilariopsis* and *Gracilaria* genus, is currently terribly chaotic (Bird 1995) and occasionally put under revision (Santelices and Doty 1989). Confusion often arises regarding any given species distribution, life-history, and even annual raw production values, or biotechnology value assessments, when said species do not hold a guaranteed identity. Therefore, all the reports which concern *Gracilaria* spp. and *Gracilariopsis* spp., whose identification was not established with confidence but performed having morphology observations alone as basis instead, place the entire findings and reports for both genera at jeopardy and may not reflect the reality. The currently untidy state of the taxonomy of *Gracilaria* and *Gracilariopsis* has even led to the release of a statement by M. D. Guiry, from *AlgaeBase* (Guiry and Guiry, 2017) found in a few *Gracilaria* and *Gracilariopsis* individual species page (including *G. longissima*), declaring that specimens reported to date as *Fucus verrucosus*, *Fucus confervoides*, and all terete *Gracilaria* from the NE Atlantic and elsewhere, actually require individual examination in order to define whether they belong to *Gracilaria* or *Gracilariopsis* genus, before assigning an individual species identity. Therefore, and although often confused with *Gracilaria gracilis* and once known as *Gracilaria verrucosa*, *G. longissima* is the current taxonomically accepted name for the species (Guiry and Guiry 2017), and the acknowledged identity of the individuals studied in the current work.

Study Site: Lagoa de Óbidos, Caldas da Rainha, Portugal

The Lagoa de Óbidos (Caldas da Rainha, Portugal) is a semi-enclosed lagoon situated in a shallow depression, which forms a natural barrier placed between the Atlantic Ocean and the riverine ecosystem of Foz do Arelho. The lagoon is extended by two branches, the Braço do Bom Sucesso by West and the Braço da Barrosa by East (Fig.: 1.1), and is bordered by the municipalities of Caldas da Rainha by North, and Óbidos by South.



Figure 1.1: Satellite image representing Lagoa de Óbidos and the main anthropological settlements surrounding the area. The red marker pinpoints the sample site.

Being the widest tidal inlet in the Portuguese shore (6.9 km² area, 2 m average depth), Lagoa de Óbidos provides shelter to a rich diversity of wildlife, including the red seaweed *G. longissima*, which grows and thrives as unattached, entangled mats on mud, and often underneath a dense meadow of sea lettuce (*Ulva lactuca*). The sampling site where *G. longissima* was collected is shown in Fig.: 1.2.



Figure 1.2: Sample site, corresponding to the red marker depicted in fig. 1. The seaweed *Gracilariopsis longissima* thrives upon a muddy substrate beneath the *Ulva lactuca* meadow.

Due to its natural tendency of closing its connection to the sea, Lagoa de Óbidos is at risk of disappear completely, and thus it currently depends on sensible human intervention to reverse this situation. The lagoon continually shifts its morphology, with channels and sandbanks under constant dynamism, mainly due to tide and wave action (Malhadas et al. 2009) although it is limited to a few hundred meters upstream of the mouth (Fortunato and Oliveira 2007). The upper lagoon is hardly affected by tides, and with freshwater also playing a minor role, there is an overall and progressive reduction of the average depth and the surface area, further aggravated by accretion and large residence times, of up to several years (Fortunato and Oliveira 2007). A number of authors suggest that such conditions do not favour pronounced variations on salinity on Lagoa de Óbidos (Carvalho et al. 2005; Santos et al. 2006).

Objectives

The main objective of the present work was to study the red seaweed *Gracilariopsis longissima* occurring in Lagoa de Óbidos, Portugal, in order to determine the growth rates of the seaweed under laboratory conditions and to evaluate its nutritional profile and bioactive capacity throughout the year.

Specific objectives:

1. To study the growth rate of *G. longissima* under distinct salinity conditions, ranging from 0 to 35‰.
2. To establish the best decontamination protocol of *G. longissima* under laboratory conditions.
3. To assess *G. longissima* nutritional profile, namely seasonal variation of protein content, and fatty acids profile.
4. To determine seasonal variations of antioxidant capacity, by total phenolic content and the DPPH radical scavenging activity.
5. To evaluate seasonal variations of antimicrobial value, namely antibacterial resistance against the Gram negative bacteria *Escherichia coli*, the Gram positive bacteria *Bacillus subtilis*, and the Gram negative marine bacteria *Vibrio alginolyticus*.

The present manuscript is structured in two main chapters, Chapter One and Chapter Two, each corresponding to a paper ready to be submitted to a scientific peer-reviewed journal. The main conclusions achieved throughout the course of this work are outlined at the end of each chapter, whereas conclusive remarks pertaining both chapters are delineated at the end of the document. In order to achieve a clean and organised layout for ease of reading, the Bibliography section encompasses references that were cited throughout the entire manuscript, and was thus placed solely at the end, instead of following each chapter. Regarding the Appendix section, it follows the Bibliography, and it is divided in two parts, Appendix A and Appendix B to match, respectively, Chapter One and Chapter Two, from where they are relevant to.

Chapter One

The role of salinity on the survival and growth performance of *Gracilariopsis longissima* (Rhodophyta, Gracilariales): an assessment performed on a laboratory scale

Abstract

The adoption of the ideal conditions is of utmost importance in any seaweed farming system, in order to achieve high biomass production and lucrative yields for the species target, which will ultimately translate into a successful and profitable business. In this sense, the survival and growth performance of the red seaweed *Gracilariopsis longissima* was evaluated, under a wide range of salinities and in small-scaled, controlled laboratory conditions. Clean *G. longissima* thalli was grown under controlled conditions ($24 \pm 1^\circ\text{C}$, photoperiod set at 12:12 (Light:Dark) and provided by daylight cool white fluorescent lamps ($10\text{-}15 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), in Von Stosch Media enriched seawater at distinct salinities, ranging from 0 to 35‰, for 44 days. Daily growth rates were assessed having the initial and final length of the thalli, measured at day 0 and day 44, respectively, and number of new ramifications were also counted. The seaweed *G. longissima* is highly tolerant to a wide range of salinities, namely from 20 to 35‰, although being unfit to survive at salinities below 20‰. The seaweed presented the maximum fitness at 35‰, observed by a growth rate of $1.611\%.\text{day}^{-1}$, and also revealed 16 new ramifications throughout the length of the assay. Insights on the influence and possible solutions on contaminations such as those caused by fungi and epiphytes, which are unquestionably hazardous to any seaweed culture system, are also provided. The present study offers therefore the first steps towards a throughout assessment of the ideal conditions, which must be applied into a culture system dedicated to the growth of *G. longissima*, in order to ensure that the maximum performance for the species is achieved.

Keywords: Red macroalgae, growth rate, ramifications, cultivation, contamination control.

Introduction

Algal culturing techniques have been described in books and papers since the early 19th century, from which Preisig and Andersen (2005) and Barsanti and Gualtieri (2014) briefly list the most noticeable advances, especially in microalgae culturing. Several methods and concepts such as media formulations, as well as reports on keeping healthy

and pure axenic cultures, have been developed and published as earlier as the late eighteen century. In regards to macroalgae, however, until the 1950s nearly all economically important seaweeds were still harvested from wild populations only, with the exception of the macroalgae *Porphyra*, the commonly known *nori*, which holds a long cultivation history dating back as early as the 17th century (Chen and Xu 2005). Concerns eventually arose regarding the risk of resource depletion due to overharvesting from 1950 onwards and the full disclosure of the complete life history of *Porphyra* has led to refinements and development of culture techniques and industry for this macroalgae, while also paving the way for the establishment and expansion of culture industries dedicated to many other economically important seaweeds (Tseng 1981; Sahoo and Yarish 2005). The overall aquatic plant farming, overwhelmingly of seaweeds, has been growing rapidly since then and it is now current practice in about 50 countries, having reached 27.3 million tonnes of production in 2014, which constituted one-quarter of the worldwide total aquaculture production by volume for this year (FAO 2016). Nowadays, large-scale seaweed cultivation techniques are standardized, routine, and economical; a successful culture takes into account several factors such as morphology and regeneration capacity of the thallus, and the synergy between irradiance, temperature, nutrients, salinity, and water movement (Sahoo and Yarish 2005). Depending on the chosen farming method, challenges may arise in the form of epiphytes (Msuya et al. 2014), fouling, and expensive infrastructures and nutrient requirements. Full knowledge of the species is crucial as well, as different taxa require distinct farming methods.

China is currently the largest mariculture producer in the world, undergoing rapid development during the last three decades, and reaching annual productions as high as 45 469.0 thousand tonnes in 2014, contributing thus to 61.62% of the world total production (FAO 2016). Gracilarioid species, to which both *Gracilaria* and *Gracilariopsis* are a part of, significantly account to these numbers, as they are one of the most cultivated seaweeds worldwide: regarding the production of farmed aquatic plants in the world, *Gracilaria* spp. reached values around the 3752 thousand tonnes in 2014, only surpassed by *Kappaphycus alvarezii* and *Euचेuma* spp. (10 992 thousand tonnes), and *Laminaria japonica* (7 655 thousand tonnes) (FAO 2016). The reasoning underlying such high values is the great value of Gracilariaceae species mainly as an agarophyte, being a tremendously valued source of hydrocolloids (Bixler and Porse 2011; Abreu et al. 2015), but also as a food and feed component (Chopin et al. 2001; Hernández et al. 2006; Mantri et al. 2009; Pereira 2011; Yarish et al. 2012; Kim and Yarish 2014; Samocha et al. 2015; Yang et al. 2015). Particularly *Gracilariopsis longissima* (S. G. Gmelin) (Steentoft et al. 1995) has been widely studied in Integrated Multi-Trophic

Aquaculture (IMTA) systems (Yang et al. 2015), with recent studies endorsing the several potential contributions this seaweed may provide to such systems, namely high performance as a biofilter agent (Hernández et al. 2005; Hernández et al. 2006; He et al. 2014), high tolerance to excessive levels of micronutrients such as copper (Brown and Newman 2003; Brown et al. 2012), ability to withstand ultraviolet radiation (Álvarez-Gómez et al. 2017), and thermal requirements for survival and growth (Steentoft and Farnham 1997).

To this date, however, and to the best of the knowledge and information gathered, studies reporting the growth performance of *G. longissima*, and even of the entire genus are scarce, when compared to the research already performed for the *Gracilaria* genus. In an attempt to fill this gap, the objectives of the present studies were to study the red seaweed *G. longissima* occurring in Lagoa de Óbidos, Portugal, in order to evaluate growth rates under distinct salinity conditions, ranging from 0 to 35‰, and therefore to determine both its salinity range of survival and the salinity value for optimal growth. The results obtained will contribute to a full assessment of the ideal set of conditions required to undertake a successful and profitable culture of this seaweed.

Materials and Methods

Sampling and Acclimatization

Specimens of *G. longissima* (Rhodophyta, Florideophyceae, Gracilariales) were harvested from Lagoa de Óbidos, in Caldas da Rainha, Portugal (39°24'18.93"N, 9°11'13.05"W) in September 2016, during low tide and transported to the laboratory in plastic containers. The classification of this seaweed was based on *AlgaeBase* (Guiry and Guiry 2017) and confirmed by botanists. In the laboratory, each plant was first washed with running seawater and cleaned thoroughly to remove debris, necrotic parts, epiphytes, and other organisms from the thalli surface. The seaweeds were then kept in constantly aerated seawater (25-30‰) during the following week, in a climatic room (24 ± 1°C) for adjustment purposes. The photoperiod was set at 12:12 (Light:Dark), with the irradiance being provided by daylight cool white fluorescent lamps (10-15 μmol photons m⁻² s⁻¹).

Selection and Isolation of Healthy Tips

A successful culture initiation requires the establishment of an axenic culture, by either spore or tip isolation. The selection, isolation, and cleaning of healthy seaweed tips was performed according to Yarish et al. (2012), being this the most critical step in culturing any seaweed. Therefore, from the previously acclimated *G. longissima* stock, fronds

exhibiting a deep dark-red colour and fleshy thalli, traits that are indicative of healthy individuals, were chosen (Fig.: 2.1). The fronds were rinsed in a series of vessels containing clean seawater, followed by a final and quick rinse of no longer than 60 seconds in clean freshwater, enough to induce osmotic shock to any adhering organisms without straining the seaweed.



Figure 2.1: Healthy frond of *Gracilariopsis longissima* collected from Lagoa de Óbidos, Portugal. The deep dark red coloration is particularly noticeable, especially in the thicker thalli.

Starter thalli were obtained by carefully cutting the tips (1-2 cm) from each cleaned and rinsed parent frond, as it is the area corresponding to the apical tissue where new and active growth happens. Each tip was individually and meticulously wiped down with sterilized cotton-tipped swabs, and subsequently dragged through an agar gel previously prepared in Petri dishes (1.0% bacteriological agar, VWR, Radnor, PA USA, in 1:1 distilled water/seawater ratio) to pull off any remaining microscopic contaminants. The agar drag was performed three times for each tip, and always through unused portions of the agar gel. All tools, seawater and distilled water used in cleaning process were previously sterilized by autoclave (121 °C, 15 minutes).

Growth Experiments

Selection of healthy fronds and isolation of tips followed the aforementioned Yarish et al. (2012) methodology. Healthy and clean seaweed tips were placed into 250 ml flat bottom flasks (8 tips per flask) and provided with sterilized seawater enriched with Von Stock solution (VSE), prepared according to Redmond et al. (2014). The components comprising the VSE media are the macronutrients nitrate and phosphate, the micronutrients iron and manganese, EDTA, and the vitamins B₁₂, thiamine, and biotin (Appendix A, table A.I). Salinity tests were carried out at 0, 2, 5, 10, 15, 20, 25, 30 and

35‰, being these values achieved by adding distilled water to filtered water (particles, sand, and UV filters were used), and checked with a refractometer (E-line refractometer, Bellingham and Stanley, China). The water was then sterilized by autoclave (121°C, 15 minutes) and further enriched with Von Stosch solution. Germanium dioxide was also added to the medium to prevent the growth of diatoms, and nystatin (Mycostatin® 100 000 UI.ml⁻¹, Bristol-Myers Squibb) to prevent and control fungi contamination (Yarish et al. 2012). Medium was changed weekly throughout the duration of the experiment, whereas nystatin was supplemented in the first week only. Cultures were kept in a climatic room (24 ± 1°C) under constant filtered aeration, with photoperiod set at 12:12 (Light:Dark) and provided by daylight cool white fluorescent lamps (10-15 μmol photons m⁻² s⁻¹) for 44 days, according to Yarish et al. (2012) and Hayashi et al. (2007b) (Fig.: 2.2). All the specimens were transferred to progressively lower or higher salinities (being 25‰ the starting point) on a weekly basis, in order to allow their adaptation and prevent stress caused solely by abrupt salinity shifts. Triplicates and controls were performed for all the assays, and clean stocks were kept as backup during the experiment. Tips that exhibit loss of pigmentation, visually observed by partial or total tip discoloration, were considered to be under stress or dead, and were thus removed from the assay.



Figure 2.2: Culture setup of *Gracilariopsis longissima* assay at different salinities, assembled in a climatic room (24 ± 1°C) with photoperiod set at 12:12 (Light:Dark) and provided by daylight cool white fluorescent lamps (10-15 μmol photons m⁻² s⁻¹).

Growth Measurements

Growth was recorded as changes in tip length and number of ramifications. Therefore, measures were performed along the main branch of each tip, and total ramifications per tip were counted; initial measurements were taken at the beginning of each assay, and final measurements taken after 44 days, for the salinities where seaweeds were

successfully thriving. Measures were determined upon full-scaled photos of *G. longissima* tips placed upon millimetric paper, in Adobe Photoshop® CC software (Adobe Systems, San Jose, CA USA). Daily growth rate calculations were performed based on the equation below, according to Mtolera et al. (1995), Gerang and Ohno (1997), Aguirre-Von-Wobeser et al. (2001), Bulboa et al. (2007), Hayashi et al. (2007a), Hayashi et al. (2007b), Hung et al. (2009) and Hayashi et al. (2011), whose formula is the one recommended to be used as the standard for seaweed growth rate determination (Yong et al. 2013).

$$\text{Daily Growth Rate (\% day}^{-1}\text{)} = \left[\left(\frac{L_t}{L_0} \right)^{\frac{1}{t}} - 1 \right] \times 100$$

Where L_0 and L_t stand as, respectively, the length measured at day 0 and day 44 of the trials, and t corresponds to the duration of the assay, in days.

Contamination Control

Seaweed cultures have often long timeframes from the excising of tips to the outplanting of individuals, and they can be easily ruined by contaminations of other microalgae, epiphytes, cyanobacteria, or fungi, which force the start of a new culture from the very beginning (Redmond et al. 2014). In this sense, contamination studies are quite helpful, as they frequently give tools and methods to prevent infection surges and subsequent damage of a seaweed culture, thus preventing financial and time losses. Therefore, in the present study additional assays were performed, to establish effective procedures against fungi contaminations.

Selection of healthy fronds and isolation of tips followed the method by Yarish et al. (2012) as previously described. After cleaning, each seaweed tip was individually immersed during 20 seconds in a fungicide or disinfectant solution, or 10 seconds for each solution when performing successive immersions in different agent solutions. Each seaweed tip was then independently placed in a test tube with 5 ml of Von Stosch media enriched seawater at 30‰ salinity and kept for four weeks. Agents tested were the antifungal medication Nystatin (Mycostatin® 100 000 UI.ml⁻¹, Bristol-Myers Squibb, New York City, NY USA) further diluted at 5, 10, and 20 ml.L⁻¹, the systemic fungicide Tocsin WG (70% p/p thiophanate-methyl, Sipcam, Lisbon, Portugal) at 1 g.L⁻¹, the preventive fungicide Pomarsol (80% p/p of thiram, Bayer, Carnaxide, Portugal) at 0.2 g.L⁻¹, and the disinfectant hydrogen peroxide (H₂O₂ 10 volumes, Continente, Matosinhos, Portugal) undiluted. Agent combinations and submersion order were the following: Tocsin WG (1

g.L⁻¹) then Nystatin (5 ml.L⁻¹), H₂O₂ then Nystatin (5 ml.L⁻¹), and H₂O₂ then Tocsin WG (1 g.L⁻¹). An additional experiment was also performed where Nystatin was added to the enriched seawater instead (at 1 ml.L⁻¹), therefore allowing the treatment to act continuously throughout the assay period. For plant fungicides, all the initial working concentrations were achieved based on manufacturer recommendations. Media was changed weekly, and controls and quadruplicates for each assay were kept. After two days, and on a weekly basis throughout the length of the four weeks assay, a selection of healthy seaweed tips was individually placed upon a potato dextrose agar (VWR, Radnor, PA USA) plate, which was then sealed and incubated at 30°C. The presence of fungi was visually confirmed following the two days of incubation on the agar. Effectiveness of any given fungicide concentration was evaluated by the seaweed fitness while on trial, and by the presence of fungi following the incubation period.

Statistical Analysis

Data is expressed as means ± standard deviation. The Student's *t*-distribution test was performed to detect significant differences on the calculated daily growth rates according to salinity, considering the level of significant difference of $p < 0.05$. The software Microsoft Office Excel 2013 was used to perform all the statistical analysis.

Results and Discussion

Growth Rate Assessment

Specimens of *G. longissima* were grown under controlled laboratory conditions at salinities ranging from 0 to 35‰, with constant surveillance of their performance during 44 days, and daily growth rates were determined. Fig.: 2.3 represents the daily growth rates calculated for seaweed growing at 20‰ and above only, corresponding to individuals which survived through the 44 days trial (see also Appendix A, Table A.II, Table A.III, and the corresponding p values of the *t*-student statistic test in Table A.IV). The figure 2.4 stands as an example of tips which remained healthy after the 44 days, where the successful performance is visually observed by the permanence of the dark rich colour throughout the assay, and by the fragmentation through vegetative propagation occurring in some tips. The highest daily growth rate value corresponds to seaweed growing at 35‰ (1.611%.day⁻¹), being the significantly highest value measured (*t*-student, $p < 0.05$), followed by seaweed growing at 30‰ (1.297%.day⁻¹). Seaweed kept at 20 and 25‰ yielded significantly lower daily growth rates than those obtained for seaweeds growing at 30 and 35‰ salinity, yet presenting no significant difference between each other (respectively, 0.888 and 0.740%.day⁻¹) (*t*-student, $p = 0.182$).

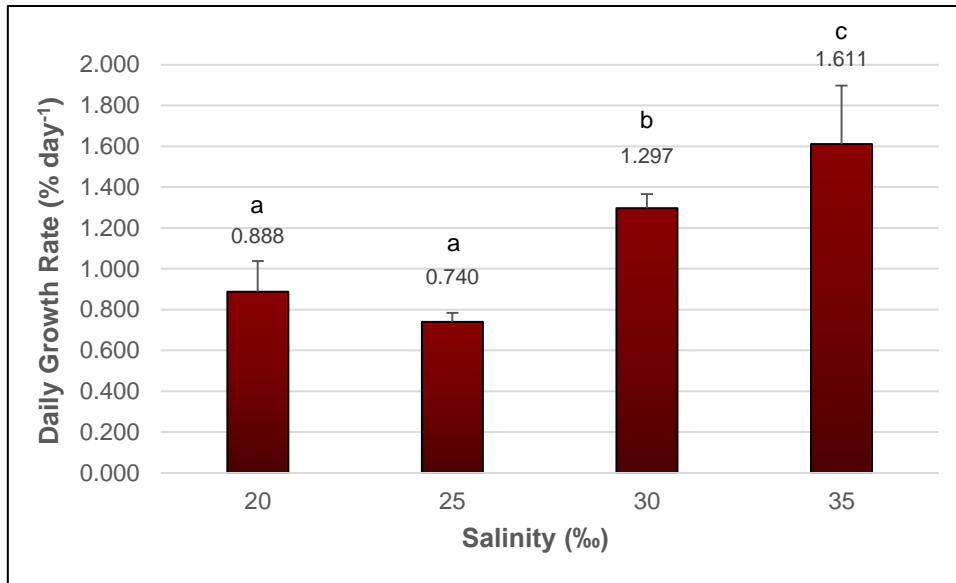


Figure 2.3: *Gracilariopsis longissima* daily growth rates (% day⁻¹) determined after 44 days, at the salinities of 20, 25, 30 and 35‰. Results are expressed as mean \pm standard deviation. $n = 3$. Letters a-c indicate statistical differences according to the *t*-student statistical test, with $p < 0.05$.

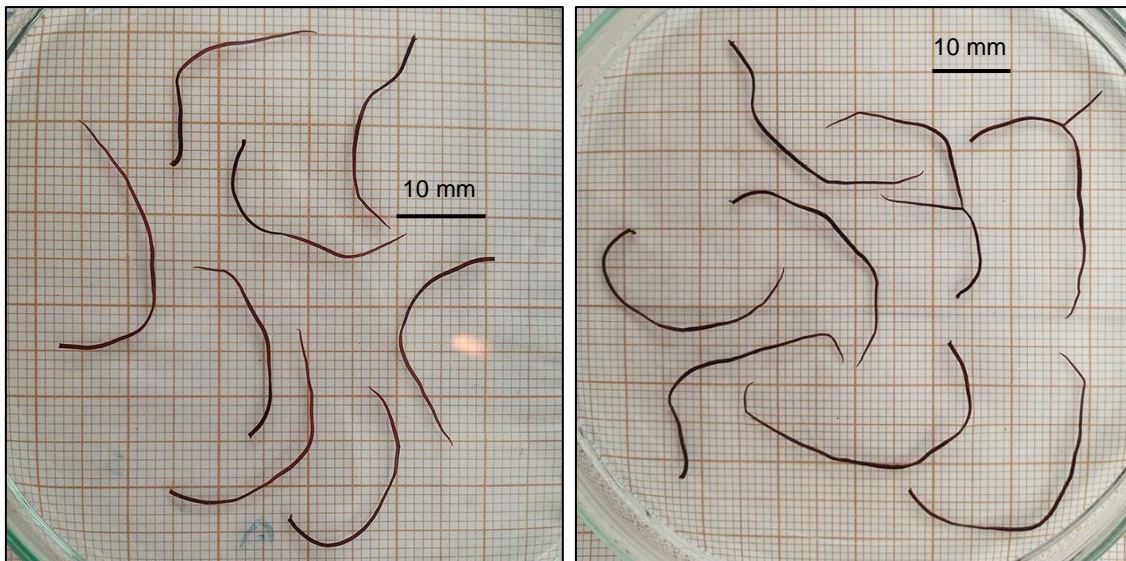


Figure 2.4: *Gracilariopsis longissima* tips thriving at 25‰ photographed at day 0 and day 44 of the trials. The successful fitness is visually observed by the unchanging deep red colour and vegetative propagation of some tips.

The number of new ramifications was also determined, according to salinity, with the results presented in Fig.: 2.5, again pertaining only to salinities where the individuals survived through the 44 days trial. The seaweed kept at 35‰ presented the highest number of new ramifications (16), followed by those kept at salinities 20 (11), 25 (4), and 30‰, the latter presenting no ramifications.

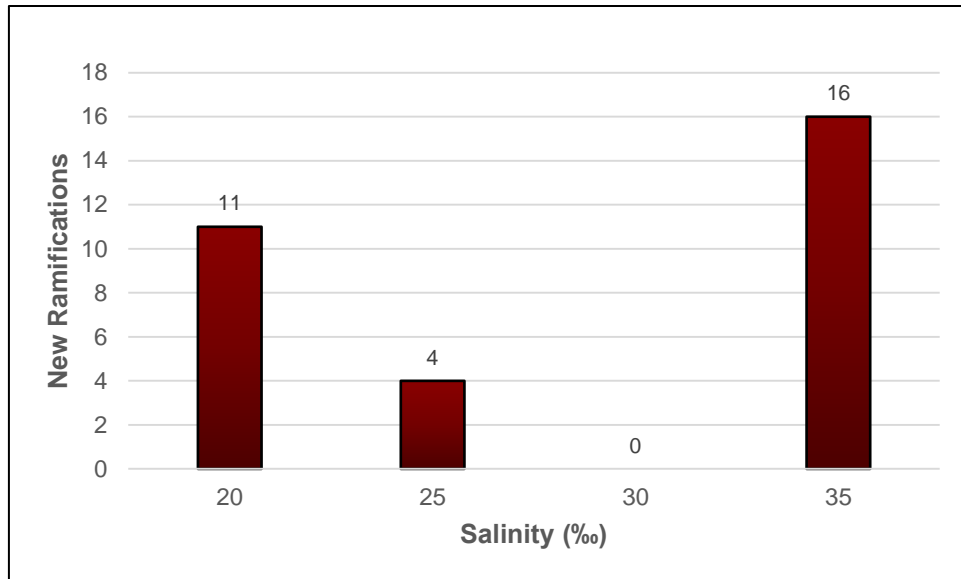


Figure 2.5: *Gracilariopsis longissima* number of new ramifications that appear throughout the essay, according to salinity. Numbers on top of each bar represent the total number of new ramifications counted for each salinity. $n = 24$.

Seaweeds kept at 15‰ and below perished within the 44 days trial, and thus their daily growth rates were not determined and data is not shown. Salinities of 15‰ and below proved to be progressively lethal to the seaweed, as the algae segments died within the first three weeks of the experiment. Salinities of 10 and 5‰ were lethal to the segments after the first two weeks of culture, whereas at salinities of 2‰ the segments died after two days in culture. Distilled water also proved to be lethal within the first 24 hours in culture, as proven by an assay with specimens transferred directly from 5 to 0‰. Figure 2.6 shows examples of the colour of seaweed tips kept at stressful conditions, exhibiting partial or total tip discoloration which corresponds to necrotic parts. The bleaching occurred starting either at the tips or in the middle section of the thalli, eventually spreading to the whole individual. Dying individuals also lost their characteristic fleshy texture, becoming flaccid and breaking down to the touch.

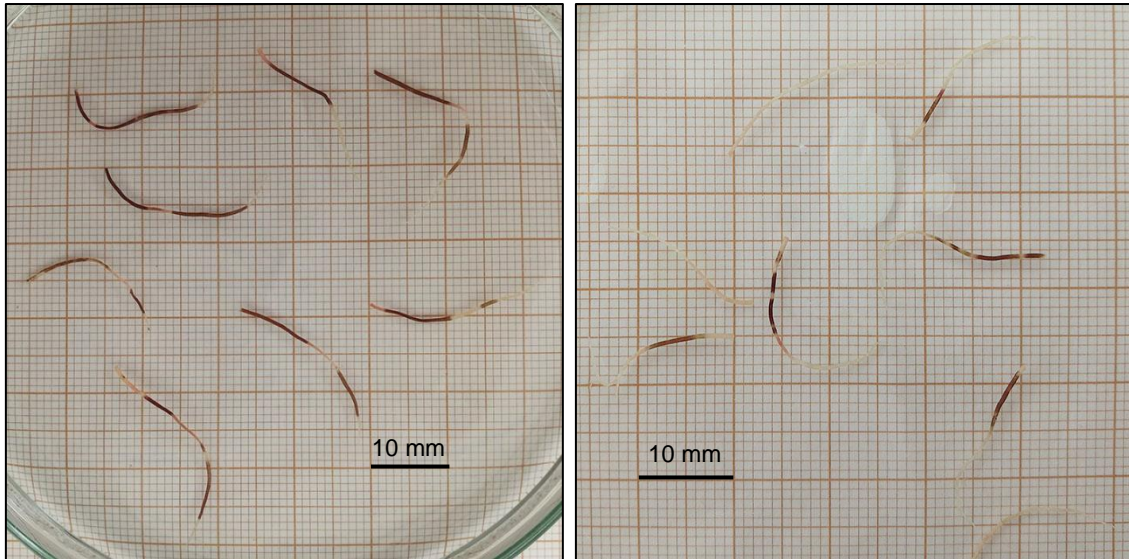


Figure 2.6: *Gracilariopsis longissima* tips photographed at the initial weeks of the 5 (left) and 10‰ (right) salinity essays. The observed discoloration correspond to a necrotic part and confirms the inability of the seaweed to thrive in these salinities.

The present study shows that *G. longissima* growth rates were expressively reliant on salinity changes. *G. longissima* appears to be better adapted to the highest salinity studied (35‰), shown by the highest daily growth rate and the number of new ramifications counted, yet it is shown that at 20 and 25‰ these organisms are capable of survival and even to perform vegetative reproduction. At 30‰, *G. longissima* seems to have not invested energy on reproduction, as it does not produce new ramifications. Such differences may be attributed to adaptation to the habitat; considering that Lagoa de Óbidos is subjected to wide shifts in salinity, with values ranging from 17 to 38‰, it is therefore expected that *G. longissima* has the ability to adapt itself and grow in a wide range of salinity to respond to such abiotic shifts. However, in the natural world lesser growth rates as a consequence of a non-optimal salinity may not allow any given species to compete successfully (Bird and McLachlan 1986). Most wild populations of marine algae are mainly confined to the intertidal region, being thus subjected to wide variations in salinity, temperature and light. Indeed, these abiotic factors have been reported to have a key role in shaping the growth and distribution of benthic marine algae (Lüning 1990; Raikar et al. 2001; Nejrup and Pedersen 2012). Specifically, salinity determine the local and regional distribution of macroalgae in coastal areas, and is subject to sudden and wide shifts in response to precipitation, tides, and wind. Generally, waters from tidal pools and semi-closed inlets are hypersaline, while the inner regions of estuaries are mainly hyposaline (Nejrup and Pedersen 2012).

The seaweed *G. longissima* from Lagoa de Óbidos is, however, and unlike most benthic marine algae, a specific case in which the organism is not only confined to a semi-enclosed, hypersaline area, but is also particularly sheltered from light as it is kept within a muddy substratum under a dense layer composed by the seaweed sea lettuce (*Ulva lactuca*). In the present study, the specific site where *G. longissima* was collected suffered deep fluctuations in salinity (from 17 to 38‰), while turbidity, substratum nature, and the presence of a *U. lactuca* layer determined the reduced light intensity received by *G. longissima* growing beneath. As such, and similarly to estuarine seaweed species, *G. longissima* seems perfectly adapted to such fluctuations, possibly having either an enhanced tolerance/elasticity to turgor pressure or cell volume changes, or the ability to perform osmotic acclimation mechanics, such as the reestablishment of the composition of cells (either by transport of ions across the cell membrane, or by the synthesis or degradation of organic osmolytes) (Kirst 1990). Such defence mechanics successfully counteract the osmotic gradient and prevent the irremediable damage caused by osmotic shock, such as cell wall disruption, impaired membrane functionality, and enzyme kinetics disturbance (Kirst 1990). However, it must be noted that such osmoregulatory processes come with an energy cost, which would otherwise be spent on growth, reproduction, and other essential metabolic processes if the seaweed was growing under the optimal salinity conditions as observed in previous studies (Hayashi et al. 2011; Nejrup and Pedersen 2012; Lawton et al. 2015). This fact stands out as a possible explanation for the lower growth values and fewer (or none at all) new ramifications obtained at salinities below 35‰: as a response to osmotic stress due to lower salinity values, *G. longissima* slew down growth and multiplication and spent energy to maintain the osmotic balance instead. Yet at extreme low values, such as 15‰ and below, such strategy only guarantee survival for a short period of time, according to Hayashi et al. (2011). In the present study, such particular survival tactic might have occurred more often in the few days following the seaweed transference to salinities progressively lower or higher, finally reaching a point of no recovery in extremely low salinity values. In light of such findings, determining not only the salinity growth range but also the optimal value (35‰) was crucial to the growth assessment of *G. longissima* under different salinities, performed in the present work, and will be of utmost importance when evaluating the yield, quality, and prospective value of the metabolites it produces.

Similar findings were also found for other Gracilariaceae by authors who studied both *Gracilaria* and *Gracilariopsis* species grown under different salinities, either in a controlled laboratory environment (Wilson and Critchley 1997; Israel et al. 1999; Raikar et al. 2001; Choi et al. 2004; Skriptsova and Nabivailo 2009; Hayashi et al. 2011; Nejrup

and Pedersen 2012), or in outdoor culturing (Israel et al. 1999). Specifically, Raikar et al. (2001) tested the influence of varying salinities to the growth rate of several *Gracilaria* species, from both tropical as well as temperate regions, finding a varied response which depended of the species considered; overall, however, and except for two isolates, *Gracilaria* spp. from temperate regions did not tolerate salinities below 15‰, while presenting abrupt increase in their growth rate at salinities between 20 to 30‰. Salinities above 30‰ were not considered by the aforementioned study. Skriptsova and Nabivailo (2009) studied both *Gracilaria* and *Gracilariopsis* species (*Gracilaria gracilis*, *Gracilaria tenuistipitata* and *Gracilariopsis bailinae*), finding that the former genus is tolerant to low salinities (10‰) whereas the latter died; the authors also determined that the fastest growth rate for *G. bailinae* was detected for 20-30‰, with values ranging from 4 to 5%.day⁻¹ increase in weight. Nejrup and Pedersen (2012) found out that *Gracilaria vermiculophylla* has an optimal growth above 15‰, and a reduced growth when exposed to lower salinities, although salinities 5‰ and below proved to be stressful to this seaweed; the authors also report that *G. vermiculophylla* growth rate was higher when placed at constant salinity, when compared to organisms growing under salinity variations. Studies specifically pertaining *G. longissima* growth performance at different salinities were not found to date.

Seaweeds often exhibit a characteristic growth, marked by a rapid initial phase followed by decreased growth rates during later stages, as the photosynthesis rate decreases due to shelf-shading of the inner parts during growth. Therefore, in order to accurately determine the growth rate of a given seaweed, Yong et al. (2013) recommend to adopt as short as a weekly time interval between data collecting, while also stating that a daily regime for data calculation should be carefully considering due to loss of precision as a result of the high sensitivity of seaweed growth to their environment. In the present study, the full 44 days were adopted as a single time interval, where only the initial and final lengths of the seaweed were gathered, in order to avoid subjecting the small and delicate seaweed fragments to stress and eventual contaminations. Optimization of the methods used must be hence considered to collect data on a weekly time interval, while reducing seaweed transport and handling to a minimum.

In the present study, individuals always grew through vegetative propagation, which is a form of clonal propagation where all the new fronds grew from one single frond. This will result in a culture with genetically identical individuals, that may be useful to achieve consistency in production (Yarish et al. 2012). However, this also results in unattached, free-floating forms, that are not suitable for some culture methods that require some form of attachment to a surface such as the suspended line culture (Yarish et al. 2012).

Moreover, the necessity to trigger the formation of reproductive structures may arise in cultures, in order to bring the life history of individuals into completion (Rueness 2005). In the present study, no individuals carrying carpospores were observed or collected regardless of season. Carpospores are fruiting bodies present in female gametophytes, resembling small dark round bumps scattered along the thalli, and from where new individuals are born as a product of sexual reproduction (Yarish et al. 2012). Thus, the success rate of *G. longissima in vitro* sexual reproduction, which would allow the development of a pure axenic culture from a single individual, the completion of its life cycle in culture, and its attachment to a substratum on certain large-scale culture systems, remains to be tested against the success of *in vitro* vegetative reproduction.

Control of Contaminants

Fungi Control

One of the main factors that can swiftly and easily destroy cultures if left unchecked, is contamination by fungi, a problem that can only be tackled pre-emptively; there seems to be no way to remediate a fungal contamination, except to restart the culture from the beginning (Redmond et al. 2014). Alternative solutions to address such contamination are thus tremendously required.

After two days of incubation in potato dextrose agar (PDA), it was visually confirmed by microscopy that fungus still lingered upon a number of seaweed thalli, and no connection between the number of infected thalli and the type of fungicide agent was found. At least one replicate for each fungicide tested presented a certain degree of contamination, although the extent of the infection was less prominent on the seaweed thalli previously treated with Nystatin, regardless of concentration. The hydrogen peroxide was the only agent that killed every seaweed tested before the end of the 4 weeks trial, while failing to completely eradicate the fungi.

Moreover, after the incubation period in PDA media, it was observed a thick, opaque and seemingly glossy layer of a dark fluid bordering the thalli surface in a number of seaweed (Fig.: 2.7), again unrelated to the fungicide agent previously used, as at least one replicate per fungicide tested showed this aspect. The prominent black colour exuding from the algal tissue was not further tested to assess its identity, however, the currently standing hypothesis supports the substance as being melanin, oozed by lingering fungi present on the algal tissue, and a consequence of a phytofungal infectious mechanism, further explained below. This finding is supported by descriptions and images found in previous works only, therefore it stands merely as a hypothesis that requires extensive further testing.

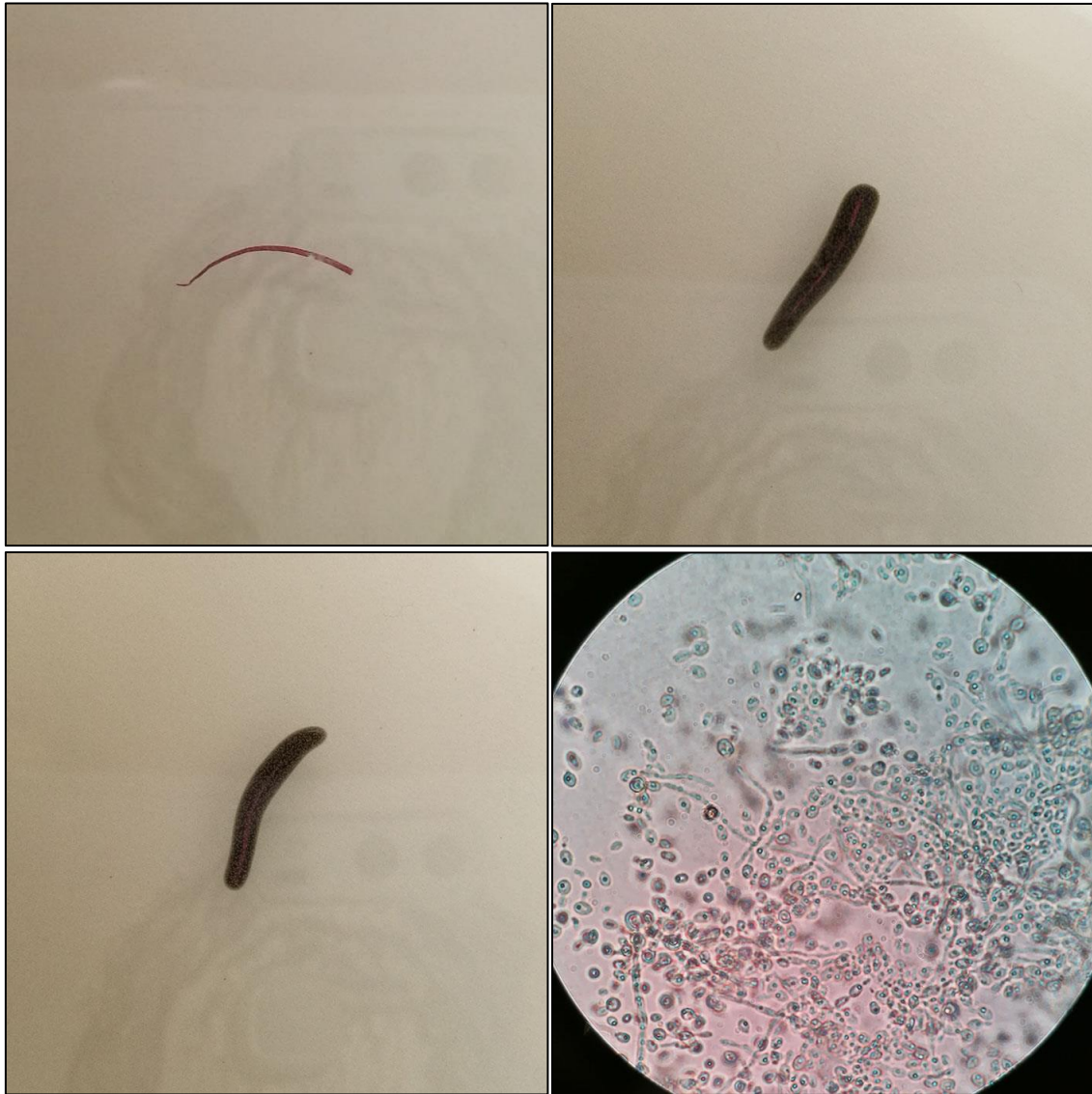


Figure 2.7: Representative images of *Gracilariopsis longissima* thalli following the two days of incubation in potato dextrose agar, after a period of (upper left) continuous treatment with Nystatin supplemented to the seawater at 1 mL.L⁻¹, (upper right) one-time treatment hydrogen peroxide and Nystatin (5 mL.L⁻¹), and (bottom left) Tocsin WG (1 g.L⁻¹) and Nystatin (5 mL.L⁻¹). A representative image obtained by microscopy (400 x) shows a section of a heavily infected thallus (bottom right).

Melanins are a large group of biologically important natural pigments, often brown or black in colour, that can be synthesized by members of all the biological kingdoms (Nosanchuk et al. 2015), and are involved in the protection against ultraviolet radiation (Eisenman and Casadevall 2012). They are formed by oxidative polymerization of phenolic or indole compounds (Langfelder et al. 2003), but further details on their chemical structure remain unanswered, due to its ubiquity, large size, insoluble nature, and heterogeneity, which prevents its study by classical biochemical methods (Eisenman and Casadevall 2012). Melanins are known to be synthesized by fungi since the early

1960s, although its involvement in infectious processes was only recently understood (Langfelder et al. 2003). Plants are reportedly infected by phytopathogens whose infection mode rely on the melanin ability to build enhanced pressure in the appressoria, which is a specialized fungal plant cell essential in the infection mechanism upon the host (Deising et al. 2000). Briefly explained, the fungal spore attaches and germinates onto the plant surface, and following the appressorium maturation, a dense layer of melanin is laid down upon this cell wall, the turgor pressure inside increases and promotes the emergence of a hyphae at the pore, which pierces through the host cuticle into the epidermal cells (Howard et al. 1991). Further insight on details such as whether the substance detected is indeed melanin and a consequence of a fungal infection upon *G. longissima*, and if so, what is its nature (DHN-melanin or DOPA-melanin according to (Langfelder et al. 2003)), can only be performed after identifying the fungus itself.

Nevertheless, if these hypotheses are confirmed, it means that the fungi remained attached to the seaweed and was not completely eradicated by the fungicide agents presently tested. These agents not only failed to completely clean the infection, but also caused detrimental effects upon the seaweed, leading to its death in a number of replicates. This outcome may have been caused by the toxicity due to the dosage applied which was, however, never higher than what was recommended by the manufacturer, taking into account that the instructions were originally meant to be applied on different contexts, namely agricultural or pharmaceutical. These agents may have, nonetheless, a preventive effect on the aggravation of fungi infections and thus have an important role in maintaining healthy cultures. It is important to keep in mind, however, aspects pertaining the dosage and frequency of the treatment even when finding an effective fungicide agent, as its toxic nature may be potentially dangerous to human health upon seaweed use. In the present study, the inability to find the most effective fungicide agent as a result of inconsistency in the results, led to choosing the nystatin product as a preventive agent, due not only to its lower toxicity associated to its intended use as a human fungicide, but also due to its visually unobserved side effects upon the seaweed. None of the seaweeds engaged in the salinity trials showed fungi contaminations along the 44 days, following the first week treatment with nystatin, leading to the hypothesis that this agent has a key role in maintaining a clean culture. Further testing needs to be performed in order to determine if treatment with this fungicide influences the growth and yield of *G. longissima* under ideal culture conditions.

It is especially worth mentioning that although fungi contamination did not constitute an issue throughout the salinity trials, a preliminary assay performed previously and in the scope of the present study, showed that *G. longissima* is quite prone to contaminations

when the initial stages are growing inside sealed Petri dishes (an approach suggested by Yarish et al. (2012) on the start-up *Gracilaria* cultures), instead of being placed into culture flasks and under constant aeration right from the start. Inside Petri dishes, with a weekly media change and under the exact conditions applied for the salinity trials, the seaweed became contaminated after roughly two weeks, leading to the hypothesis that the aeration itself has a prominent role in preventing the proliferation of fungi. Aeration is a critical component of any culture, as not only it increases the availability of carbon dioxide and stabilizes pH values, but also simulates natural water currents, which promote the seaweed suspension, prevent self-shading, increase light exposure, and ultimately enhance the photosynthetic process (Yarish et al. 2012). In the present work, it is also suggested that the resulting air bubbles from the aeration, by keeping both the seaweed and water continuously in motion, may have prevented the adherence and proliferation of eventual contaminants upon the seaweed tissue.

Epiphyte Control

Algal epiphytes are ubiquitous, naturally occurring among natural seaweed populations, and play an important ecological roles in coastal benthic communities (Potin 2012). They constitute, however, a major concern in mariculture as they affect the growth and productivity of the host, eventually destroying cultures and causing extreme economical losses. This problem has given grounds on recent research on the matter (Hensley et al. 2013; Anderson and Martone 2014). Epiphytes can belong to either Chlorophyta, Phaeophyceae, and Rhodophyta macroalgae groups, but diatoms and cyanobacteria are also capable of epiphytism (Pickering et al. 1993). The epidemiology behind epiphytism can occur in several ways according to the extent of the infection, namely (1) weak or strong attachment to the thalli surface without further damage upon the host tissues, (2) infiltration into the outer layer of the host wall without further damage upon the cortical cells, (3) deep infiltration into the host cell wall and disruption of cortical tissues, (4) and deep penetration into the cortex and medullary tissue with further destruction of the host cells (Leonardi et al. 2006).

In the present study, a number of start-up cultures were affected by epiphytism, a phenomenon illustrated by figure 2.8, which shows a red epiphyte growing attached to the thalli of *G. longissima* tips. This contamination constituted a problem which heavily affected the cultures standing at 30‰, therefore a few solutions were sought out to eliminate the contaminant, based on herbicide treatment and light intensity. The epiphyte persisted even after treatment with the contact herbicide diquat (200 g.l⁻¹ diquat dibromide, Selectis, Setúbal, Portugal) tested at several diluted solutions starting with the initial concentrations of 2 and 5 ml.l⁻¹, the latter proving to be lethal for *G. longissima*

after one week, whereas the epiphyte remained healthy. Assays performed by halving the light intensity upon the contaminated cultures did not yield satisfactory results as both the epiphyte and host persevered, whereas doubling the light intensity proved to enhance even further the growth of the epiphyte while ultimately killing the host.

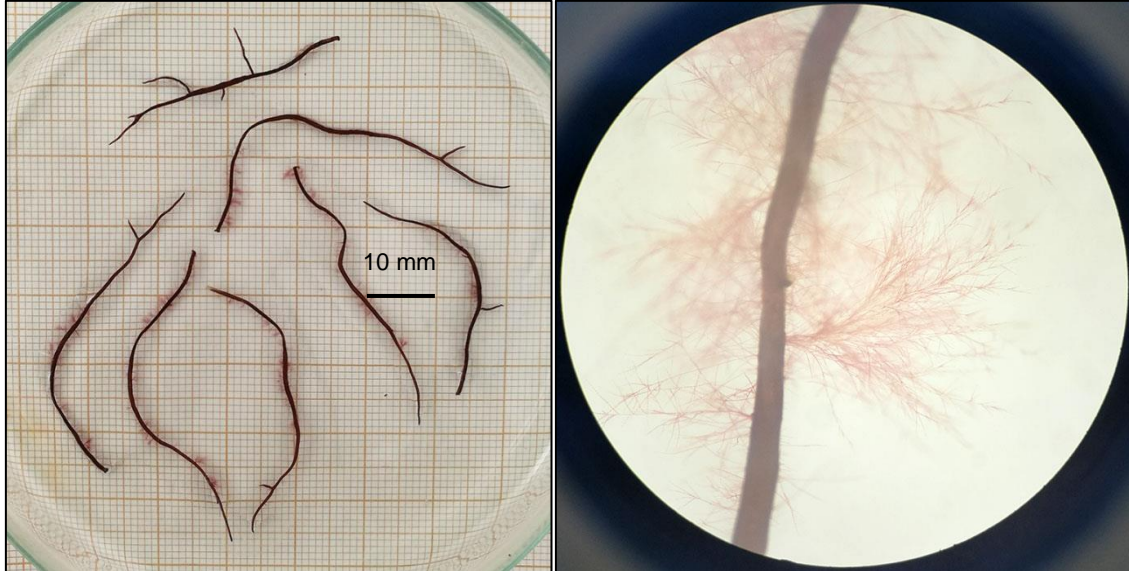


Figure 2.8: *Gracilariopsis longissima* thalli (darker and thicker filament) infected by an unidentified red epiphyte (several slim, pale red filaments attached to the darker thalli), placed upon milimetric paper (left) and under a stereomicroscope, amplification 17x (right).

Common red macroalgae epiphytes affecting Gracilariaceae have already been identified by previous research, namely the genera *Polysiphonia*, *Antithamnionella*, *Chondria*, and *Ceramium* (Kuschel and Buschmann 1991; Pickering et al. 1993; Fletcher 1995; Muñoz and Fotedar 2010). Although the epiphyte detected in the present work remains unidentified, it is expected to be a Rhodophyta from the Order Ceramiales, due to its prominent red colour, small branched filaments and perfect adaptability to the very same conditions to which *G. longissima* thrives, namely temperature, salinity, and nutrient selection. The epiphyte contaminated cultures kept between 25 and 35‰, showed higher incidence at 30‰, but seemingly absent from cultures kept at 20‰ and below. However, and although the contaminated organisms were promptly discarded and considered in neither the growth nor the number of ramifications assays, it was not possible to determine with certainty if the epiphyte was nevertheless infecting the inner tissues, even if no attached forms were visually observed upon the thalli. A visually hidden infection mode might explain why no new ramifications were detected for the seaweed growing at salinities of 30‰, although its growth was higher than for those seaweeds kept in lower salinities. The organisms kept at 30‰ might have possibly

invested their energy on defence processes against the invasive, yet unseen epiphyte, which in turn was also depriving the host from the nutrients that were provided to it on a weekly basis. Therefore, identification of both the epiphyte and its epidemiological action is of paramount importance, to further understand its effect upon the host, and ultimately allowing the execution of management tools for any given cultivated seaweed (Leonardi et al. 2006).

Additional Considerations

In the natural environment, low light do not usually limit the growth and development of *Gracilaria* species, trait determined by the turbid waters that usually illustrate their habitat, according to Beer and Levy (1983). Considering the particular light conditions in which *G. longissima* from Lagoa de Óbidos thrives, stated above, it is put forward, in the present study, the hypothesis that the same may be applied to *Gracilariopsis* species as well. Nevertheless, it is essential to perform growth studies at different light intensities to access the range at which *G. longissima* presents its optimal growth. Raikar et al. (2001) detected an increase in growth rates for all *Gracilaria* species considered, with the optimal light range varying with the species considered. While growing luxuriantly in the intertidal zone and also in pools (Boras de Zaiuso 1987), young thalli are not commonly seen in tide pools, fact clarified by Jones (1959) who reported that exposure to full light in tidal pools has deleterious effects on *Gracilaria*, especially on young organisms. In the present study, *G. longissima* grew under cool white lighting, which has been regarded as the best light source for most seaweed growth systems (Redmond et al. 2014); however, light emitting diode (LED) technology will be regarded as being the most economic and efficient light source for indoor seaweed cultivation, playing a role not only in growth rates but also in production yields, as anticipated by Kim et al. (2015) who studied the influence of mixed colours and combinations of both fluorescent and LED lightings on the growth and pigment content of *Gracilaria tikvahiae*. The selective use of wavelengths to control pigment production and morphogenesis is unquestionably fascinating, yet the authors also bring to attention that studies regarding LED light sources in algal research are still scarce. In this sense, *G. longissima* growth and yield production under the influence of selective LED wavelengths is certainly a topic worth exploring.

It is worth mention as well the role of the culture media in indoor cultivation of seaweeds. Seaweeds require a wide selection of nutrients to grow, divided into three categories: macronutrients, micronutrients or trace elements, and vitamins. Nitrogen (N) and

phosphorus (P) are critical macronutrients required by seaweeds, limiting growth and yields in nature. In aquaculture, the addition of N and P to a system causes carbon (C) to become limited, being thus mandatory to add this macronutrient as well. In indoor cultivation of red seaweeds, the nutrient media von Stosch enrichment medium (VSE) is commonly used (Kim 2012; Kim and Yarish 2014; Redmond et al. 2014), and it was the current choice for the present study. VSE contains several important nutrients including the macronutrients nitrate and phosphate, the micronutrients iron and manganese, EDTA, and the vitamins B₁₂, thiamine, and biotin. However, as pointed out by Kim and Yarish (2014), when considering a commercial scale, VSE is not a feasible nutrient media due to its high price and time consuming costs, therefore alternatives must be considered. Both *Gracilaria* and *Gracilariopsis* species have a high bioremediation potential (Vergara et al. 1995; Hernández et al. 2006; Yarish et al. 2012; He et al. 2014), therefore alternative nutrient sources such as culture waste from fish or shellfish, sewage, or fermentation residue from anaerobic digesters, as suggested by Yarish et al. (2012), are well worth considering. Future testing of common and commercially available plant fertilizers is also a possibility to ponder, not only to assure economically viable options, but also to assess whether there are variations in *G. longissima* growth and nutritional profile reflected by the use of distinct nutrient media. A similar study was performed by Kim and Yarish (2014) where they report the effect of distinct commercial fertilizers in the growth of *Gracilaria tikvahiae*, finding that parameters such as growth, productivity, tissue C and N contents, and C : N ratio are significantly affected by nutrient conditions.

Seaweeds presenting fast growth rates provide competitive and economic advantage in algal culture, being one of the most relevant features when selecting species for commercial farming (Borowitzka 1992) . The first steps towards a successful commercial farming of *G. longissima* have been thus given in the present study, at a laboratory scale, by evaluating its growth rate and performance at a wide range of salinities (0 to 35‰). The high salinity tolerance observed for *G. longissima* establishes this red seaweed as a potential candidate not only for indoor, but also outdoor culture in temperate regions, being its cultivation possible at sites where salinity fluctuations take place, such as river mouths, semi-enclosed systems, and also in the open sea; however, care has to be taken in outdoor culture scenarios, where extended rain periods may decrease the salinity to values not tolerated by the seaweed (Macchiavello et al. 1998). Being a native species of Lagoa de Óbidos, *G. longissima* also holds the advantage of posing no environmental risk that is commonly associated with the introduction of alien seaweed strains into local environments, consequently posing serious ecological threats to native

species, habitats, and ecosystems. Examples of such cases are the well documented case of the red seaweed *Asparagopsis armata*, a species endemic to the southern hemisphere and native to Australia, but introduced into the Atlantic and Mediterranean in the 1920s, and even currently cultivated in seaweed farms in Ireland (Kraan and Barrington 2005); or *G. vermiculophylla*, originally described from Japan and native of Asian intertidal zones and river mouths, but recently reported from European waters (Rueness 2005). The abovementioned species are both reportedly observed in Portuguese shores as well (Rueness 2005; Araújo et al. 2009; Abreu et al. 2011a; Lopes et al. 2011).

In the present study, growth performance and response of *G. longissima* was determined by the growth rate formula stated by (Mtolera et al. 1995; Gerang and Ohno 1997; Aguirre-Von-Wobeser et al. 2001; Bulboa et al. 2007; Hayashi et al. 2007a; Hayashi et al. 2007b; Hung et al. 2009; Hayashi et al. 2011). However, in the assessment of production efficiencies, Lapointe and Ryther (1978) report that seaweed product yield is more significant than daily growth rate, and a number of authors (Skriptsova and Nabivailo 2009; Hayashi et al. 2011; Lawton et al. 2015) found out that higher growth rates do not necessarily parallel with higher production yields. Generally, for any given seaweed species, growth rates and product yield profile are affected by the same factors (Yong et al. 2013). Seaweed metabolism is shaped by parameters such as temperature, salinity, light and nutrients, which force the organisms to adjust to the ever-shifting natural conditions. Consequently, seaweed often produce a wide variety of biologically active secondary metabolites in order to thrive successfully (Plaza et al. 2008). Therefore, a full nutritional assessment should be performed to seaweeds kept at different conditions, such as light, temperature and salinity, to find whether an optimal growth does correspond to its maximum nutritional value. As a dynamic system, aquatic environments are subjected to temperature, salinity and light intensity shifts. Response to these abiotic factors is a fundamental assessment that must be carried out when harvesting and selecting fast growth individuals for culture systems.

Conclusion

The first steps towards a successful cultivation of *G. longissima* have been given in the present work, containing a throughout assessment of its growth and vegetative reproduction performance under a wide range of salinities. The present study also identifies obstacles that might arise when starting and keeping a healthy culture, such as fungi and epiphyte contaminants, providing preventive solutions and further

approaches that may have to be adopted in order to prevent the loss of a culture. To further optimize *G. longissima* growth rates however, it is crucial to understand the nutrient physiology of the species, with focus on nutrient assimilation, storage, and uptake rates, critical tissue nutrient concentrations, and growth rates (Harrison and Hurd 2001). Such concepts are applied in culture systems in order to manipulate growth rates by changing nutrient loading rates (Smit et al. 1997; Navarro-Angulo and Robledo 1999), control epiphytes by nutrient pulsing (Friedlander et al. 1991; Pickering et al. 1993), and enhance compound formation by nutrient manipulation (Dawes 1995; Vergara et al. 1995). A successful seaweed cultivation thriving under the most optimal conditions not only guarantees that the maximum growth rate is achieved, but also preserves the yield and quality of the metabolites produced. Further studies will highlight temperature and light ranges that must be considered when regarding the ideal culture conditions that must be applied for a fruitful growth and development of high-quality, healthy specimens.

Chapter Two

The nutraceutical profile of *Gracilariopsis longissima* (Rhodophyta, Gracilariales), a natural source of potentially valuable compounds

Abstract

Seaweeds have been acknowledged as a natural source of compounds with potential relevance in several fields of science and health, drawing the attention of researchers in recent years. In the present study, a comprehensive assessment of the nutritional value (ash, protein, and lipid profile), antioxidant capacity (total phenolic content and DPPH radical scavenging activity), and antimicrobial activity (by the agar diffusion assay) of *Gracilariopsis longissima* collected from Lagoa de Óbidos, Portugal, was performed, with insights regarding seasonal variability also included. Results indicate that *G. longissima* has a low content in ash (7.05% in dry weight), a variable protein content according to season (11.19% of dry weight in July, 27.04% of dry weight in January), and a total fat content of 1.67 g.100⁻¹ g dry seaweed. Arachidonic acid methyl ester (20:4 ω6) stood out as the prevalent polyunsaturated fatty acid (43.84% of total FAs), while palmitic acid methyl ester (16:0) was the predominant saturated fatty acid (36.78% of total FAs). Regarding antioxidant activity, *G. longissima* generated low amounts of phenolic content (ranging from 0.54 to 1.90 mg GAE.g⁻¹, according to month), and a weak scavenging activity was detected (ranging from 3.93 up to 8.92%, according to month). *G. longissima* possesses an interesting antibacterial activity against all the bacteria considered, namely *Escherichia coli*, *Bacillus subtilis*, and *Vibrio alginolyticus* (inhibition halos with 9-10, 14-15, and 15-16 mm, respectively), with no differences detected between seasons for any bacteria studied. Overall, *G. longissima* stands as a potential natural source of compounds, with its nutritional value demonstrated by its high protein and low lipid content, while holding promise as well as an antibacterial agent, being thus relevant for a wide range of biotechnological applications. However, in order to consider any recommendations pertaining the regulated adoption of *G. longissima* as a nutraceutical agent, further tests are required to assess the bioavailability of its nutrients and phytochemicals, as well as to evaluate the eventual toxicity that can be associated to the compounds present in this algal source.

Keywords: ash, total proteins, lipids profile, antioxidant, antibacterial, seasonality.

Introduction

Seaweeds are globally distributed, thriving in cold, temperate, and tropical zones, playing vital roles in sustaining the biodiversity and functional ecology in marine ecosystems (Bracken and Williams 2013). Algae have a great taxonomic diversity, and often live under the influence of continuously shifting environmental variations such as light, salinity and temperature; some species also thrive in complex habitats, and under extreme conditions, which shape their metabolism to provide the ability to adapt to these conditions. Adaptation mechanisms often include the production of a wide variety of biologically active secondary metabolites, rendering seaweeds as an interesting natural source of compounds potentially valuable as functional ingredients (Plaza et al. 2008). The research of the nature, quality and quantity of the compounds produced by such a diverse group is grounds for limitless and encouraging prospects and possibilities, being still a vast untapped field to the date (Lordan et al. 2011).

Nevertheless, seaweeds have been explored since the dawn of human existence as a healthy nutritional source, and they are nowadays known to contain dietary fibers, carbohydrates, minerals, vitamins, and essential amino acids, while having low caloric content (Holdt and Kraan 2011; Lordan et al. 2011; Pereira 2011; Pereira 2016). Seaweeds do also have a prominent role in nutraceutical and cosmeceutical industries, being rich in phycocolloid compounds such as carrageenan, alginate, and agar, which are the primary metabolites most widely explored, due to their importance as gelling, thickening, and stabilizing agents (Bixler and Porse 2011; Carvalho and Pereira 2015; Pereira 2016). Other primary metabolites are polysulfated polysaccharides such as fucoidan and galactosyl glycerol, among others, with confirmed biological properties such as antibacterial (Kasanah et al. 2015; Pérez et al. 2016), antifungal (Peres et al. 2012), antiviral (Chen et al. 2013), antioxidant (Yang et al. 2012; Yeh et al. 2015; Pinteus et al. 2017), anti-inflammatory (D'Orazio et al. 2012; Lee et al. 2013), and anti-tumour activities (Horta et al. 2014; Rodrigues et al. 2015a), and anticoagulant activities. Seaweeds also produce important secondary metabolites such as carotenoid, xanthophyll, chlorophyll, and phycobiliprotein pigments (Rowan 2011), polyphenols (Sabeena Farvin and Jacobsen 2013), terpenoids, vitamins (Škrovánková 2011), alkaloids, and fatty acids (Kumari et al. 2013; Schmid et al. 2014). The abovementioned research concerns a particular group of compounds, yet bibliography is also rich in stimulating research concerning the overall diversity and benefits of marine bioactive compounds obtained from seaweeds (Holdt and Kraan 2011; Brownlee et al. 2012; Domínguez 2013; Ibañez and Cifuentes 2013; Kadam et al. 2013; Andrade et al. 2013; Brown et al. 2014; Cardoso et al. 2014; Fan et al. 2014; Cardoso et al. 2015; Hamed et

al. 2015; Rodrigues et al. 2015b; Fleurence and Levine 2016). This diverse biochemistry is known to present interspecific, intra-annual and inter-annual variability (Khairy and El-Shafay 2013).

The overall chemistry of red seaweeds is known to be more diverse than in other algae groups (Kasanah et al. 2015), and a number of authors suggest Rhodophyta are the most nutritionally rich species regarding carbohydrate, protein, and energy value (Renaud and Luong-Van 2006). Red seaweeds have the widest diversity in secondary metabolites, being thus the most important source of biologically active metabolites in comparison to green and brown seaweeds. Rhodophyta are the main producers of halogenated compounds (Cabrita et al. 2010), with a remarkable diversity of biological properties such as antibacterial, antifungal, antiviral, antitumor, anti-inflammatory, antioxidative, anticoagulant, ichthyotoxic, cytotoxic, insecticidal, antiproliferative, antifouling, and antifeedant activities (Blunt et al. 2012). Additionally, red seaweeds are reportedly a good source of protein, rendering it as a worthy candidate in the search of new sources to tackle protein malnutrition issues (Pereira 2016). Moreover, the specific structure and function of phycoerythrin, the main light harvesting protein-pigment of photosystem II in Rhodophyta, renders it as an extremely valuable component for a wide range of application, such as a fluorescent probe and food colorant (Dumay et al. 2014).

Furthermore, all living organisms require lipids as components of membranes, energy storage compounds, and as cell signaling molecules (Wells et al. 2017). In human nutrition, health concerns and the wish of leading a healthier lifestyle by the general public, have led into a growing interest on vitally important functions of lipids and fatty acids, which can be classified into saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and the long-chain polyunsaturated fatty acids (PUFA). Consumption of food with high content in saturated fatty acids is generally associated with the development of cardiovascular diseases, although recent research accuses carbohydrates as the main responsible for this condition (Hamed et al. 2015). Seafood, however, is known to be substantial rich in mono- and polyunsaturated fatty acids, which are considered beneficial as long as they are not oxidized (Hamed et al. 2015). Particularly the polyunsaturated acids are found in significantly higher levels in seaweeds than in land vegetables (Gressler et al. 2010). Although vital to human health, polyunsaturated fatty acids cannot be synthesized by the human organism, and thus food constitutes the only source for this group of essential fatty acids (Lunn and Theobald 2006). Particularly, the omega-3 (ω 3) fatty acids such as eicosapentaenoic acid (EPA, 20:5 ω 3) and docosahexaenoic acid (DHA, 22:6 ω 3), with confirmed benefits to human health, have a suppressive effect onto medical disorders such as coronary artery

disease, hypertension, cancer, depression, inflammatory and autoimmune diseases, obesity, and mental illnesses (Simopoulos 2008; Wall et al. 2010; Cottin et al. 2011; Schmid et al. 2014; Röhrig and Schulze 2016; Simopoulos 2016), stressing the importance of adopting a balanced ω_3/ω_6 ratio in diets; in this sense, macroalgae have generally a quite balanced ratio and therefore value as a healthy component in diets.

Seaweeds are a source of bioactive compounds useful as antioxidant agents which are also present in human skin and possess innate defense mechanisms against the oxidation of lipids and proteins in the cells, and the ability to prevent DNA damage, s. Such agents are able to scavenge reactive oxygen species (ROS), and reactive nitrogen species (RNS), avoiding thus cell deterioration (Xu et al. 2017). However, such skin natural defenses can be overrun by oxidative stress, triggered by ultraviolet radiation, alcohol, smoking, or environmental toxins, which prompt the production of excessive ROS and RNS (Masaki 2010; Xu et al. 2017) with adverse effects to the organism. Examples of such effects are damage to protein and lipid membranes, DNA breakdown, and apoptosis/necrosis induction; these consequently lead to photoaging complications, from basic skin wrinkling and dryness to inflammation, acne lesions, melanogenesis, skin cancer, and chronic and degenerative diseases (Masaki 2010; Wang et al. 2015).

Finally, there are several venues to trail in the fight against infectious diseases, with protective agents such as vaccines and antibiotics commonly made available by pharmaceutical industries. However, new antimicrobial resistance mechanisms against such agents are increasing each year, emerging and spreading globally, due to either natural resistance, genetic mutation, or acquired resistance of the infectious organism due to misuse and overuse of antimicrobials (WHO 2016). There is thus an urgent need for novel antimicrobial agents, from new and alternative sources, to be at disposal in the combat of emerging and reemerging infectious diseases. In this sense, the marine environment may provide a great diversity of potential sources of bioactive compounds with interesting activities (Blunt et al. 2012; Hamed et al. 2015), and marine algae constitute a generous portion of this diversity. The value of seaweeds as therapeutic agents has been the focus of published studies since the 50s (Pratt et al. 1951), and seaweeds currently stand as a worth exploring source of novel bioactive compounds in health and disease prevention (Brown et al. 2014; Fleurence and Levine 2016).

A thorough assessment of the nutritional profile and chemical composition of any potential food source is mandatory, before being authorised to be used for human consumption. Regulation concerning the use of seaweeds for this purpose was first established by France (Mabeau and Fleurence 1993). Portugal holds specific regulations

for seaweed harvesting (Ministério da Marinha 1964) and farming (European Union 2009; Governo da República 2017). Human and animal consumption of seaweeds in Portugal is currently regulated at an European level (European Union 1997), acknowledging merely 22 seaweed species as edible. However, the Portuguese shores are home to several extensively studied macroalgae species, and their nutraceutical value is widely acknowledged and strongly acclaimed (Lopes et al. 2011; Mendes et al. 2013; Andrade et al. 2013; Rodrigues et al. 2015a; Rodrigues et al. 2015b; Raja et al. 2016; Pinteus et al. 2017). The objectives of the present studies were to perform a comprehensive study regarding the red seaweed *Gracilariopsis longissima* (S. G. Gmelin) (Steentoft et al. 1995) occurring in Lagoa de Óbidos, Portugal, in order to give the first steps towards a full evaluation of its (1) nutritional value, namely protein content and fatty acids profile, (2) antioxidant capacity, by detection of phenolic compounds and analysis of the DPPH radical scavenging activity, and (3) antimicrobial value by the agar diffusion assay, to assess the seaweed resistance against the pathogenic bacteria *Escherichia coli* and *Bacillus subtilis*, from food origin, and the marine bacterial pathogen *Vibrio alginolyticus*. Protein, antioxidant, and antimicrobial analysis were performed in samples collected throughout the year.

Materials and Methods

Sampling and Storage

G. longissima thalli were collected from Lagoa de Óbidos, in Caldas da Rainha, Portugal (39°24'18.93"N, 9°11'13.05"W) in April, July and October of 2016, January and February of 2017, during low tide and transported to the laboratory in plastic containers. The classification of this seaweed was based on *AlgaeBase* (Guiry and Guiry 2017) and confirmed by botanists. In the laboratory, each organism was first washed with running seawater and cleaned thoroughly to remove necrotic parts, epiphytes, and other organisms from the surface. Individuals were quickly rinsed with distilled water; healthy portions were selected either to be stored in plastic bags at -20°C, or to be freeze dried at -80°C (Scanvac Coolsafe 55-4, Frilabo, Portugal). The freeze-dried seaweed was shredded with a food processor, cautiously sealed in plastic tubes and stored at -20°C until further analysis.

Moisture, Organic, and Ash Content

Moisture, organic matter and ash contents were determined only for the February sample, as it was the only month when an adequate amount of biomass suitable for analysis was collected. Content in moisture was determined by drying a weighted portion

of fresh seaweed at 60°C (Binder, Tuttlingen, Germany) for at least 24 h; the moisture content was expressed as a percentage of fresh weight. Total ash was determined by incineration of the previously dried and weighted portions of algal material in a muffle furnace (Nabertherm, Lilienthal, Germany) at 450°C for at least 5 h, and the ash content was expressed as a percentage of dry weight (AOAC 2016). Due to the critical nature of the procedure, the assay was performed with eight replicas of the same sample.

Total Protein Content

Nitrogen content was determined for all the samples by the Kjeldahl method (Kjeldahl 1883), adapted from where crude protein content is estimated by multiplying the nitrogen content by a conversion factor of 6.25 for photosynthetic organisms (Greenfield et al. 2003; Denis et al. 2010; Munier et al. 2015; Rodrigues et al. 2015b; AOAC 2016). The protein is thus accurately determined, as the conversion factor is based on the quantification of total amino acids (Angell et al. 2016). Briefly, a previously weighted freeze-dried seaweed sample (0.5 g) was transferred to digestion tubes, where it was digested in sulfuric acid (ProLabo, Paris, France) with two catalyst tablets (VWR, Radnor, PA USA) (Panreac, Barcelona, Spain). This step was performed in a Kjeldahl digester apparatus (Foss™ Digester 2006, Hillerød, Denmark) for 30 minutes at 200°C followed by 90 minutes at 400°C, to let the nitrogen present in the sample to be converted into ammonia. After the sample was allowed to cool at room temperature, 80 ml of distilled water was slowly poured into the digestion tube, which was then distilled into a trapping solution in a Kjeldahl distillation apparatus (Foss™ Kjeltec 2006, Hillerød, Denmark). Finally, the ammonia was quantified by titration with 0.1 M hydrogen chloride (HCl, Merck, Darmstadt, Germany). Controls for all samples were achieved with sulfur acid and the catalyst tablets only, and all reactions were performed in triplicate. The protein content was calculated according to the following equation (AOAC 2016):

$$\text{Total Protein Content (\%)} = \frac{[(V_{\text{sample}} - V_{\text{control}}) \times n \times 6.25 \times 0.014]}{m} \times 100$$

where V_{sample} corresponds to the volume of HCl (ml) spent in the titration of the sample, V_{control} corresponds to the volume of HCl (ml) spent in the titration of the control, n corresponds to the concentration of the HCl solution used in the titration (mol.L^{-1}), and m corresponds to the initial weight of the seaweed sample.

Total Fatty Acid Content

Preparation of crude fat was done following a modified version of Bligh and Dyer (1959). Freeze-dried seaweed (2.5 g) was weighted and dissolved in 15 ml methanol:chloroform

(2:1 v/v), and vortex-stirred for 5 minutes afterwards. To the solution 2 ml of saturated solution of sodium chloride (NaCl) was added, before being vortex-stirred for 1 minute. Then, 5 ml of ultrapure water and 5 ml chloroform were added to the mixture, which was subsequently vortex-stirred again for 2 minutes, and followed by centrifugation at 6000 rpm, 4°C, during 10 min. Next, the bottom layer was carefully collected into an anhydrous sodium sulfate column, with a previously weighted vial placed underneath to recover the filtrated solvent. Next, the solvent was completely evaporated at 40°C (Laborota 4000, Heidolph, Schwabach, Germany), and the vial weighted afterwards. Total fat content was calculated according to the equation:

$$\text{Total Fat Content (g.100 g}^{-1}\text{ edible weight)} = \frac{F_w - I_w}{S_w} \times 100$$

where I_w is the initial weight of the vial (g), F_w is the final weight of the vial (g), and S_w is the freeze-dried sample weight (g).

Fatty Acid Profile

Crude fat (5 mg) was dissolved in 2.5 ml acetyl chloride methanol (1:20 v/v) and vortex-stirred until complete dissolution of the fat. The fat solution was then heated at 80°C for 1 h in a water bath, and 0.5 ultrapure water and 1 ml *n*-heptane were added afterwards, followed by centrifugation at 1500 x *g* for 5 minutes. The upper phase was filtered and collected into GC vials. The extract sample was tested in duplicate.

Total fatty acid profile was performed by gas chromatography quantification of their methyl esters (FAMES) for the February sample, with a Finnigan Ultra Trace gas chromatograph equipped with a Thermo TR-FAME capillary column (30 m x 0.25 mm ID, 0.25 µm film thickness) and a flame ionization detector. Helium was used as the carrier gas with a flow rate of 1.5 ml.min⁻¹, and air and hydrogen were supplied to the detector at flow rates of, respectively, 350 ml.min⁻¹ and 35 ml.min⁻¹. The injection volume was 1 µl and the injector, operating in splitless mode, and the detector were set at the temperatures 250 and 260°C, respectively. The column temperature profile was set at 60°C for 1 min, followed by a 15°C.min⁻¹ raise to 150°C and held for 1 min, followed by a 5°C.min⁻¹ raise to 220°C and held for 10 min. Peak integration and areas were determined with XCalibur™ software (Thermo Scientific™, Waltham, MA USA). FAMES were identified by the comparison of retention times acquired with those obtained by Simões et al. (2014). Fatty acid content (% total fat) was quantified by applying the corrective factor of 0.80 as proposed by Greenfield et al. (2003).

Preparation of Ethanolic Extracts

In order to access the antioxidant and antimicrobial activity of *G. longissima*, extracts of this seaweed had to be prepared in advance; in the present study, this was achieved by using ethanol as the organic solvent. Therefore, freeze-dried seaweed (1 g) was weighted, suspended in 20 ml ethanol 96%, vortex-stirred for 30 min, and then centrifuged at 10 000 rpm during 10 min. The supernatant was carefully transferred into a previously weighted tube afterwards, while the pellet was subjected again to suspension in ethanol, vortex-stirring, and centrifugation, as mentioned above. These steps were repeated until the supernatant showed a bleached colour, being thus discarded along with the pellet. The previously collected supernatants were then evaporated at 40°C (Heidolph, Schwabach, Germany) to completely remove ethanol residues, transferred into a previously weighted tube, re-dissolved in DMSO (VWR, Radnor, PA USA) at a concentration of 100 g.l⁻¹, and finally stored at -20°C until further analysis.

Antioxidant Activity

Total Phenolic Content

Total polyphenols content was determined for all the samples by colorimetric method of Folin-Ciocalteu proposed by Singleton et al. (1965) adapted to a microscale version, using gallic acid as standard. Briefly, 2 µl of *G. longissima* ethanolic extract was mixed with 158 µl ultrapure water, 10 µl of Folin-Ciocalteu reagent (PanReac, Barcelona, Spain), and allowed to stand at room temperature for 2 min. Then, 30 µl sodium carbonate solution (20% p/v) (Merck, Darmstadt, Germany) was added to the mixture, which was then incubated at room temperature, in the dark, for 90 min, and the absorbance read at 750 nm afterwards (Synergy H1 Hybrid Reader, BioTek® Instruments, Winooski, VT USA). A calibration curve was determined by measuring the absorbance of known concentrations of gallic acid solutions (0.01 to 1 mg.ml⁻¹) (Sigma-Aldrich, Darmstadt, Germany), following the above procedure as if it were a sample; from the calibration curve, the total content of phenolic compounds was quantified. Results are expressed as mg of Gallic Acid Equivalent per g of dry weight extract (mg GAE.g⁻¹). Controls were also executed, and all samples were performed in quadruplicate.

DPPH Radical Scavenging Activity

The DPPH compound (2,2-diphenyl-1-picrylhydrazyl) is a stable free-radical molecule, characterized by the delocalization of an unpaired valence electron over the whole molecule. DPPH has the ability to scavenge radicals, becoming neutralized when accepting hydrogen atoms from a donor; as this colorimetric reaction can be monitored

by spectrophotometry, the DPPH is widely known as a reliable monitor of antioxidant assays (Sharma and Bhat 2009; Raja et al. 2016). In the present study, the DPPH free radical scavenging ability of *G. longissima* was monitored according to an adapted version of Duan et al. (2006) into a microscale version. Concisely, 2 µl of the previously prepared seaweed ethanolic extract was added to 198 µl of a freshly prepared solution of DPPH radical (Sigma-Aldrich, Darmstadt, Germany) dissolved in ethanol (0.1 mM). The microplate sample was left to react at room temperature in the dark for 30 min, and its absorbance was read at 517 nm afterwards (Synergy H1 Hybrid Reader, BioTek® Instruments, Winooski, VT USA). All samples were performed in quadruplicate. The ability to scavenge the DPPH radical was determined by following the formula:

$$\text{Scavenging Effect (\%)} = 100 - \frac{\lambda_{\text{sample}}}{\lambda_{\text{control}}} \times 100$$

Where λ_{sample} is the absorbance of the sample (the absorbance of 2 µl sample in 198 µl DPPH, minus the absorbance of 2 µl sample in 198 µl EtOH), and the λ_{control} is the absorbance of the DMSO (the absorbance of 2 µl DMSO in 198 µl DPPH, minus the absorbance of 2 µl DMSO in 198 µl EtOH).

Antimicrobial Activity

Culture of Test Microorganisms

To assess the antimicrobial activity of the seaweed extracts, the following microorganisms were tested: the Gram negative *Escherichia coli* (ATCC 5922) which is commonly found in the lower intestine of endothermic organisms, the Gram positive *Bacillus subtilis* (ATCC 6633) usually found in soil and the gastrointestinal tract of ruminants and humans, and the Gram negative marine bacterium *Vibrio alginolyticus* (CECT 521) which is a common fish pathogen. The test bacteria stored at -80°C were revived by inoculating 100 µl of stock bacteria into appropriate and previously autoclaved culture media: Luria-Bertani Broth (Scharlau, Barcelona, Spain) for both *E. coli* and *B. subtilis* growth, and Tryptic Soy Broth (VWR, Radnor, PA USA) with 1% sodium chloride for *V. alginolyticus*. Bacteria was then incubated at 37°C (*E. coli* and *B. subtilis*) and 30°C (*V. alginolyticus*), at 150 rpm (Stuart, Staffordshire, UK). The overnight cultures were adjusted to 0.5 of McFarland standards (McFarland 1907), which corresponds to about $5 \cdot 10^5$ CFU ml⁻¹, by diluting the bacteria onto a saline solution prepared with sodium chloride (0.85%) in distilled water.

Agar Diffusion Assay

The antimicrobial activity was determined according to the agar diffusion method (Bauer et al. 1966) for the April, July, October and January samples. Sterile paper discs of 7 mm

diameter (Filtres FIORONI, Ingré, France) were impregnated with 10 µl of the previously prepared seaweed ethanolic extract and left to air-dry at room temperature for 1 minute. Discs impregnated with DMSO only (10 µl) were used as a negative control, to check whether this solvent influences antibiotic activity, whereas chloramphenicol antimicrobial susceptibility test discs (30 µg/disc, Thermo Fisher Scientific, Waltham, MA USA) represented the positive control.

Autoclaved Mueller-Hinton agar (VWR, Radnor, PA USA) plates were seeded with 100 µl of test bacteria suspension; for the marine bacteria *V. alginolyticus*, Mueller-Hinton agar prepared in 1.5% sodium chloride was used instead. Sterile swabs were used to seed the test bacteria and assure a uniform covering. The impregnated discs with seaweed extract and controls were carefully laid onto the agar surface. All tests were performed under sterile conditions and in triplicate for each bacterial strain. The agar plates were then incubated for 24 h at 37°C (*E. coli* and *B. subtilis*) and 30°C (*V. alginolyticus*). Evidence of antibacterial activity was evaluated by measuring the diameter (in mm) of the clear circles shaped around the discs, corresponding to the microbial growth inhibition zone.

Statistical analysis

Data is expressed as means \pm standard deviation. The Student's *t*-distribution test was performed to analyse all data, considering the level of significant difference of $p < 0.05$. The software Microsoft Office Excel 2013 was used to perform all the statistical analysis.

Results and Discussion

Moisture, Organic, and Ash Content

The determination of the total amount of minerals present within a food source is commonly made by quantification of its ash content, defined by the inorganic residue left remaining after removal of the water and organic matter, by either ignition or complete oxidation of organic matter, present in a food sample (Harris and Marshall 2017). In the present study, the seaweed *G. longissima* presents a high moisture content (83.60% in total fresh weight), relatively to its organic (15.24% in total fresh weight) and ash content (1.16% in total fresh weight) (Fig.: 3.1, see also Appendix B, Table B.I). Specifically, and regarding total dry weight, this red seaweed presents a high organic matter content (92.95% in dry weight) and a relatively very low content in ash (7.05% in dry weight).

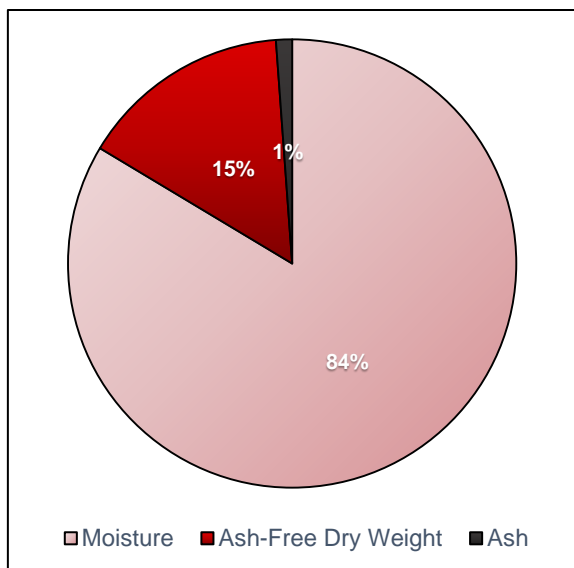


Figure 3.1: Moisture, organic, and ash content proportions (% in rounded values) obtained from the red seaweed *Gracilariopsis longissima* samples collected in February 2017. Moisture (in light red) accounts for 83.60% of total weight, while organic (red slice) and ash contents (black slice) account for 15.24% and 1.16% of total weight, respectively.

A number of authors who also quantified the ash content by AOAC methodology detected variable numbers among macroalgae. Higher ash contents were found by Gressler et al. (2010) for other Gracilariaceae, namely *Gracilaria dominguensis* (23.8% in dry weight) and *Gracilaria birdiae* (22.5% in dry weight). Rodrigues et al. (2015b) report an ash content value ranging from 20.52 to 35.99% of dry weight for several green, brown and red seaweeds, including *Gracilaria gracilis* (with 24.8% ash in dry weight). Renaud and Luong-Van (2006) determined an ash content comparatively higher, of 49.3% for *Gracilaria salicornia*. Other red seaweed groups also present a higher content in ash in comparison to *G. longissima*, namely *Grateloupia turuturu* with more than 18% ash in dry weight (Denis et al. 2010), *Porphyra* sp. with 19.07% ash in dry weight (Sánchez-Machado et al. 2004), and *Mastocarpus stellatus* and *Gigartina pistillata* with respectively 24.99 and 34.56% of dry weight (Gómez-Ordóñez et al. 2010). The above cited authors mention the possibility of data variations due to species identity, seasonal period, geographical location, and environmental growth conditions. Therefore, it is possible that the moisture, organic, and ash proportions obtained for *G. longissima* are closely related to seasonal variations, which influence the water temperature and salinity, and sunlight available; this hypothesis will be tested in the future with seaweed samples collected during the year. Reports or studies concerning the ash content specifically in *G. longissima* were not found to date in literature. Given that the ash proportion of any given food source may contain important microelements to human and animal nutrition (Paiva et al. 2014), future studies pertaining the ash composition in *G. longissima* must be performed.

Total Protein Content

Nowadays the global interest in the sustainable production of biomass for the production of protein, has promoted research specifically focused on the potential of seaweeds as protein providers (Lourenço et al. 2002; Harnedy and Fitzgerald 2011; Samarakoon and Jeon 2012). These bioactive peptides, usually composed of 2 to 20 amino acid residues, may be involved in numerous and important biological functions, such as antioxidant, anticancer, antihypertensive, antiatherosclerotic, and immunomodulatory processes (Fan et al. 2014).

In the present research, the seaweed *G. longissima* collected from Lagoa de Óbidos holds a remarkable total protein content according to season (Fig.: 3.2, and Appendix B, Table B.II). The peak of the cold season, January, stood out as the month when the harvested *G. longissima* yielded the highest amount of protein (27.04% of dry weight), significantly higher than the values obtained for the remaining months, except that of February (Appendix B, Table B.III). Conversely, the *G. longissima* samples collected in July presented the lowest value in protein (11.19% of dry weight), which was statistically significantly lower than all the other months. Statistic tests did not detect significant differences (*t*-student, $p > 0.05$, Appendix B, Table B.III) among the *G. longissima* samples collected in April, October, and February, where the values ranged from 24.13 (October) to 25.59 (February) of dry weight.

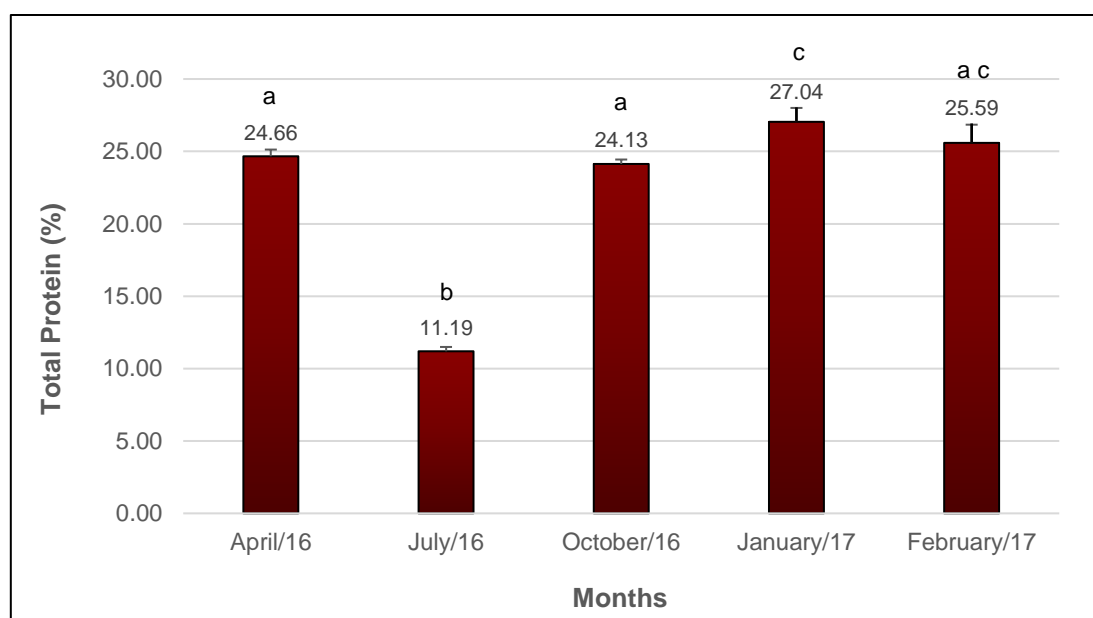


Figure 3.2: Total protein content (% of dry weight) obtained from the red seaweed *Gracilariopsis longissima* samples collected throughout the year. Results are expressed as means. Vertical line above each bar represents the standard deviation. $n = 3$. Letters a-c indicate statistical differences according to the *t*-student statistical test, with $p < 0.05$.

The total protein value range matches those obtained from other macroalgae researchers. Seaweeds have variable protein content between *taxa*, though the highest content is generally found in red seaweeds, such as *Porphyra tenera* with values reaching 47% (Pereira 2016). Authors such as Rodrigues et al. (2015), who assessed the protein content in red, brown and green macroalgae in the Central West Coast of Portugal, found the highest content in all red seaweeds considered, *Osmundea pinafitida* (Ceramiales), *G. turuturu* (Halymeniales) and *G. gracilis* (Gracilariales), with values ranging from 20.2 to 23.8% of dry seaweed. Renaud and Luong-Van (2006) calculated the protein content of tropical marine macroalgae, finding the highest protein values in Rhodophyta species (*Acanthophora muscoides*, *Bostrychia tenella*, *Laurencia majuscula* and *Wrangelia plumosa*), although the mean value obtained (8.0% dry weight) is lower than those usually reported for red seaweeds. The authors also report protein values for two *Gracilaria* species, namely *G. salicornia* and an unidentified *Gracilaria* spp., with respectively 6 and 7% of dry weight. The red seaweeds *P. tenera*, *G. gracilis*, and *Palmaria palmata* are especially rich in protein, holding values as high as 47.5% (Fujiwara-Arasaki et al. 1984), 45% (Francavilla et al. 2013) and 30% (Hagen Rødde et al. 2004) dry weight, respectively, which are values comparable to those reported for widely known and explored protein providers such as soybean (48 to 50% of dry weight) (García et al. 1997).

Overall the total protein content value found for *G. longissima* collected in Lagoa de Óbidos present very consistent values throughout the year, supported by statistical analysis, except for July which corresponds to the peak of the warm season. Generally, and according to Wells et al. (2017), the macroalgae protein content decreases during periods of nutrient limitation, such as coastal and lake thermal stratification phenomena during summer. The temperate Portuguese summers typically have high temperatures, lack strong winds and rain, and according to Verburg and Hecky (2009) these factors deeply affect vertical water mixing, nutrient transport and renewal across water layers in lakes and coastal regions. Similarly, and geographically close to the sampling location of the present study, Galland-Irmouli et al. (1999) estimated values significantly higher in the winter-spring seasons (21.9% of dry weight), than those of summer-autumn period (11.9% of dry weight) obtained from *P. palmata* sampled at the French Brittany Coast; these results are explained by the authors by the higher nutrient availability in seawater during winter and early spring, which reflects upon the seaweed protein content. Also geographically close and with findings quite similar to the present study, Francavilla et al. (2013) report a marked and significant seasonal dependency in the protein content determined for *G. gracilis* harvested at the Lesina Lagoon (Southern Adriatic Coast,

Italy); the authors list January as the month when the seaweed yielded the highest protein value (45% dry weight), whereas individuals collected in July presented the lowest value (31% dry weight). However, these seasonal patterns are not shared for a number of studies focused on seasonal variations on seaweed protein content, as particularly unique seasonal patterns were also detected on the literature (Kaehler and Kennish 1996; Galland-Irmouli et al. 1999; Renaud and Luong-Van 2006; Khairy and El-Shafay 2013; Francavilla et al. 2013). To name an example, Renaud and Luong-Van (2006) detected significantly higher percentages in tropical Rhodophyta collected in the hot and wet tropical Australian summer (mean 8.4% dry weight) than those sampled in the cool and dry winter (mean 7.6% dry weight). Given that such studies are performed worldwide, the divergence in the seasonal pattern between results is most likely a reflection of not only the species studied, but also the specific regional climate of the sampling area.

Analyses of total protein content in algae open venues in the search and discovery of new protein sources. In the present study, *G. longissima* from Lagoa de Óbidos collected in the winter season presents nearly the same protein value as the red seaweed *P. palmata*, mentioned above as being an especially rich protein source. The values obtained during the winter season are also comparable to other reputable protein sources such as peanuts (23 to 28%), cooked beef (17 to 41%), traditional cheddar (25 to 27%), while being higher than egg white (7.0%), bread (7 to 11%), and a number of seeds and vegetables, such as chia seeds (16%) (Health Canada 2015). Considering that there are potential benefits associated with the consumption of protein from a proper combination of animal and vegetal sources (Hoffman and Falvo 2004), *G. longissima* from Lagoa de Óbidos might potentially be adopted in the preparation of healthy diet practices, as an alternative to known and popular protein-rich sources, such as the aforementioned. This is especially true for *G. longissima* harvested in winter, or cultivated under artificial conditions that replicate a winter season in the natural environment. The determination of the content, proportion, digestibility, and bioavailability of the amino acids that compose the protein fraction of this seaweed will permit further assessments of its nutritional value and quality (Gressler et al. 2010; FAO 2011; Francavilla et al. 2013), and must thus be carried out in the future.

Fatty Acid Content and Profile

Seaweeds are known for their extremely low fat content (1 to 5%), poorly contributing thus as energy providers (Lordan et al. 2011). This proportion is mainly represented by phospholipids and glycolipids (Holdt and Kraan 2011). This content may vary significantly throughout the year, as shown by Francavilla et al. (2013) for *G. gracilis*, though authors

such as Renaud and Luong-Van (2006) found no seasonal differences in the total lipid content of the 30 seaweeds studied. Among the three macroalgae groups, lowest fat contents are generally reported for red seaweed, such as *G. salicornia* (1.3 g.100⁻¹ g dry seaweed) (Renaud and Luong-Van 2006), *O. pinnatifida* and *G. gracilis* (0.6-3.6 g.100⁻¹ g dry seaweed) (Francavilla et al. 2013; Rodrigues et al. 2015b), and *Porphyra dioica* (Schmid et al. 2014). The total fat content of *G. longissima* presently obtained was 1.67 g.100⁻¹ g dry seaweed, which stands within the range of values abovementioned.

The fatty acid methyl ester (FAME) profile of the lipid extracts obtained from *G. longissima* samples collected in Lagoa de Óbidos in February is shown in Table III.I, whereas the chromatogram of the fatty acid methyl esters is represented in Figure 3.3, with peak values standing as retention times. Ten fatty acids were detected: the saturated fatty acids myristic, palmitic, and stearic acids; the monounsaturated fatty acids palmitoleic, oleic, and vaccenic acids; and the polyunsaturated fatty acids linoleic, arachidonic, eicosatrienoic, and eicosapentaenoic acids. Polyunsaturated fatty acids and saturated fatty acids showed similar proportions, and accounted for the majority of the total fatty acids (FAs) obtained (48.26 and 42.53% of total FAs, respectively). Monounsaturated fatty acids present a lower proportion with 7.06% of total FAs. Arachidonic acid methyl ester (20:4 ω6) stood out as the prevalent PUFA (43.84% of total FAs), while palmitic acid methyl ester (16:0) was the predominant saturated fatty acid (36.78% of total FAs), which renders both components as being the main fatty acids composing the lipid fraction of *G. longissima*; however, due to the low-fat content of this seaweed (1.67 g.100⁻¹ g) these methyl esters merely represent, respectively, 0.576 and 0.489 g of the total edible weight. The remaining fatty acid methyl esters proportions were entirely residual in comparison, with values ranging from 0.21% for linoelaidic acid, to 4.83% for oleic acid, of the total FAs.

Table III.I: Fatty acid profile of total lipids extracted from the red seaweed *Gracilariopsis longissima* samples collected in February 2017.

Fatty Acid	Peak Area (% of total fat)	Content (g·100⁻¹ g edible weight)	Common Name	Family
14:0	2.69 ± 0.13	0.036 ± 0.013	Myristic Acid	SFA
16:0	36.78 ± 0.47	0.489 ± 0.142	Palmitic Acid	SFA
16:1 ω7	0.36 ± 0.07	0.005 ± 0.002	Palmitoleic Acid	MUFA
18:0	3.06 ± 1.47	0.044 ± 0.032	Stearic Acid	SFA
18:1 ω9	4.83 ± 0.10	0.064 ± 0.018	Oleic Acid	MUFA
18:1 ω7	1.87 ± 0.09	0.025 ± 0.006	Vaccenic Acid	MUFA
18:2 ω6	0.69 ± 0.13	0.009 ± 0.005	Linoleic Acid	PUFA
18:2 ω6 trans	0.21 ± 0.10	0.003 ± 0.002	Linoelaidic Acid	PUFA
20:4 ω6	43.84 ± 3.71	0.576 ± 0.127	Arachidonic Acid	PUFA
20:3 ω3	3.21 ± 0.11	0.043 ± 0.011	Eicosatrienoic Acid	PUFA
20:5 ω3	0.32 ± 0.01	0.004 ± 0.001	Eicosapentaenoic Acid	PUFA
SFAs	42.53 ± 1.14	0.569 ± 0.186		
MUFAs	7.06 ± 0.12	0.094 ± 0.027		
PUFAs	48.26 ± 3.60	0.636 ± 0.146		
PUFAs ω6	44.73 ± 3.48	0.586 ± 0.131		
PUFAs ω3	3.53 ± 0.12	0.047 ± 0.013		
Unsat./Sat.	1.30 ± 0.12	0.017 ± 0.004		
16:1	0.36 ± 0.07	0.005 ± 0.002		
18:1	6.70 ± 0.19	0.089 ± 0.024		
Ratio ω6/ω3	12.69 ± 0.55	0.167 ± 0.043		

Values are expressed as mean ± standard deviation. SFA = saturated fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid.

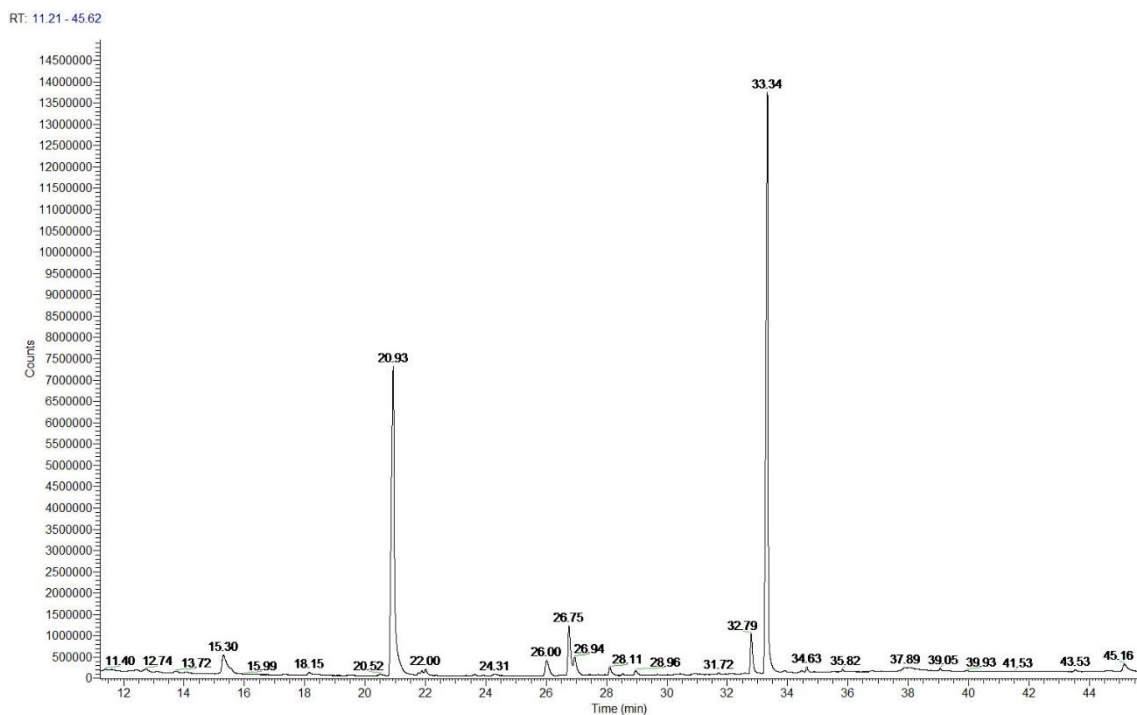


Fig. 3.3: Chromatogram of the fatty acid methyl esters (FAMES) of a freeze-dried sample of the red seaweed *Gracilariopsis longissima*. Peak represent the fatty acid methyl esters obtained, whereas peak values represent retention times.

The fatty acid profile of *G. longissima* lipidic extract obtained in the present study resembles those of several other macroalgae. Jamieson and Reid (1972) studied 12 Rhodophyta, finding that the major constituent fatty acids are generally the palmitic acid and eicosapentaenoic acid, while the arachidonic acid methyl ester (20:4 ω 6) stood out as the prevalent PUFA, whereas other polyunsaturated acids were generally present in amounts less than 5%. More recently, Schmid et al. (2014) analyzed the fatty acid profile of 16 species of macroalgae from the Irish west Coast at two seasons, finding high variability between and within algal groups, and listing the palmitic, oleic, linoleic, arachidonic, and eicosapentaenoic acids, as the most common, similarly to the present findings. Moreover, the authors found significant differences in total fatty acids between seasons, although it remains to be tested whereas the fatty acid content also reveals significant differences. Among *Gracilariaceae*, Gressler et al. (2010) also list both the palmitic and the arachidonic acids as the predominant fatty acids present in *G. dominguensis* and *G. birdiae*, although the specific proportions point out the palmitic acid as the predominant fatty acid (accounting for 65.4 and 56.9%, respectively, of total FA for these species), while on the present study the prominent role is taken by the arachidonic acid. Also similarly to the present study, Francavilla et al. (2013) list arachidonic and palmitic acids as the most abundant fatty acids detected in *G. gracilis*

(16-33 and 26-38%, respectively), and several other fatty acids in relative low concentrations, overall finding a correlation between relative concentrations and seasonality. Wen et al. (2006) detect a total protein content of 0.87% for *Gracilaria lemaneiformis*, comprised by a high proportion of palmitic acid (39%), while linoleic and oleic acids comprise a high proportion of the unsaturated fatty acids (61% of total FA). For *G. longissima*, Stabili et al. (2012) report the predominance of the palmitic acid methyl ester (42% of total FA) however, it is also pointed out the prominence of the saturated fatty acids parcel (67.30%) in relation to monounsaturated and polyunsaturated fatty acids (16.16 and 16.54%, respectively), a finding not presently observed for *G. longissima* collected from Lagoa de Óbidos.

The ratio of $\omega 6$ to $\omega 3$ fatty acids stood at 12.69 in the current study, which is surprisingly high for a macroalgae species according to previous studies; one exception is the report by Francavilla et al. (2013) who also detected a high $\omega 6/\omega 3$ ratio for *G. gracilis*, also collected in winter (20.48%). It must be also mentioned, however, that the authors detected a significantly lower $\omega 6/\omega 3$ for the remaining months studied (3.95 to 8.96%). Generally, studies report this ratio to stand close to 1 for *G. longissima* (Stabili et al. 2012) and for seaweeds in general (Schmid et al. 2014). A balanced value close to 1 corresponds to the maximum recommended ratio value in healthy human diet, although in western diets this value nowadays can reach values as high as 20 (Simopoulos 2016). In this sense, *G. longissima* does not seem to be a suitable candidate when the objective is to adopt a balanced diet due to its high $\omega 6/\omega 3$ ratio; however, before rushing to such hypothesis, it must be noted that samples from February only were analyzed, lacking thus a thorough analysis performed throughout the year. The examples from other authors cited above show that the fatty acid profiles change according to macroalgae species; yet in fact, even within species, each seaweed population has its very own fatty acid profile, with a generally greater range of fatty acids present than in higher plants (Jamieson and Reid 1972). The fatty acid profile is also shaped by its geographic distribution and temporal variation (Colombo et al. 2006), where environmental conditions, such as temperature, salinity, photoperiod, and nutrient availability play a key role (Stabili et al. 2012; Rodrigues et al. 2015b). Generally, PUFA are more abundant in cold water algae, with a lower $\omega 6/\omega 3$ fatty acid ratio, and higher content of unsaturated fatty acids, in comparison with warm water algae (Schmid et al. 2014). Therefore, harvesting season must always be taken into account when evaluating total fatty acid content and FAME profiles, as several authors detected differences between seasons for many seaweed groups studied (Venkatesalu et al. 2012; Kendel et al. 2013; Francavilla et al. 2013; Schmid et al. 2014). Hypothesis arise then, considering the above

stated, of whether *G. longissima* fatty acid profile and proportion change according to season, similarly to what was observed in previous studies with other seaweed species.

It must be noted that, before comparing results between studies, the difference between drying procedures (simple dry or freeze-dry), and the lipid extraction methods adopted by different authors must be always considered. The analytical methods used by phycologists in the assessment of lipids and fatty acid profiles in algae are often diverse and poorly described, and the potential variability of the results can happen due to the variety of protocols for lipid extraction and fractionation, and FAME setup before gas chromatography analysis (Guihéneuf et al. 2015). Discrepancies regarding geographic location and sample season must also be taken into account. As an example, while the *G. longissima* presently studied was collected during winter in Lagoa de Óbidos, which is in contact with the Atlantic Ocean, Stabili et al. (2012) collected their samples during summer in the Mar Piccolo of Taranto, a basin in contact with the Mediterranean Sea. These factors alone, season and geography, may explain the differences in the fatty acid profile obtained for the same algal species. Hypothesis arise on whether this profile may be efficient in identifying not only species, but subspecies and populations.

Antioxidant Activity

Macroalgae living in shallow waters and thus exposed to air and UV radiation can suffer the formation of ROS, so they are innately protected by antioxidant defense systems present in their cells (Kelman et al. 2012). This antioxidant ability can be quantified, and may happen due to one or a combination of various mechanisms of antioxidant action, such as the colloidal properties of the substrates, conditions and stages of oxidation, and the localization of antioxidants in different phases (Frankel and Meyer 2000). For this reason, and also due to the lack of standardized and approved methods to quantify the antioxidant activity (Prior et al. 2005), many authors recommend to perform more than one type of assay for this purpose (Frankel and Meyer 2000; Pinchuk et al. 2012). In the present study, two assays based on electron transfer (Huang et al. 2005), such as the total phenols by Folin-Ciocalteu reagent and the DPPH radical scavenging activity, were performed on ethanolic extracts of *G. longissima* collected throughout the year.

Total Phenolic Content

A number of compounds have been reported to have antioxidant effects, summarized by Masaki (2010), namely ascorbic acid, tocopherols, carotenoids, and phenols. From this list, the last two are naturally produced by macroalgae, among other photosynthetic organisms. Specifically, phenolic compounds are an important group of chemical compounds in seaweeds (Lordan et al. 2011), whose quantity and quality varies between

species from any given group, with red algae generally having the lowest phenolic content. Phenols are vital in algal cell defense against abiotic and biotic stress, owing to their ability to their antioxidant, antimicrobial, and antiviral properties; greater antioxidant properties are reportedly the result of lower degrees of polymerization (Rodrigues et al. 2015b). Phenols are also responsible for a variety of physiological effects, either toxic or curative, on human health (Onofrejová et al. 2010).

In the present study, *G. longissima* presents generally low amounts of phenolic compounds, as shown in Figure 3.4 (see also Appendix B, Table B.IV, with the corresponding *t*-student *p* values represented in Appendix B, Table B.V). Results range from 0.54 to 1.90 mg GAE.g⁻¹ according to the sampling month, and in the following order: February > July > October > January > April. For April the lowest amount of phenolic compounds (0.54 mg GAE.g⁻¹) was detected, being this result statistically different from the remaining months except January (1.18 mg GAE.g⁻¹) (*t*-student, *p* = 0.121). July and February presented the highest values, and similar content in phenolic compounds, with respectively 1.81 and 1.90 mg GAE.g⁻¹ with no statistical differences detected between both months (*t*-student, *p* = 0.810). October presented a phenol concentration of 1.41 mg GAE.g⁻¹, being no statistically different from July and January samples (*t*-student, *p* = 0.211 and *p* = 0.456, respectively). The lack of statistical significant differences between most of the samples stands as proof that seasonal variation is not enough to completely shape the amount of phenolic compounds present in *G. longissima* at any given season. Also interestingly, and as mentioned, February and July have a similar phenolic content, yet these two months stand in opposing ends on a seasonal scale, being February one of the coldest months and July one of the warmest. Such result hints to the possible role of temperature-induced stress (due to, respectively, cold and hot temperatures) in sculpting the phenolic production in these samples in a similar manner, a hypothesis that can only be established with further testing.

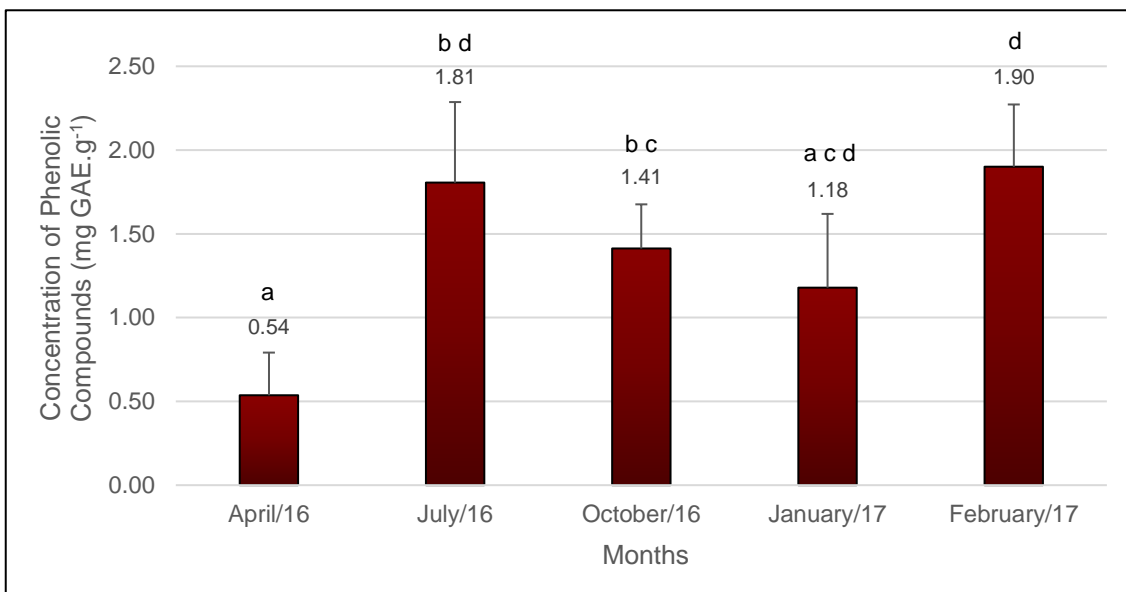


Figure 3.4: Concentration (mg GAE.g⁻¹) of the total phenolic compounds obtained from the red seaweed *Gracilariopsis longissima* samples collected throughout the year. Results are expressed as means. Vertical line above each bar represents the standard deviation. $n = 4$. Letters a-d show statistical differences according to the t -student statistical test, with $p < 0.05$.

DPPH Radical Scavenging Activity

To assess the antioxidant activity of a given compound, the DPPH scavenging ability is one of the best known and frequently chosen analysis (Sharma and Bhat 2009; Ghosh et al. 2015; Pękal and Pyrzynska 2015; Raja et al. 2016).

The Figure 3.5 shows the DPPH scavenging activity against the ethanolic extracts of *G. longissima*, where the values range from 3.93 to 8.92%, in the following order, from highest to lowest: February > January > July > October > April (see also Appendix B, Table B.VI, with the p values from the t -student statistic test depicted in Table B.VII). The DPPH showed the highest scavenging ability against the February extracts with 8.92%, a result significantly different from the remaining months except January, with 7.14% (t -student, $p = 0.060$). The DPPH scavenging ability against the April extracts presented the lowest value (3.93%), however, statistical analysis did not find significant differences between the scavenging ability of DPPH of the ethanolic extracts of April, July and October, which present the lowest overall values ranging from 3.97 to 4.06%. It is noteworthy that, with the strong absorption band centered at 520 nm, the DPPH radical presents a deep dark violet colour in solution, becoming pale yellow following radical scavenging, enabling the reaction to be followed spectrophotometrically. This colour change was not visually observed in the samples analyzed, which is indicative that the DPPH trapped low amounts of free radicals from the ethanolic extracts of *G. longissima*, standing thus in concordance with the low values presently obtained.

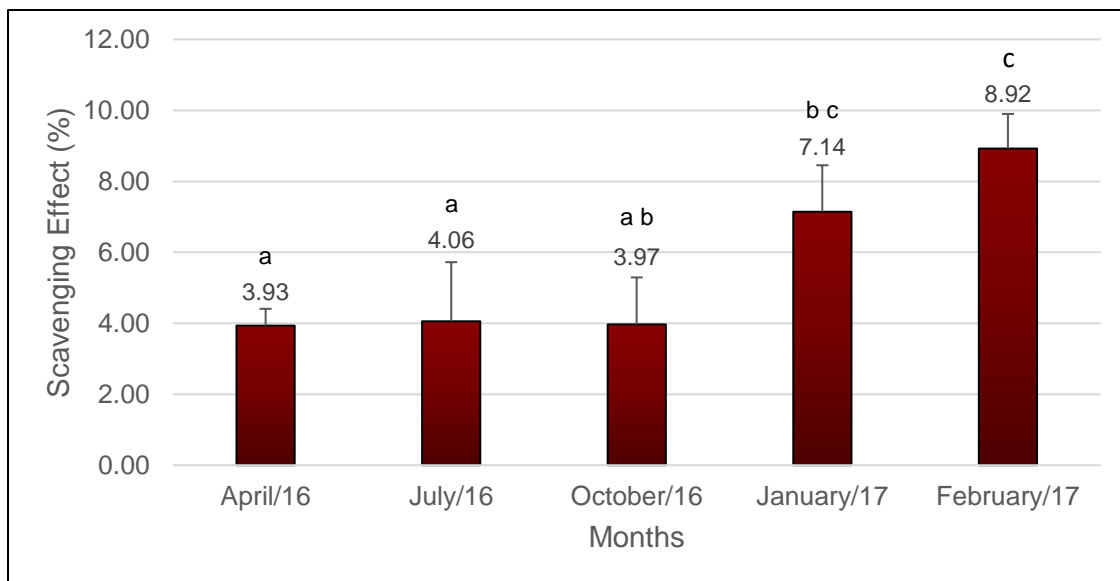


Figure 3.5: DPPH Scavenging effect (%) obtained from ethanolic extracts from the red seaweed *Gracilariopsis longissima* samples collected during the year. Results are expressed as means. Vertical line above each bar represents the standard deviation. $n = 4$. Letters a-c indicate statistical differences according to the t -student statistical test, with $p < 0.05$.

In recent years research has been performed to discover and understand the nature of the natural antioxidants in algae, to ultimately mitigate the degenerative effects of oxidative stress upon human health (Cornish and Garbary 2010; de Almeida et al. 2011; Brownlee et al. 2012; Kelman et al. 2012; Lee et al. 2013; Horta et al. 2014; Chan et al. 2015; Machu et al. 2015). Although no studies regarding the ability or the nature of any potential antioxidant compounds produced by *G. longissima* have been performed to date, the family Gracilariaceae has been one of the red seaweed groups to receive such attention (Duan et al. 2006; Yang et al. 2012; Francavilla et al. 2013; Wu et al. 2015; Yeh et al. 2015). Specifically, and to cite an example, Francavilla et al. (2013) performed a detailed antioxidant study on water, *n*-hexane, ethyl acetate, and methanolic extracts of *G. gracilis* samples collected throughout the year in Lesina Lagoon (Southern Adriatic Sea, Italy), by evaluating the total phenolic content and the DPPH radical scavenging ability among other antioxidant methods. The authors report a low yield of phenolic content on methanol extracts from all the extracts studied, with the lowest concentration reported for October (2.3 mg GAE.g⁻¹), and yet still higher than the highest value obtained for *G. longissima* in the present study, corresponding to February. Regarding the DPPH assay, the authors report finding seasonal differences for all months for the ethyl acetate extracts, a result not observed regarding other extracts such as the methanolic extracts, where the spring and winter samples showed similar values. Overall, however, and for

all assays, the authors report high antioxidant and radical scavenging activities in the summer extracts.

A number of authors such as Raja et al. (2016) report interesting results regarding the total phenolic content and DPPH scavenging effect in brown and green seaweeds (*Eisenia arborea* with 80.11 mg GAE.g⁻¹, DPPH scavenging effect with 29.54%, and *Codium fragile* and *Ulva prolifera* with respectively 8.62 and 19.9 mg GAE.g⁻¹, and 8.09 and 5.47% scavenging effect), being these results closer to the present findings for *G. longissima*. Regarding other food sources, the total phenolic content obtained for *G. longissima*, regardless of the season factor, yielded values standing much below of those obtained for many other compounds. To mention a few examples, 28 products (fruits, vegetables, and grain products) studied by Velioglu et al. (1998) yielded phenolic contents ranging from 169 to 10548 mg.100 g⁻¹, the latter corresponding to red onion scale, and 14 Chinese medicinal plants studied by Zhang et al. (2011) also presented comparatively high phenolic content, such as *Sophora japonica* with 91.33 mg GAE.g⁻¹. It must be noted, however, that the results obtained for the present study are not comparable with the examples mentioned above, even those within Gracilariaceae species, due to several aspects. Although the species factor may be the sole responsible for the discrepancy found in the antioxidant activity measure, other considerations may also be accountable: the choice of the antioxidant capacity evaluation method as mentioned in the beginning of the present section, the type of extract studied, and even factors pertaining the species geographic location and type of habitat, such as temperature shifts, light radiation, nutrient availability, and even anthropogenic pressures and environmental pollution (which eventually trigger the natural defenses of the seaweed as consequence of stress). Regarding the selection of the extraction procedure, this is surely a critical step to perform, as the properties of the extracts depend on the conditions in which they were prepared (Mendes et al. 2013). Researchers often prepare the seaweed extracts with water, ethanol, methanol, n-hexane, acetone, or ethyl acetate, or even aqueous mixture of solvents (Francavilla et al. 2013; Chan et al. 2015). The seaweed extracts in the present study were prepared with ethanol as the organic solvent, similar to previous studies (Mendes et al. 2013; Andrade et al. 2013; Chan et al. 2015). In light of the present findings and those from the abovementioned studies, *G. longissima* does not seem to stand as a potential source of natural bioactive compounds with antioxidant activities. However, it is worth considering in the future to perform a comparative study to evaluate the antioxidant and antimicrobial properties of a selection of extracts obtained from *G. longissima*, to assess whether different extraction methods lead to divergent results. Such approach would be similar to that of previous studies

focused on seaweeds, which report a close association between antioxidant activity and the type of extract analyzed (Francavilla et al. 2013; Chan et al. 2015; Raja et al. 2016). The present antioxidant study must also be repeated by surveying aqueous mixtures of solvent extracts, instead of pure solvent extracts, in light of the findings of Jiménez-Escrig et al. (2012), who report higher efficiency in the analysis of polyphenol compounds on samples extracted with aqueous mixtures of organic solvents, and Pérez-Jiménez and Saura-Calixto (2006), who state an overall higher antioxidant activity also for the same category of extracts.

Regarding the geographic location and type of habitat, populations of the same seaweed species often thrive in very distinct and unique habitats (Guiry and Guiry 2017). Therefore, the sampling sites where a species can be collected for study are extremely diverse regarding biotic and abiotic conditions, and this might reflect upon the results obtained. In the present study, the populations of *G. longissima* from the semi-enclosed Lagoa de Óbidos grow in mudflats characterized by extreme salinity shifts (ranging from 15 to 38‰), nearly buried under dense patches of sea lettuce (*Ulva lactuca*) that broadly grow and create lengthy green “meadows” along the shoreline. This extensive growth is aggravated by nitrogen levels that often increase dramatically in estuaries, due to anthropogenic activities such as fertilizer leeching and sewer discharges (Cole et al. 1993). Lagoa de Óbidos is one regional summer hotspot, locally renowned, appealing, and sought out in the warm season, bringing in an human activity surge due to tourism and its inherent business opportunities; it also stands close to settlements, farmlands and factories punctuating the upland area, all accountable for the nitrogen increase in the water, especially in early summer when farmlands are being cultivated. Sea lettuce blooms are promoted this way, establishing a dense layer that blocks even further the light from *G. longissima*, which in turn does not need to activate its antioxidant defenses as it is sheltered from ultraviolet radiation. Therefore, *G. longissima* antioxidant ability may be exceptionally low due to this particularly unique set of conditions alone. Yet this hypothesis can only be confirmed when more studies focused on the antioxidant ability of this species from other parts of the world are performed.

Antimicrobial Activity

The screening of antimicrobial effects in order to discover new antibiotic sources is now commonly practiced, and it usually reveals remarkable results; it is quite common to find differences in the antimicrobial capacity of seaweeds according factors such as season, geographic location, and classification (green, brown, and red) (Carvalho and Pereira

2015). Among Rhodophyta, several seaweed groups have shown potential as antibacterial agents, such as the genera *Laurencia* (Rhodomelaceae, Ceramiales), *Acanthophora* (Rhodomelaceae, Ceramiales), and *Gracilaria* (Gracilariaceae, Gracilariales), among other species. The antibacterial compounds for these red seaweeds are the focus of an extensive and comprehensive review by Kasanah et al. (2015). The genus *Gracilariopsis* is not mentioned on this report, however, preliminary research on *G. longissima* potential as an antimicrobial agent has been performed recently (Stabili et al. 2012; Cavallo et al. 2013), with lipidic algal extracts of the red seaweed. Work performed with ethanolic extracts of *G. longissima* seems to be inexistent to the best of the present knowledge, and therefore a reliable comparison of the results presently found from those obtained by the abovementioned authors is not currently possible.

The ethanolic extracts of *G. longissima* possess a very interesting antibacterial activity against the infectious agents *E. coli*, *B. subtilis* and *V. alginolyticus* (Fig.: 3.6 and 3.7, and Appendix B, Table B.VIII). For *E. coli*, the inhibition halo measured around 10 mm for all the months studied, except January, with 9 mm. For *B. subtilis*, the inhibition halo values stood around 14 mm for April and January, and 15 mm for July and October. For *V. alginolyticus*, values were around 15 mm for April, and 16 mm for the remaining months studied. The *t*-student statistical analysis determined that there are no difference between months, for any bacteria studied (Appendix B, Table B.IX). The chloramphenicol controls were comparatively higher for each organism studied, with inhibition halos standing around 22 mm for *E. coli*, 32.5 mm for *B. subtilis* and 30.5 mm for *V. alginolyticus*, while the negative controls proof that DMSO is not accountable for the activity detected.

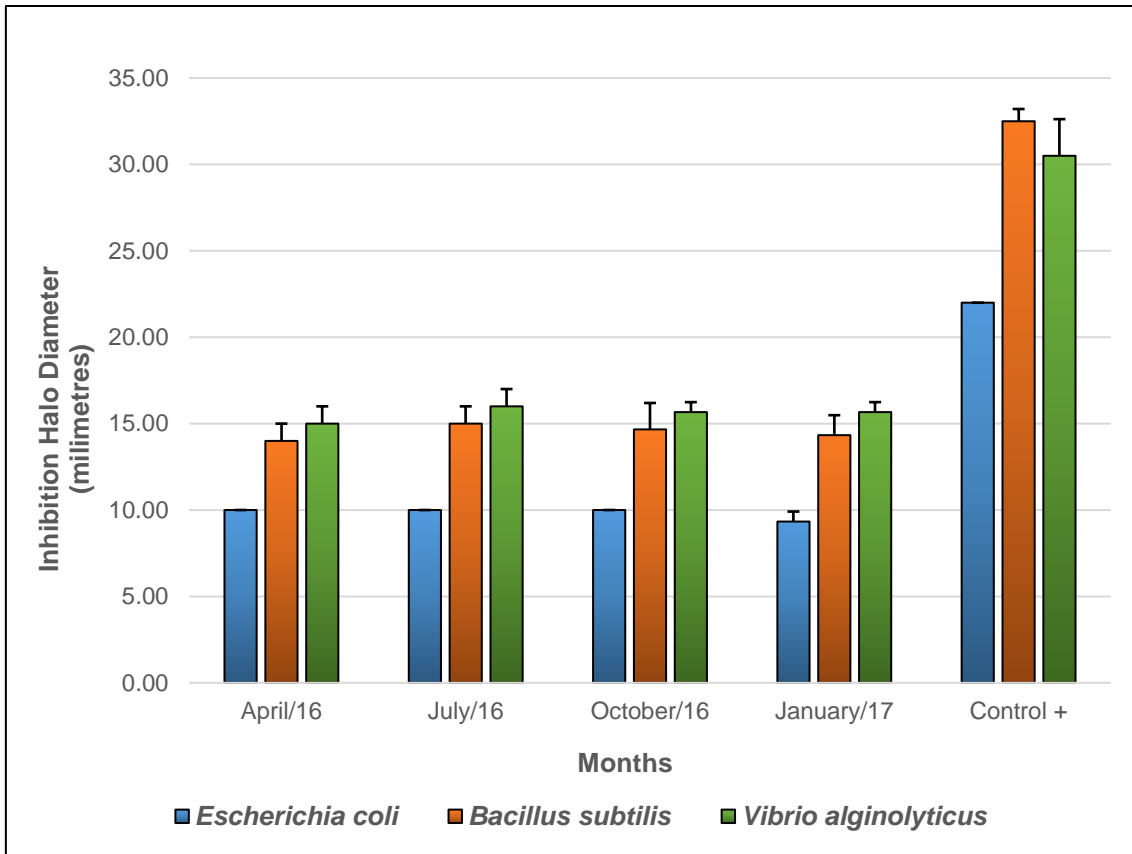


Figure 3.6: Antibacterial activity of the ethanolic extract of *Gracilariopsis longissima* samples collected in April, July, October, and January, against the infectious bacteria *Escherichia coli* (blue), *Bacillus subtilis* (orange), and *Vibrio alginolyticus* (green). Results are the means expressed in millimetres, and correspond to the inhibition halo diameter measured around the disc placed upon each organism culture spread. Positive control is represented by chloramphenicol. Vertical line above each bar represents the standard deviation. $n = 3$

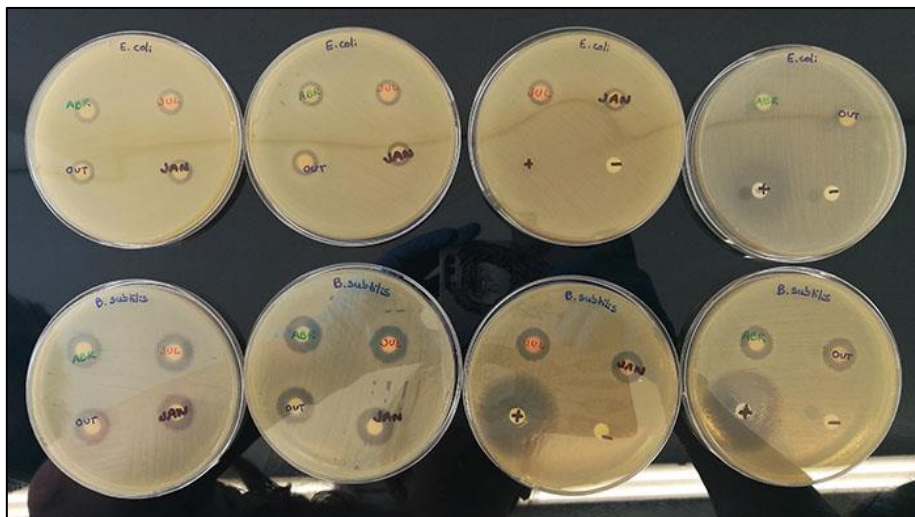


Figure 3.7: Representative images of the inhibition halos obtained with the ethanolic extract of *Gracilariopsis longissima* samples collected in April, July, October, and January, against the growth of *Escherichia coli* and *Bacillus subtilis*. Positive control was obtained with chloramphenicol, whereas negative control is represented by DMSO.

Interestingly, the values found for *B. subtilis* and *V. alginolyticus* are reasonably similar, although the organisms are quite different regarding their source (food and marine origin, respectively) and their cell wall structure (gram-positive and gram-negative, respectively). On the other hand, and although *E. coli* and *V. alginolyticus* are both gram-negative bacteria, they incite different antibacterial activity strengths from the *G. longissima* extracts. Antimicrobial activity in *G. longissima* was also monitored by Stabili et al. (2012) against several strains of *Vibrio* spp. and *Candida* spp., from lipid extracts obtained from samples collected in the Mediterranean Sea by summer. Interestingly, although *G. longissima* showed no activity against *Candida* spp. in their study, it showed a remarkable distinct activity against *Vibrio* spp., which depended of the *Vibrio* species studied, with values as low as 0 and 0.8 mm for *Vibrio salmonicida* and *Vibrio fluvialis*, respectively, and as high as 25 mm for *V. alginolyticus*, whereas in the present study, a diameter of only 15 to 16 mm was obtained for this organism. This difference can be explained, however, by the type of the extract employed. The lipidic extract employed by the mentioned authors may have a very effective component, such as a particular fatty acid, responsible for the strong activity detected.

Resistance developed by *Vibrio* sp. in aquaculture systems is a major concern and cause for serious financial loss. The commercial synthetic antibiotics available on the market often produce undesirable side effects such as antibiotic resistance, toxicity to cultivated specimens, and environmental pollution potentially hazardous to human and animal health. Therefore, there is an urgent necessity of research investment into novel and natural antibiotic agents to control fish and shellfish diseases such as vibriosis (Defoirdt et al. 2011) and to produce new probiotic feed for fish. Many species of the genus *Vibrio* are allegedly responsible for the most common disease outbreaks in aquaculture systems worldwide (Novriadi 2016), and *V. alginolyticus* is one such species (Kalatzis et al. 2016). Hence, the results obtained presently regarding the *G. longissima* activity against *V. alginolyticus* stand as a testimonial of the potential of this seaweed as a worth exploring natural source in vibriosis disease control. Moreover, there were statistically no significant differences observed in the antibacterial activity values obtained between months, yielding thus no consequence in choosing a specific harvest period for *G. longissima*, when the sole purpose is the exploitation of this seaweed as an antimicrobial agent. Questions remain, however, as to which specific compound is responsible for the observed antibacterial activity, and in light of Stabili et al. (2012) findings, whether a lipidic extract of the *G. longissima* collected in Lagoa de Óbidos might be even more suitable as an antibacterial agent, than the ethanolic extract presently studied.

Conclusive Remarks

The potential of the red seaweed *G. longissima* from Lagoa de Óbidos was studied in order to access its value as a nutraceutical natural source. The dry matter was analysed to access its proteic and lipidic value, whereas the corresponding ethanolic extract was screened for antioxidant and antibacterial activities. Overall, *G. longissima* is a valuable natural source, with its nutritional value attested by its high protein and low lipid content, while holding promise as well as an antibacterial agent, being thus relevant for a wide range of biotechnological applications. While research has been already made focused on a particular group of bioactive compounds in *G. longissima* (Mollet et al. 1998; Stabili et al. 2012; Cavallo et al. 2013), to this date reviews or extensive research performed on the overall bioactive compounds and activities in *G. longissima* are still lacking. Such compilation of data has already been done for the genus *Gracilaria* (de Almeida et al. 2011), and even for species within this genus such as *G. gracilis* (Francavilla et al. 2013). Reports focused on seasonal variation upon the biochemical composition of macroalgae are sampled from diverse parts of the world, each with its own seasonal climatic pattern, shaping in unique ways the biochemistry of seaweed populations (Renaud and Luong-Van 2006; Hung et al. 2009; Denis et al. 2010; Venkatesalu et al. 2012; Kendel et al. 2013; Khairy and El-Shafay 2013; Schmid et al. 2014). Geographical location is thus a key factor to consider when assessing the seasonal variability in the biochemical composition of macroalgae, which must not be overlooked when comparing studies. It is therefore essential to briefly describe the climatic pattern of the sample sites on the time of sampling, as terms such as “winter” and “summer” do not completely describe the climatic scenario on a given place, at a particular time. Depending on the geographic region, seasons are shaped more or less dramatically by climate, which can even suffer deviations each passing year, especially in recent times due to climatic change.

Therefore, and relevant to the present study, it must be noted that Portugal is mainly characterized by a temperate Mediterranean climate characterized by mildly temperate wet winters, and dry and hot summers (IPMA 2017). Specifically in 2016, summer months were particularly hot and dry, being July classified as such, with two specific heat waves occurring during this month. The maximum temperature achieved values not felt since July 1931, whereas the mean temperatures were only surpassed by those of July 1989. Air temperature mean was 24.33°C, expressively higher than normal, whereas the mean precipitation values were lower than normal, with a mean value of 13.8 mm. Regarding the other months considered, IPMA (2017) generally classifies them as normal regarding temperature and precipitation, although exceptions are described for precipitation values in April, classified as extremely rainy, and October and January, both

classified as very dry. All the climatic patterns above mentioned were also reported for the littoral west of the Portuguese Centro region, where Lagoa de Óbidos is located. This particular seasonal pattern possibly echoed upon *G. longissima* biochemical composition, especially regarding protein content, while seemingly having no effect upon its antimicrobial and antioxidant activities, the latter being generally low nevertheless. Overall, *G. longissima* seems to hold more value as a potential nutraceutical source if harvested in winter, but such hypothesis is based merely on total protein evidence. Further test is required, namely regarding the detection and evaluation of an eventual seasonal pattern in fatty acid content and profile, and ash content and mineral composition, in order to pinpoint the season(s) leading to the highest yield for any given compound, or group of compounds.

Although the potential nutritional or bioactive content of several algal species has been extensively reported on literature, the amount of studies focused on the bioavailability of nutrients and phytochemicals from algal sources are still poor in comparison (Wells et al. 2017). Bioavailability is an important concept in pharmacology, defined as the rate and extent to which the bioactive substance is absorbed and becomes available at the site of action, and in nutrition, defined as the food fraction used by the body. This concept thus addresses a group of processes such as the release from a food matrix, absorption, distribution, metabolism, and elimination phases (LADME) (Rein et al. 2013). Wells et al. (2017) state that studies pertaining the bioavailability of a given substance are often performed under short-term *in vitro* conditions, which restrains a flawless appreciation of their value as a nutritional agent. These authors also critically review the current unbalanced situation regarding the status on algal research, stressing out the different food components related to the reported efficacy of algal as a nutritional source. As such, future studies pertaining the nutraceutical potential of *G. longissima* from Lagoa de Óbidos must include the bioavailability of the compounds with nutritional value obtained from this macroalgae, in order to consider further recommendations related to the adoption of *G. longissima* in the human diet.

Paracelsus (1493-1541), a renowned physician, alchemist, and astrologer of the German Renaissance, stated once “*All things are poison and nothing is without poison; only the dose makes a thing not a poison*”, a statement regarded now as the classic toxicology maxim. The adoption of any new therapeutic agent, or food source, in the human health and diet must be approached sensibly, as with drug and food consumption there is always some degree of danger associated. Such risk is not only related to the chemicals themselves, but also with the dosage associated when taken by the organism. Although the literature is rich and diverse regarding any given seaweed potential as a natural

nutraceutical source, research is still comparatively poor regarding any eventual adverse effects to human health upon its consumption (Wells et al. 2017). Although not being the scope of the present study, it is important to mention in the present context that the seaweed *G. longissima* is reportedly efficient as a biofilter and bioremediator (Brown and Newman 2003; Hernández et al. 2006; He et al. 2014). The ability of seaweeds to uptake metals is relevant to detect anthropogenic signatures in coastal areas, however it is also a feature that clashes with their potential application on the human diet; while under normal conditions, metal integration improves their nutritional quality, excessive uptake can lead to toxicity (Wells et al. 2017). As such, and although constituting a promising natural food source as already suggested above, questions including the eventual toxicity of the compounds present in *G. longissima* have certainly to be answered.

Final Remarks and Future Research

Although the most pertinent conclusions have already been outlined in the respective sections of each chapter, a few extra points have still to be considered.

As stated before, the taxonomy of the Gracilariaceae family is currently terribly chaotic (Bird 1995). Most authors do rely on visual identification only, or do not give details on how the identification was performed; therefore, it is to be considered the possibility of incorrect identifications among studies, not only within *Gracilaria* and *Gracilariopsis* genus, but also among both groups as well. Morphological confirmation is not enough to achieve full certainty of the species identification within the *Gracilariaceae* group, due to the extent of morphological discrepancies found in images submitted to credited databases such as *AlgaeBase*, even within a single gracilarioid species. For example, and according to Guiry and Guiry (2017), older published works may have possibly studied *Gracilariopsis longissima* under the name *Gracilaria verrucosa* or *Gracilaria gracilis*, or the other way around. Evidently the present work is no exception to this, and questions remains about the true identity of the seaweed presently studied, even though it was confirmed by botanists. Therefore, the first and foremost priority in further studies concerning the gracilarioid of Lagoa de Óbidos, will be to achieve an irrefutable species identification, by means of histological observation of the tissues having as a working background the observations from Fredericq and Hommersand (1990), and by *rbcL* sequence analyses, by taking into consideration the most recent taxonomic treatment adopted for *Gracilariopsis* genus (Gurgel et al. 2003) to support the visual confirmation.

The greatest asset of the Gracilariaceae family, and the main reason why its species are highly regarded, explored, and cultivated, is related to the quality of their phycocolloids. Phycocolloids are polymers composed of chemically modified sugars, being the main structural components of seaweed cell walls. Specifically the alginates are extracted primarily from brown seaweeds, and agar and carrageenan extracted from red seaweeds (Pangestuti and Kim 2015). Together, they make up the largest market for algal extracts. To outline a few examples, the widely commercialized sugar gel agar is largely obtained from *Gracilaria lemaneiformis*, also known as *Gracilaria tikvahiae* in the western North Atlantic, being the most sought out seaweed species for this purpose (Santelices and Doty 1989). A few other gracilarioids are reported to hold potential value as provider of high-quality phycocolloids, such as *G. gracilis* (Skriptsova and Nabivailo 2009), *Gracilaria firma* (Araño et al. 2000), and *Gracilaria vermiculophylla* (Villanueva et al. 2010), to name a few. Regarding microbiological grade agar, only a low number of gracilarioids meet the requirements demanded by certain particular blends, and as such

this agar grade is mostly explored from other seaweed sources, such as *Gelidium* and *Pterocladia* (Santelices and Doty 1989). The assessment of the agar and carrageenan quality of *G. longissima* was not the scope of the present work, yet it is one of the main studies scheduled to be carried out in the near future. Such is justified by the considerable biotechnological and economical value that most Gracilariaceae studied to date hold as agarophyte, possibly extendable to *G. longissima* from Lagoa de Óbidos.

As stated by Stabili et al. (2012), the use of a natural product in medicine and pharmacy requires large amounts of biological material. In the context of seaweeds, such large amounts can be obtained by harvesting, or cultivation of the source. Yet as stated before, the massive harvesting of any given resource, including any species of macroalgae, quickly leads to its depletion, a situation that is only desirable if the source happens to be an invasive species, such as the red seaweed *Asparagopsis armata* or *G. vermiculophylla* intrusive presence in European waters, both discussed in Chapter One. Fortunately, from a biotechnological perspective, these species are also potential sources of interesting bioactive compounds (Villanueva et al. 2010; Lopes et al. 2011; Pinteus et al. 2015), which gives ground to justifiable extensive harvesting activities in areas where these species pose a threat to the local ecosystems. This way, two objectives are met: the invasive agent is not only removed from the environment, but also explored for its biotechnological potential. Regarding native seaweed species, the adoption of a cultivation indoor or outdoor system for the target species instead of harvesting the corresponding natural source, is a solution to consider. This way, condition cultures can even be manipulated in order to yield maximum growth and product yield, while leaving the natural populations untouched and preserved. Such solution also solves the problem of harvesting seaweeds that might pose a threat to human health upon consumption, due to the high toxicity levels of certain elements related to their bioremediation efficiency.

Currently, no doubt remains pertaining the role of the environment in shaping the balance of the biochemical elements of *G. longissima*, and therefore its overall nutraceutical profile, to a more or less extent depending on the specific compound under analysis. The factor “season” is of utmost importance, and shape a great number of factors on a regional level, such as temperature, sunlight, salinity, and available nutrients, all of these acting synergistically and modelling biomes in very unique ways. Several examples detailing how seasonality affect a number of compounds present in seaweeds were already put forward in Chapter Two. Such results are absolutely relevant in outlining not only the ideal harvest season of any given seaweed to obtain the maximum yield possible for the target compound, but also in delineating culture conditions in order to replicate

specific natural climate accountable for such high yields. In this sense, the two main research topics investigated presently, growth rates and potential biotechnological value of the native seaweed *G. longissima*, provide results that supplement each other, working in close cooperation to deliver the first steps towards a successful high-performance farming of a native species. Critical thinking and further studies outlined throughout the present work will complement the results already achieved, in order to reach a greater perspective towards the accomplishment of such an enterprise.

The field of algal research offers exciting opportunities for research and collaboration between phycologists dedicated to specific fields of study. During the course of the present work, the extensive bibliographic research offered an opportunity to find excellent reviews pertaining the prospects offered by any given seaweed species (or macroalgae in general) as a nutraceutical natural source, most of them cited throughout this document. However, “potential” is a word that is constantly present in every work focused on this particular subject (including the present one), leaving room to many enquiries about the actual applicability of the studied source on the human diet, namely its toxicity and bioavailability. As such, it is pertinent to continue the present studies by answering questions regarding the toxicity that may be associated with the consumption of fresh or processed *G. longissima*, as well as the bioavailability of its nutrients and phytochemicals, explained further in Chapter Two. Meanwhile, studies focused on achieving the ideal sustainable conditions to obtain the maximum growth rate and production yields must also be performed. The seaweed *G. longissima* collected from Lagoa de Óbidos looks strikingly promising as a protein source, and also as an antibacterial agent against *Vibrio*. Yet as outlined in previous chapters, these results are still the first step of a throughout assessment of the evaluation of this seaweed as a nutraceutical agent. No steps were taken yet regarding productivity yields under manipulated conditions, and the results outlined in Chapter Two reflect only the natural conditions in the time of harvest. All the aforementioned questions that were put forward in the course of the present work, and still linger to this date, offer excellent opportunities for a synergetic collaboration between several fields of science dedicated to the study of *G. longissima*, and it is envisioned that the first steps taken in the present work pave the way to thrilling and promising research opportunities in the very near future.

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Appendix

Appendix A

Table A.I: Composition of the stock solutions required for Von Stosch's Enriched Seawater Medium (VSE), for use with red algae, as proposed by Redmond et al. (2014). For 1 L of seawater, 1 ml of each of the above stock solution is added. Solutions 3 and 4 were mixed immediately prior to addition to seawater.

Ingredients	Quantity (g.L⁻¹ distilled water)
Solution 1: Nitrogen	
Ammonium Chloride (NH ₄ Cl)	26.75
Solution 2: Phosphate	
Sodium Phosphate. Dibasic, 12-hydrate, crystal (Na ₂ HPO ₄ ·12H ₂ O)	0.4
Solution 3: Iron	
Ferrous Sulfate (FeSO ₄ ·7H ₂ O)	0.278
Solution 4: EDTA	
Disodium Ethylenediamine Tetra Acetate (Na ₂ EDTA)	3.72
Solution 5: Manganese	
Manganese Chloride (MnCl ₂)	0.0198
Solution 6: Vitamins	
Thiamine	0.2
Biotin	0.001
Vitamin B ₁₂	0.002

Table A.II: Length, in millimetres, of *Gracilariopsis longissima* tips determined at the beginning (day 0) and ending (day 44) of each salinity trials (20 to 35‰). Dish ID stands for the identity of each petri dish. To each tip a randomly assigned number was attributed; as such, each number regards solely the tip it was assigned to, serving merely to ease the measuring process. Therefore, each number assigned to a "day 0" tip, may or may not correspond to that very same number in the "day 44" tip from any given Petri dish. The "growth" lane corresponds to the difference between each "initial" and "final" means, per salinity, and corresponds to the values used to calculate the Daily Growth Rate (% day⁻¹).

		20‰ day 0			25‰ day 0			30‰ day 0			35‰ day 0	
Tip	Dish ID	1	2	3	4	5	6	7	8	9	10	11
		1	31.92	26.27	27.26	20.02	28.55	35.07	16.96	10.37	8.47	21.16
	2	30.84	20.65	28.28	23.98	22.90	23.64	11.69	14.11	8.60	20.46	20.59
	3	24.31	12.82	26.54	22.83	20.54	30.85	14.35	16.18	10.92	19.25	19.15
	4	17.49	22.79	22.15	25.17	31.68	27.33	9.29	7.64	8.08	22.77	15.31
	5	22.09	27.68	22.24	27.63	35.13	27.95	15.31	13.41	19.80	19.18	16.47
	6	23.64	18.97	24.91	27.51	32.84	26.79	12.77	16.38	15.17	18.40	16.47
	7	25.60	20.25	21.90	25.76	21.40	28.30	8.09	12.66	15.17	19.58	21.60
	8	25.10	24.71	22.60	20.16	32.74	24.84	17.25	16.12	12.45	16.61	
	Mean	25.12	21.77	24.49	24.13	28.22	28.10	13.21	13.36	12.33	19.68	18.27
		20‰ day 44			25‰ day 44			30‰ day 44			35‰ day 44	
Tip	Dish ID	1	2	3	4	5	6	7	8	9	10	11
	1	27.05	34.66	33.19	33.25	47.92	39.19	19.42	25.55	25.86	40.16	30.28
	2	43.67	28.08	37.75	24.74	45.96	43.64	16.05	19.01	18.99	36.88	36.14
	3	37.86	29.11	48.42	34.28	39.1	38.99	22.51	26.04	21.68	35.82	35.46
	4	32.70	27.74	43.41	32.84	42.09	36.17	21.24	23.77	23.57	46.37	25.95
	5	36.07	36.20	32.04	34.46	30.81	35.55	25.15	24.74	16.52	45.12	42.31
	6	30.42	27.23		37.48	29.52	47.33	28.85	25.37	25.18	38.53	35.38
	7	33.71	29.58		37.50	29.85	36.88	24.53		15.45	36.97	47.71
	8	45.90	33.10		26.82	48.70	38.17	22.37		28.74	32.30	
	Mean	35.92	30.71	38.96	32.67	39.24	39.49	22.52	24.08	22.00	39.02	36.18
	Growth	10.80	8.95	14.48	8.54	11.02	11.39	9.30	10.72	9.67	19.34	19.33
	Mean ± SD	11.41 ± 2.82			10.32 ± 1.55			9.90 ± 0.75			19.34 ± 3.95	

Table A.III: Daily Growth Rate (% day⁻¹), according to salinity, calculated in agreement with Mtolera et al. (1995), Gerang and Ohno (1997), Aguirre-Von-Wobeser et al. (2001), Bulboa et al. (2007), Hayashi et al. (2007a), Hayashi et al. (2007b), Hung et al. (2009) and Hayashi et al. (2011), for *Gracilariopsis longissima* samples growing on a laboratory scale, having as basis the difference between the tip lengths measured at the beginning and ending of a 44 days trial (Table II, Appendix A). $n = 3$ for salinities 20 to 30‰, and $n = 2$ for salinity 35‰.

Salinity (‰)	Sample 1	Sample 2	Sample 3	Mean	SD
20	0.816	0.785	1.061	0.888	0.151
25	0.691	0.752	0.777	0.740	0.044
30	1.219	1.348	1.324	1.297	0.069
35	1.568	1.654		1.611	0.287

Table A.IV: *t*-student statistical analysis, with a probability value of $p < 0.05$, performed upon the Daily Growth Rate (% day⁻¹) data presented on Table III, for *Gracilariopsis longissima* samples growing on a laboratory scale, having as basis the tip lengths measured at the beginning and ending of a 44 days trial (Appendix A, Table II). p -values below 0.05 indicate that there are statistical differences between pairs, and are showed in bold. Redundant values are greyed out. $n = 3$ for salinities 20 to 30‰, and $n = 2$ for salinity 35‰.

Salinity (‰)	20	25	30	35
20		0.182	0.042	0.046
25	0.182		0.001	0.009
30	0.042	0.001		0.043
35	0.046	0.009	0.043	

Appendix B

Table B.I: Values, in percentage, obtained for the moisture, organic, and ash content determined for *Gracilariopsis longissima* samples collected from Lagoa de Óbidos, in February 2017. AFDW stands for Ash-Free Dry Weight and corresponds to the organic content. Means and standard deviation (SD) are also represented. $n = 13$.

	Moisture (%)	AFDW per Dry Weight (%)	AFDW per Fresh Weight (%)	Ash per Dry Weight (%)	Ash per Fresh Weight (%)
<i>Gracilariopsis longissima</i> (February 2017)	83.58	93.15	15.29	6.85	1.12
	83.80	93.01	15.07	6.99	1.13
	83.75	92.63	15.05	7.37	1.20
	84.02	92.27	14.75	7.73	1.24
	83.68	93.30	15.22	6.70	1.09
	83.03	92.86	15.76	7.14	1.21
	84.15	93.11	14.75	6.89	1.09
	83.40	93.02	15.44	6.98	1.16
	82.53	92.28	16.12	7.72	1.35
	83.75	93.26	15.16	6.74	1.10
	83.86	93.27	15.06	6.73	1.09
	83.65	92.95	15.20	7.05	1.15
	83.60	93.18	15.28	6.82	1.12
Mean	83.60	92.95	15.24	7.05	1.16
SD	0.42	0.35	0.37	0.35	0.08

Table B.II: Mean values ($n = 3$) obtained for the protein content (%) determined by the Kjeldahl method (Kjeldahl 1883), in *Gracilariopsis longissima* collected from Lagoa de Óbidos in 2016 and 2017. 1, 2 and 3 corresponds to the assays performed in triplicate for each month.

	April/16	July/16	October/16	January/17	February/17
1	25.01	11.03	24.49	28.15	24.30
2	24.84	11.02	23.96	26.41	26.81
3	24.14	11.54	23.96	26.57	25.66
Mean	24.66	11.19	24.13	27.04	25.59
SD	0.46	0.30	0.30	0.96	1.26

Table B.III: *t*-student statistical analysis, with a probability value of $p < 0.05$, performed upon the protein content (%) determined by the Kjeldahl method (Kjeldahl 1883), in *Gracilariopsis longissima* collected from Lagoa de Óbidos according to month. *p*-values below 0.05 indicate that there are statistical differences between pairs, and are indicated in bold. $n = 3$.

Months	April/16	July/16	October/16	January/17	February/17
April/16		0.001	0.123	0.034	0.379
July/16	0.001		0.001	0.002	0.003
October/16	0.123	0.001		0.017	0.242
January/16	0.034	0.002	0.017		0.367
February/16	0.379	0.003	0.242	0.367	

Table B.IV: Concentration (mg GAE.g⁻¹) of the total phenolic compounds obtained from the red seaweed *Gracilariopsis longissima* samples collected throughout the year. Results are expressed as means. 1, 2, 3 and 4 corresponds to the essays performed in quadruplicate for each month. $n = 4$.

	April/16	July/16	October/16	January/17	February/17
1	0.56	2.15	1.15	1.72	1.48
2	0.48	1.39	1.53	0.86	2.36
3	0.24	2.29	1.73	1.36	2.00
4	0.86	1.39	1.25	0.78	1.76
Mean	0.54	1.81	1.41	1.18	1.90
SD	0.25	0.48	0.26	0.44	0.37

Table B.V: *t*-student statistical analysis, according to month, with a probability value of $p < 0.05$, performed upon the total phenolic compound values obtained from the red seaweed *Gracilariopsis longissima* samples, collected throughout the year. p -values below 0.05 indicate that there are statistical differences between pairs, and are indicated in bold. $n = 4$.

	April/16	July/16	October/16	January/17	February/17
April/16		0.033	0.037	0.121	0.014
July/16	0.033		0.211	0.010	0.810
October/16	0.037	0.211		0.456	0.030
January/17	0.121	0.010	0.456		0.142
February/17	0.014	0.810	0.030	0.142	

Table B.VI: DPPH Scavenging effect (%) obtained from ethanolic extracts from the red seaweed *Gracilariopsis longissima* samples collected throughout the year. Results are expressed as means. 1, 2, 3 and 4 corresponds to the essays performed in quadruplicate for each month. $n = 4$.

	April/16	July/16	October/16	January/17	February/17
1	4.05	5.99	4.69	8.74	10.19
2	4.02	2.89	5.47	5.63	8.84
3	4.40	4.89	2.77	7.49	7.82
4	3.27	2.45	2.95	6.71	8.84
Mean	3.93	4.06	3.97	7.14	8.92
SD	0.47	1.67	1.32	1.31	0.97

Table B.VII: *t*-student statistical analysis, according to month, with a probability value of $p < 0.05$, performed upon the DPPH scavenging effect values obtained from the red seaweed *Gracilariopsis longissima* samples, collected throughout the year. *p*-values below 0.05 indicate that there are statistical differences between pairs, and are indicated in bold. $n = 4$.

	April/16	July/16	October/16	January/17	February/17
April/16		0.873	0.962	0.015	0.003
July/16	0.873		0.939	0.004	0.009
October/16	0.962	0.939		0.053	0.003
January/17	0.015	0.004	0.053		0.060
February/17	0.003	0.009	0.003	0.060	

Table B.VIII: Antibacterial activity of the ethanolic extract of *Gracilariopsis longissima* samples collected in April, July, October, and January, against the infectious bacteria *Escherichia coli*, *Bacillus subtilis*, and *Vibrio alginolyticus*. Results are expressed in millimetres, and correspond to the inhibition halo diameter measured around the disc placed upon each organism culture spread. Positive control is represented by chloramphenicol, whereas negative control is represented by DMSO. 1, 2, and 3 corresponds to the essays performed in triplicate for each month. $n = 3$.

		<i>April/16</i>	<i>July/16</i>	<i>October/16</i>	<i>January/17</i>	<i>Control +</i>	<i>Control -</i>
<i>Escherichia coli</i>	1	10	10	10	10	22	0
	2	10	10	10	9		0
	3	10	10	10	9		0
	Mean	10.0	10.0	10.0	9.3	22.0	0.0
	SD	0.0	0.0	0.0	0.6		
<i>Bacillus subtilis</i>	1	14	16	16	15	32	0
	2	15	15	15	15	33	0
	3	13	14	13	13		0
	Mean	14.0	15.0	14.7	14.3	32.5	0.0
	SD	1.0	1.0	1.5	1.2	0.71	
<i>Vibrio alginolyticus</i>	1	15	15	16	16	29	0
	2	16	16	15	15	32	0
	3	14	17	16	16		0
	Mean	15.0	16.0	15.7	15.7	30.5	0.0
	SD	1.0	1.0	0.6	0.6	2.12	

Table B.IX: t -student statistical analysis, according to month, with a probability value of $p < 0.05$, performed upon the antibacterial activity of the ethanolic extract of *Gracilariopsis longissima* samples collected in April, July, October, and January, against the infectious bacteria *Escherichia coli*, *Bacillus subtilis*, and *Vibrio alginolyticus*. x corresponds to pairs of months with equal antibacterial activity values, and thus no statistical analysis was performed upon those. $n = 3$.

		<i>April/16</i>	<i>July/16</i>	<i>October/16</i>	<i>January/17</i>
<i>Escherichia coli</i>	<i>April/16</i>		x	x	0.184
	<i>July/16</i>	x		x	0.184
	<i>October/16</i>	x	x		0.184
	<i>January/17</i>	0.184	0.184	0.184	
<i>Bacillus subtilis</i>	<i>April/16</i>		0.225	0.423	0.423
	<i>July/16</i>	0.225		0.423	0.184
	<i>October/16</i>	0.423	0.423		0.423
	<i>January/17</i>	0.423	0.184	0.423	
<i>Vibrio alginolyticus</i>	<i>April/16</i>		0.423	0.529	0.529
	<i>July/16</i>	0.423		0.667	0.667
	<i>October/16</i>	0.529	0.667		x
	<i>January/17</i>	0.529	0.667	x	